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Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade

Dung T. Le,¹,²,³ Jennifer N. Durham,¹,²,³ Kellie N. Smith,¹,³ Hao Wang,³ Bjarne R. Bartlett,²,4 Laveet K. Aulakh,³,⁴ Steve Lu,²,4 Holly Kemberling,³ Cara Wilt,³ Brandon S. Luber,³ Fay Wong,²,³ Nilofer S. Azad,¹,³ Agnieszka A. Rucki,¹,³ Dan Laheru,³ Ross Donehower,³ Atif Zaheer,³ George A. Fisher,³ Todd S. Crocenzi,⁷ James J. Lee,³ Tim F. Greten,⁶ Austin G. Duffy,⁹ Kristen K. Ciombor,¹⁰ Aleksandra D. Eyring,¹¹ Bao H. Lam,¹¹ Andrew Joe,¹¹ S. Peter Kang,¹¹ Matthias Holdhoff,³ Ludmila Danilova,¹,³ Leslie Cope,¹,³ Christian Meyer,⁹ Shibin Zhou,¹,³,⁴ Richard M. Goldberg,¹² Deborah K. Armstrong,⁷ Katherine M. Bevar,⁹ Amanda N. Fader,¹ⁱ Janis Taube,¹,³ Franck Housseau,¹,³ David Spetzler,¹⁴ Nianqing Xiao,¹⁴ Drew M. Pardoll,¹,² Nickolas Papadopoulos,⁵,⁴ Kenneth W. Kinzler,⁷,³,⁴ James E. Shihleman,¹⁵ Bert Vogelstein,¹,³,⁴ Robert A. Anders,¹,³,¹⁵ Luis A. Diaz Jr,¹,²,³,°†‡

The genomes of cancers deficient in mismatch repair contain exceptionally high numbers of somatic mutations. In a proof-of-concept study, we previously showed that colorectal cancers with mismatch repair deficiency were sensitive to immune checkpoint blockade with antibodies to programmed death receptor–1 (PD-1). We have now expanded this study to evaluate the efficacy of PD-1 blockade in patients with advanced mismatch repair–deficient cancers across 12 different tumor types. Objective radiographic responses were observed in 53% of patients, and complete responses were achieved in 21% of patients. Responses were durable, with median progression-free survival and overall survival still not reached. Functional analysis in a responding patient demonstrated rapid in vivo expansion of neoantigen-specific T cell clones that were reactive to mutant neopeptides found in the tumor. These data support the hypothesis that the large proportion of mutant neoantigens in mismatch repair–deficient cancers make them sensitive to immune checkpoint blockade, regardless of the cancers’ tissue of origin.

Therapy with immune checkpoint inhibitors has uncovered a subset of tumors that are highly responsive to an endogenous adaptive immune response (1). When the interaction between the checkpoint ligands and their cognate receptors on the effector cells is blocked, a potent and durable antitumor response can be observed, and on occasion this response can be accompanied by severe autoimmunity (2–5). These findings support the notion that many cancer patients contain in their immune system the capacity to react selectively to their tumors, ostensibly through recognition of tumor-specific antigens.

The molecular determinants that define this subset of tumors are still unclear; however, several markers, including PD-1 ligand (PD-L1) expression, RNA expression signatures, mutational burden, and lymphocytic infiltrates, have been evaluated in specific tumor types (6–10). Although such markers appear to be helpful in predicting response in specific tumor types, none of them have been evaluated prospectively as a pan-tumor biomarker. Another potential determinant of response is mutation-associated neoantigens (MANAs) that are encoded by cancers (11–14). Mismatch repair–deficient cancers are predicted to have a very large number of MANAs that might be recognized by the immune system (15–18). This prediction led us to conduct a small phase 2 study, focused on 11 patients with colorectal cancers, which demonstrated that PD-1 blockade was an effective treatment for many patients with these tumors (19). Since the initiation of that trial, other studies have shown that the number of mutations in mismatch repair–deficient colorectal cancers correlates with the response to PD-1 blockade, providing further support for a relationship between mutation burden and treatment response (20).

The genomes of mismatch repair–deficient tumors all harbor hundreds to thousands of somatic mutations, regardless of their cell of origin. We therefore sought to investigate the effects of PD-1 blockade (by the anti–PD-1 antibody pembrolizumab) in mismatch repair–deficient tumors independent of the tissue of origin. In the current study, we prospectively evaluated the efficacy of PD-1 blockade in a range of different subtypes of mismatch repair–deficient cancers (ClinicalTrials.gov number NCT01876511).

Eighty-six consecutive patients were enrolled between September 2013 and September 2016 (table S1). The data cutoff was 19 December 2016. All patients received at least one prior therapy and had evidence of progressive disease prior to enrollment. Twelve different cancer types were enrolled in the study (Fig. 1). All enrolled patients had evidence of mismatch repair deficiency as assessed by either polymerase chain reaction or immunohistochemistry. For most cases, germline sequencing of MSH2, MSH6, PMS2, and MLH1 was performed to determine whether the mismatch repair deficiencies were associated with a germline change in one of these genes (i.e., whether the patients had Lynch syndrome) (table S2). Germline sequence changes diagnostic of Lynch syndrome were noted in 32 cases (48%), with MSH2 being the most commonly mutated gene. In seven additional cases where germline testing was not performed, the patient reported a family history consistent with a diagnosis of Lynch syndrome.

Adverse events during treatment were manageable and resembled those found in other clinical studies using pembrolizumab (table S3). Although 74% of patients experienced an adverse effect, most were low-grade. Endocrine disorders, mostly hypothyroidism, occurred in 21% of patients and were easily managed with thyroid hormone replacement.

Seventy-eight patients had disease that could be evaluated by Response Evaluation Criteria in Solid Tumors (RECIST) (Table 1). Objective radiographic responses were noted in 46 of the 86 patients (53%; 95% confidence interval [CI], 42 to 64%), with 21% (n = 18) achieving a complete radiographic response. Disease control (measured as partial response + complete response + stable disease) was achieved in 66 of the 86 patients (77%; 95% CI, 66 to 85%). Radiographic responses could be separated into two classes. First, in 12 cases, scans at 20 weeks showed stable disease, which eventually converted to an objective response (measured as tumor size reduction in response to therapy, according to RECIST criteria). Second, in 11 additional cases, we observed
an initial partial response or stable disease at the 20-week scan that later converted to a complete response while treatment was continued. The average time to any response was 21 weeks; the average time to complete response was 42 weeks (Fig. 1). Of note, the objective response rate was similar between colorectal cancer and other cancer subtypes. Specifically, we observed objective responses in 52% (95% CI, 36 to 68%) of patients with colorectal cancers and in 54% (95% CI, 39 to 69%) of the patients with cancers originating in other organs (tables S4 and S5). There was also no significant difference in the objective response rate between Lynch syndrome–associated and non-Lynch syndrome–associated tumors [46% (95% CI, 30 to 63%) versus 59% (95% CI, 41 to 76%), respectively; \( P = 0.27 \)].

Neither median progression-free survival (PFS) nor median overall survival (OS) has yet been reached (median follow-up time of 12.5 months; Fig. 1), and the study is ongoing. However, the estimates of PFS at 1 and 2 years were 64% and 53%, respectively. The estimates of OS at 1 and 2 years were 76% and 64%, respectively, which is markedly higher than expected considering the advanced state of disease in this cohort (21). The PFS and OS were not significantly different in patients with colorectal cancers relative to those with other cancer types (fig. S1). Neither PFS [hazard ratio (HR) = 1.2 (95% CI, 0.582 to 2.512); \( P = 0.61 \)] nor OS [HR = 1.71 (95% CI, 0.697 to 4.196); \( P = 0.24 \)] were influenced by tumors associated with Lynch syndrome.

Eleven patients achieved a complete response and were taken off therapy after 2 years of treatment. No evidence of cancer recurrence has been observed in those patients with an average time off therapy of 8.3 months. Seven other patients had residual disease by imaging, but pembrolizumab was discontinued after reaching the 2-year milestone or because of intolerance to therapy. To date, the average time off

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**Fig. 1.** Patient survival and clinical response to pembrolizumab across 12 different tumor types with mismatch repair deficiency. (A) Tumor types across 86 patients. (B) Waterfall plot of all radiographic responses across 12 different tumor types at 20 weeks. Tumor responses were measured at regular intervals: values show the best fractional change of the sum of longest diameters (SLD) from the baseline measurements of each measurable tumor. (C) Confirmed radiographic objective responses at 20 weeks (blue) compared to the best radiographic responses in the same patients (red). The mean time to the best radiographic response was 28 weeks. (D) Swimmer plot showing survival for each patient with mismatch repair–deficient tumors, indicating death, progression, and time off therapy. (E and F) Kaplan-Meier estimates of progression-free survival (E) and overall patient survival (F).
therapy for this group was 7.6 months. As of the data cutoff, none of these patients has shown evidence of progression since discontinuation of pembrolizumab.

Twenty patients with measurable radiographic disease underwent pertussis disease biopsies between 1 month and 5 months after the initiation of therapy. Twelve of these biopsies demonstrated no evidence of tumor cells and were shown to have varying degrees of inflammation, fibrosis, and mucin, consistent with an ongoing immune response (fig. S2). The other eight cases showed residual tumor cells. The absence of cancer cells in posttreatment biopsies was a strong predictor of PFS [HR for PFS = 0.189 (95% CI, 0.046 to 0.767); P = 0.012], with median PFS of 25.9 months versus 2.9 months for biopsies with evidence of residual tumor. Although there was no significant difference in OS between patients whose biopsies were positive or negative for tumor cells, median OS has not yet been reached in patients with negative biopsies (table S6).

Primary clinical resistance to initial therapy with pembrolizumab, as measured by progressive radiographic disease on the first study scan, was noted in 12 patients (14%) (Table 1). After determining the exomic sequences of tumor and matched normal DNA from three of these patients, we compared the results with those of their primary tumors (fig. S3 and table S7). In the first case, the primary duodenal tumor and brain metastasis shared 397 nonsynonymous somatic mutations, providing unequivocal evidence that the metastasis was derived from the primary duodenal tumor rather than from an independent tumor. Moreover, the metastasis harbored 1000 nonsynonymous new mutations not present in the primary tumor, while the primary tumor harbored 964 mutations not present in the metastasis (table S9). In the second case, the primary colorectal tumor and brain metastasis shared 848 nonsynonymous somatic mutations, similarly providing unequivocal evidence of a genetic relationship between the two lesions. The brain metastasis harbored 221 nonsynonymous mutations not present in the primary colorectal tumor, while the primary tumor harbored 100 mutations not present in the metastasis (table S10). Of note, the brain metastases from both of these patients contained mutations in the B2M gene. In the patient with the colorectal tumor, a truncating mutation (L15Ffs*41) in the B2M gene was identified in the metastasis but not in the primary tumor. The primary duodenal tumor harbored a truncating mutation in β2-microglobulin (V69Wfs*34), whereas the metastasis retained this mutation and acquired a second B2M mutation (12L-P; table S7).

We also evaluated the exomes of three primary tumors from patients who originally had stable disease by RECIST criteria at 20 weeks, but whose disease progressed within 8 months of initiating therapy. The average mutational burden was 1647 for this group, similar to those of the other patients described above. Interestingly, two of these three tumors harbored mutations of B2M (table S7).

We next sought to directly test the hypothesis that checkpoint blockade induces peripheral expansion of tumor-specific T cells and that mismatch repair–deficient tumors harbor functional MANA-specific T cells. Deep sequencing of T cell receptor CDR3 regions (TCR-seq) has emerged as a valuable technique to evaluate T cell clonal representation in both tumors and peripheral blood. We performed TCR-seq on tumors from three responding patients (obtained from archival surgical resections) and identified intratumoral clones that were selectively expanded in the periphery (Fig. 2A). These clones were present at very low frequency (often undetectable) in the peripheral blood before pembrolizumab treatment, but many rapidly increased after treatment initiation, followed by a contraction that generally occurred before radiologic responses were observed. To characterize functional T cell clones specific for mutant peptides, we obtained peripheral blood from one of the patients (subject 19). We tested the patient's posttreatment peripheral blood for reactivity against the 15 top candidate MANAs as identified via a neoantigen prediction algorithm [specified by the patient's human leukocyte antigen (HLA) class I alleles; see supplementary materials] with an interferon-γ (IFN-γ) ELISpot assay. Counts of spot-forming cells or cytokine activity analyses revealed T cell responses against 7 of 15 peptides (Fig. 2, B and C). We next interrogated the expanded lymphocyte populations against these seven peptides with TCR-seq. Clonal T cell expansion was noted in response to three of the seven peptides (Fig. 2D), with specificity demonstrated by a lack of expansion in response to any other peptide tested (fig. S4). In the peripheral blood, T cell expansion to these three mutant peptides resulted in 142 unique TCR sequences, seven of which were found in the tumor sample (two from MANA1, three from MANA2, and two from MANA4) (Fig. 2D). Of note, the mutant peptides that scored positive all resulted from frameshift mutations—the type of mutation that is most characteristic of mismatch repair–deficient cancers.

All seven of the MANA-reactive TCRs were detectable in peripheral blood at very low frequency (less than 0.02%) before treatment. However, four of the clones rapidly increased in frequency in the peripheral blood after anti–PD-1 treatment (Fig. 2E). Similar to results from the three patients

<table>
<thead>
<tr>
<th>Type of response</th>
<th>Patients (n = 86)</th>
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</thead>
<tbody>
<tr>
<td>Complete response</td>
<td>18 (21%)</td>
</tr>
<tr>
<td>Partial response</td>
<td>28 (33%)</td>
</tr>
<tr>
<td>Stable disease</td>
<td>20 (23%)</td>
</tr>
<tr>
<td>Progressive disease</td>
<td>12 (14%)</td>
</tr>
<tr>
<td>Not evaluable</td>
<td>8 (9%)</td>
</tr>
<tr>
<td>Objective response rate</td>
<td>53%</td>
</tr>
<tr>
<td>95% CI</td>
<td>42 to 64%</td>
</tr>
<tr>
<td>Disease control rate</td>
<td>77%</td>
</tr>
<tr>
<td>95% CI</td>
<td>66 to 85%</td>
</tr>
<tr>
<td>Median progression-free survival time</td>
<td>NR</td>
</tr>
<tr>
<td>95% CI</td>
<td>14.8 months to NR</td>
</tr>
<tr>
<td>2-year progression-free survival rate</td>
<td>53%</td>
</tr>
<tr>
<td>95% CI</td>
<td>42 to 68%</td>
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<tr>
<td>Median overall survival time</td>
<td>NR</td>
</tr>
<tr>
<td>95% CI</td>
<td>NR to NR</td>
</tr>
<tr>
<td>2-year overall survival rate</td>
<td>64%</td>
</tr>
<tr>
<td>95% CI</td>
<td>53 to 78%</td>
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</table>
Fig. 2. TCR clonal dynamics and mutation-associated neoantigen recognition in patients responding to PD-1 blockade. (A) TCR sequencing was performed on serial peripheral T cell samples obtained before and after PD-1 blockade. Tumor tissue with mismatch repair deficiency was obtained from three responding patients. Shown for each patient are 15 TCR clones with the highest relative change in frequency after treatment (left) that were also found in the original tumor (right panels). (B) Whole-exome sequencing was performed on tumor and matched normal tissue from patient 19. Somatic alterations were analyzed using a neoantigen prediction pipeline to identify putative MANAs. Reactivity to 15 candidate MANAs was tested in a 10-day cultured IFN-γ ELISpot assay. Data are shown as the mean number of spot-forming cells (SFC) per 10⁶ T cells (left) or mean cytokine activity (right) of triplicate wells ± SD. *P < 0.05, **P < 0.01, ***P < 0.001. (C) Seven candidate MANAs were selected for TCR analysis on the basis of ELISpot reactivity. (D) MANA-specific T cell responses were identified against three of seven candidate MANAs (MANA1, MANA2, and MANA4) after 10 days of in vitro stimulation (left panels). MANA-specific clones were identified by significant expansion in response to the relevant peptide and no significant expansion in response to any other peptide tested (fig. S3). Data are shown as the relative change in TCR clone frequency compared to the frequency of that clone after identical culture without peptide. These T cell clones were also found in the original tumor biopsy (right panels). (E) Frequency of MANA-specific clones, carcinoembryonic antigen (CEA), and radiographic response in the tumor [from (D)] were tracked in the peripheral blood before treatment and at various times after pembrolizumab treatment. Time is shown in weeks after the first pembrolizumab dose. (F) In vitro binding and stability assays demonstrate the affinity kinetics of each relevant MANA and the corresponding wild-type peptide (when applicable) for their restricting HLA class I allele. The A*02:01-restricted influenza M GILGFVTL epitope was used as a negative control for each assay; known HLA-matched epitopes were used as positive controls when available. Data are shown as counts per second with increasing peptide concentration for binding assays (top) or counts per minute over time for stability assays (bottom). Data points indicate the mean of two independent experiments ± SD. Amino acid abbreviations: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.
analyzed above, the frequencies of these functionally validated MANA-specific T cell clones peaked soon after treatment and corresponded with normalization of the systemic tumor marker, predating objective radiographic response by several weeks. This peak in T cell clonal expansion was followed by decreases in frequency, reminiscent of T cell responses to acute viral infections (Fig. 2E). Because all the MANAs were from frameshift mutations, only MANA2 had a similar wild-type counterpart (differing in the two C-terminal amino acids). The corresponding wild-type peptide bound to HLA with less than 1% of the affinity of the mutant peptide counterpart (Fig. 2F), consistent with the mutation conferring HLA binding.

To estimate the proportion of cancer patients for whom the results of this study might be applicable, we evaluated 12,019 cancers representing 32 distinct tumor types for mismatch repair deficiency using a next-generation sequencing–based approach (Fig. 3). In accordance with a recent independent estimate using a different approach (23), we found that >2% of adenocarcinomas of the endometrium, stomach, small intestine, colon and rectum, cervix, prostate, bile duct, and liver, as well as neuroendocrine tumors, uterine sarcomas, and thyroid carcinomas, were mismatch repair–deficient. Across these 11 tumor types, 10% of stage I to stage III cancers and 5% of stage IV cancers were mismatch repair–deficient. This represents roughly 40,000 annual stage I to III diagnoses and 20,000 stage IV diagnoses in the United States alone. Because genetic and immunohistochemical tests for mismatch repair deficiency are already widely available, these results tie immunity, cancer genetics, and therapeutics together in a manner that will likely establish a new standard of care. In the future, testing for mismatch repair deficiency in patients who are refractory to other treatments might be considered in order to identify those who may benefit from PD-1 pathway blockade, regardless of tumor type.

REFERENCES AND NOTES

The data reported are tabulated in the main text and supplementary materials. The raw TCR RNA sequence data have been deposited into the ImmuneACCESS project repository of the Adaptive Biotech database, under the following link: https://clients.adaptivebiotech.com/pub/diaz-2017-science. We thank K. Helwig for administrative support, C. Blair for outstanding technical assistance, and E. H. Rubin, R. Dansey, and R. Pemerton at Merck & Co. Inc. (Kenilworth, N.J.) for supporting this research. Funded by the Swim Across America Laboratory at Johns Hopkins, the Ludwig Center for Cancer Genetics and Therapeutics, the Howard Hughes Medical Institutes, the Bloomberg-Kimmel Institute for Cancer Immunotherapy at Johns Hopkins, the 2017 Stand Up To Cancer Colon Cancer Dream Team, the Commonwealth Fund, the Banyan Gate Foundation, the Lustgarten Foundation for Pancreatic Cancer Research, the Bloomberg Foundation, the Sol Goldman Pancreatic Cancer Research Center, Merck & Co., Inc., Gastrointestinal SPORE grant P50CA052524, and NIH grants P30CA066973, CA136572, CA23460, CA201981, CA67941, CA46958, and CA57345. L.D., D.L., B.V., N.P., and K.W.K. are inventors on a patent application (PCT/US2015/060331 or WO2016077553 A1) submitted by Johns Hopkins University that covers checkpoint blockade and microsatellite instability. L.D., B.V., N.P., and K.W.K. are founders of PapGene and Personal Genome Diagnostics (PGxD). L.D. is a consultant for Merck, Illumina, PGxD, and Cell Design Labs. PGxD and PapGene, as well as other companies, have licensed technologies from Johns Hopkins University, on which L.D., B.V., N.P., and K.W.K. are inventors. Some of these licenses and relationships are associated with equity or royalty payments. The terms of these arrangements are being managed by Johns Hopkins and Memorial Sloan Kettering in accordance with its conflict-of-interest policies.

SUPPLEMENTAL MATERIALS

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Materials and Methods
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AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE
Recurrent infection progressively disables host protection against intestinal inflammation

Won Ho Yang,1,2,3 Douglas M. Heithoff,1,3 Peter V. Aziz,1,2,3 Markus Sperandio,4 Victor Nizet,5 Michael J. Mahan,1,2,3 Jamey D. Marth1,2,3*

Intestinal inflammation is the central pathological feature of colitis and the inflammatory bowel diseases. These syndromes arise from unidentified environmental factors. We found that recurrent nonlethal gastric infections of Gram-negative Salmonella enterica Typhimurium (ST), a major source of human food poisoning, caused inflammation of murine intestinal tissue, predominantly the colon, which persisted after pathogen clearance and irreversibly escalated in severity with repeated infections. ST progressively disabled a host mechanism of protection by inducing endogenous neumidase activity, which accelerated the molecular aging and clearance of intestinal alkaline phosphatase (IAP). Disease was linked to a Toll-like receptor 4 (TLR4)–dependent mechanism of IAP desialylation with accumulation of the IAP substrate and TLR4 ligand, lipopolysaccharide-phosphate. The administration of IAP or the antiviral neumidase inhibitor zanamivir was therapeutic by maintaining IAP abundance and function.

Inflammation of the intestinal tract is the defining feature of colitis and the human inflammatory bowel diseases (IBDs), including Crohn’s disease and ulcerative colitis (UC). In these syndromes, chronic inflammation disrupts intestinal homeostasis and provokes immune-mediated tissue damage (1–3). The origins of these diseases remain mysterious and involve one or more environmental factors (4). Among multiple human monozygotic twin comparisons, the genetic contribution to the origin of UC is about 20%, whereas in Crohn’s disease, genetics may play a larger though often minor role (5). Considering the possible environmental origins of disease, pathogenic infection has been studied as a factor in precipitating intestinal inflammation (6). Notably, bacterial infections have been linked to seasonal increases in hospital admissions involving intestinal inflammation and IBD (7).

Small-inoculum bacterial infections that are brief and self-limited are likely to be the most common infections, and they may frequently go unreported, potentially leading to an under-appreciation of the numbers of infections among individuals. We hypothesized that there may be cumulative effects of repeated small-inoculum and subclinical infections, which, if true, may be detected in a model of human food poisoning. We designed a study using recurrent low-titer non-lethal gastrointestinal infection by the bacterium Salmonella enterica Typhimurium (ST), a common human pathogen. Nontyphoidal Salmonella (NTS) produces a greater human disease burden than any other foodborne bacterial pathogen in the United States, causing more than a million illnesses annually (8, 9). Globally, NTS causes 93.8 million cases and 155,000 deaths each year (10) and is responsible for up to 50% of bacteremias in young children from developing countries (11, 12).

Results

ST infection elicits intestinal inflammation by diminishing host intestinal alkaline phosphatase levels

Beginning at 8 weeks of age, wild-type C57BL/6J mice were infected by gastric intubation with 2 × 108 ST colony-forming units (CFU) every 4 weeks for six consecutive months. After infection, ST was detected transiently and predominantly in the small intestine and some lymphoid tissues. The pathogen was cleared by the host to undetectable levels by 21-days post-infection monitoring, which further noted the absence of overt symptoms or mortality among the animals (fig. S1, A and B). The onset of disease required more than a single infection with this low titer, whereas multiple signs of disease were evident among most of the animals before the fourth infection. Phenotypes consistent with the onset of intestinal dysfunction included weight loss, reduced colon length, altered stool consistency (diarrhea), and the presence of fecal blood (Fig. 1, A to D, and fig. SIC). By 20 weeks of age and before the fourth infection, signs of disease were present among most of the animals and further included an epithelial barrier defect (Fig. 1E). At 32 to 48 weeks, rectal prolapse was observed among some animals undergoing recurrent infections (Fig. 1F). The frequency of disease symptoms escalated with successive recurrent infections and persisted for at least 5 months after the cessation of infections.

Reductions in alkaline phosphatase (AP) activity and intestinal AP (IAP) abundance were detected in both the small intestine and intestinal contents, whereas levels of duodenal tissue mRNA encoding IAP were unchanged (Fig. 1, G to I, and fig. S2A). Mammalian IAP is produced exclusively by enterocytes of the duodenum and is released from the cell surface into the lumen where it can dephosphorylate and thereby detoxify the lipopolysaccharide (LPS) endotoxin of Gram-negative bacteria (13–16). We found that oral supplementation with calf IAP (cIAP) maintained normal AP activity levels in the intestinal tract among animals receiving recurrent ST infections (Fig. 1D). Analysis of LPS isolated from the intestinal tract at 20 weeks of age before the fourth infection and after ST clearance revealed a fourfold increase in endogenous LPS-phosphate levels in the context of a 50% increase in total LPS, both of which were maintained close to normal levels in mice receiving cIAP treatment (Fig. 1K). Augmentation of AP activity by cIAP treatment also protected against the development of disease symptoms encompassing weight loss, colon length, diarrhea, fecal blood, and epithelial barrier function (Fig. 1L).

Inflammatory cytokines associated with intestinal tissue inflammation include chemokine ligand 5 (CCL5), interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ). Recurrent ST infection progressively increased inflammatory cytokine mRNA expression levels predominantly in the colon, unless cIAP treatment was provided (Fig. 1M). Histopathological changes were also predominantly seen in the colon, including an infiltration of leukocytes into the lamina propria, which included neutrophils, monocytes, and T cells, as well as an erosion of the epithelial barrier, and reduced goblet cell numbers, whereas a much lesser effect was observed in the small intestine and, in addition, only in the ileum (Fig. 1N and figs. S2B and S3). Thus, recurrent nonlethal gastrointestinal infection of ST diminished the expression of host IAP activity that normally confers host protection against intestinal inflammation and tissue damage predominantly in the colon. Studies were further undertaken to identify the mechanisms that regulate IAP function.

IAP deficiency is linked to an accelerated rate of desialylation and endocytic localization

IAP is synthesized as a glycosylphosphatidylinositol (GPI)–linked glycoprotein residing on the
enterocyte cell surface of the duodenum until it is released into the intestinal lumen by phospholipase activity (13, 17). IAP production was investigated by pulse-chase experiments of ex vivo primary enterocyte cultures derived from small intestinal tissue. Normal rates of IAP synthesis and appearance at the cell surface were observed among all enterocyte cultures regardless of previously cleared infections (Fig. 2A). In contrast, a significant decrease in IAP cell surface half-life with reduced IAP abundance in the culture media was measured among enterocyte samples from mice that had cleared multiple infections (Fig. 2, B and C). Colocalization studies revealed increased colocalization of IAP with markers of early endosomes and lysosomes coincident with reduced cell surface IAP abundance (Fig. 2, D and E).

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**Fig. 1. Recurrent ST infection diminishes the abundance and protective role of IAP.** Wild-type (WT) mice were analyzed during a course of recurrent ST infection (2 × 10³ CFU) or uninfected [phosphate-buffered saline (PBS)] at indicated time points (arrows). (A) Body weight (ST, n = 20; PBS, n = 19). (B) Colon length (n = 40 per condition). (C) Diarrhea and stool consistency (ST, n = 19; PBS, n = 13). (D) Fecal blood (ST, n = 19; PBS, n = 13). (E) Intestinal epithelial barrier function (n = 8 per condition) at 20 weeks of age before the fourth infection. FITC, fluorescein isothiocyanate. (F) Rectal prolapse (ST, n = 30; PBS, n = 20) at 32 to 48 weeks of age or 4 to 20 weeks after last ST infection (representative image). (G) AP activity (n = 40 per condition). (H) Immunoblot blot analysis of IAP at 20 weeks of age before the fourth infection (n = 8 per condition). (I) Relative IAP abundance (n = 40 per condition). (J) AP activity ± cIAP (n = 40 per condition). (K) LPS abundance and phosphate released from LPS (n = 8 per condition) at 20 weeks of age. (L) Body weight (n = 10 per condition), colon length (n = 8 per condition), diarrhea (ST, n = 23; PBS, n = 14; ST + cIAP, n = 19; PBS + cIAP, n = 15), fecal blood (ST, n = 23; PBS, n = 14; ST + cIAP, n = 19; PBS + cIAP, n = 15) at 48 weeks of age (20 weeks after last ST infection), and intestinal epithelial barrier function (n = 8 per condition) at 20 weeks of age before the fourth infection. (M) Cytokine mRNA expression (n = 30 per condition). (N) Hematoxylin and eosin (H&E)–stained intestinal tissues at 48 weeks of age (20 weeks after last ST infection). L, intestinal lumen; E, epithelial layer; C, crypt; G, goblet cell; S, submucosa; I, infiltration of leukocytes. Graphs are representative of 16 fields of view (n = 4 per condition). All scale bars, 100 μm. Error bars represent means ± SEM. **P < 0.001, *P < 0.01, and *P < 0.05; Student’s t test (A, B, E, and G to I) or one-way analysis of variance (ANOVA) with Tukey’s multiple comparisons test (J to N).
IAP is glycosylated during its synthesis in the secretory pathway. We next analyzed the glycan linkages attached to enterocyte IAP using analytical lectins, including the α2-3 sialic acid–specific lectin MacKayia amurensis lectin II and the galactose–specific lectins Erithrina cristagalli agglutinin and Ricinus communis agglutinin. These lectins have been validated biochemically and genetically, although their binding does not provide full structural resolution of all glycan types and linkages present. A significant reduction of terminal sialic acid linkages coincident with the exposure of underlying galactose linkages was measured in the context of recurrent infection (Fig. 2F). No comparable changes to other specific glycan linkages were detected, including the siaylation state of core 1 O-glycans, or changes in abundance of α2–6–linked sialic acids, using peanut agglutinin and Sambucus nigra lectins, respectively. In the absence of infection, we observed a progressive desialylation of the glycans attached to nascent IAP on the enterocyte cell surface, indicative of a feature of its normal molecular aging. This basal rate of IAP desialylation was significantly increased by recurrent ST infection and was concurrent with increased IAP internalization and degradation in enterocytes (Fig. 2G). Although IAP deficiency appeared to be the predominant factor in disease onset, as indicated by the effects of cIAP treatment, multiple enterocyte cell surface glycoproteins were observed to be desialylated and internalized from the cell surface of endocytes, including sucrase–isomaltase, dipetidyl peptidase 4, and lactase (fig. S4). These results suggest that the presence of one or more sialyltransferases establishes the normal half-lives (and consequent abundance) of enterocyte cell surface glycoproteins, including IAP.

**ST3Gal6 is responsible for IAP sialylation in protecting against intestinal inflammation**

The ST3Gal6 sialyltransferase generates α2–3 sialic acid linkages on glycoproteins and is highly expressed in the intestinal tract (18, 19). In mice lacking a functional St3gal6 gene, we detected a significant reduction in AP activity and IAP abundance, whereas mRNA encoding IAP was unchanged (Fig. 3, A and B, and fig. S5A). Although LPS levels in the intestinal tract were similar at 20 weeks of age (before a fourth ST infection) (Fig. 3C), the absence of ST3Gal6 was further investigated. The impact of IAP deficiency caused by the absence of ST3Gal6 was also deficient with increased galactose exposure (Fig. 3D), similar to findings in wild-type mice experiencing recurrent ST infection. Glycer alterations were further detected histologically among small intestinal epithelial cells (fig. S5B). Diminished IAP sialylation in ST3Gal6 deficiency was linked to reduced IAP cell surface residency and increased IAP colocalization with markers of endosomes and lysosomes (Fig. 3, E to G). Thus, IAP sialylation by ST3Gal6 functions to maintain IAP half-life and abundance in the intestinal lumen. The absence of IAP deficiency caused by the absence of ST3Gal6 was further investigated.

Mice aging in the absence of ST3Gal6 was compared with that of wild-type littermates in the presence and absence of recurrent ST infection and cIAP therapy. Spontaneous phenotypes detected in uninfected mice lacking ST3Gal6 included reduced body weight, reduced colon length, diarrhea, the presence of fecal blood, and epithelial barrier dysfunction, all of which were exacerbated by recurrent ST infection. The addition of cIAP to drinking water normalized AP activity levels during adult life and reduced or eliminated signs of disease (Fig. 4, A to C). Similarly, increased LPS-phosphate levels were closely associated with
Reduced IAP and the onset of disease symptoms, whereas total LPS increased only modestly in the intestinal contents of infected mice (Fig. 4D). LPS is generated predominantly in the colonic microbiota, which serve as markers or effectors of intestinal inflammatory disease (20). A survey of microbiota using 16S ribosomal DNA (rDNA) probes revealed a 50% increase of intestinal bacterial load attributed primarily to the Gram-negative Enterobacteriaceae, consistent with the magnitude of increase of total LPS. These findings were present among both uninfected St3gal6-null mice and wild-type littersmates subjected to recurrent ST infection (Fig. 4E). The temporal acquisition of these microbiota changes observed at 32 weeks was found to emerge progressively among wild-type mice receiving periodic ST infections and was coincident with the emergence and increase in the severity of disease symptoms (Fig. S6). These findings are also consistent with reports of altered commensal microbiota populations that can contribute intestinal inflammation and that often include elevated levels of Enterobacteriaceae (3, 21).

St3gal6 deficiency spontaneously increased inflammatory cytokines in the intestinal tissues of uninfected animals, and this was further exacerbated by recurrent ST infection (Fig. 4F). Similarly, histopathological findings in the absence of ST3Gal6 correlated with elevated inflammatory marker expression and leukocyte infiltration, epithelial layer discontinuity, and reduced goblet cell numbers, which were also increased in severity by recurrent ST infection (Fig. 4G). These results demonstrated that the ST3Gal6 sialytransferase is essential to support normal IAP sialylation and expression and thereby host protection against spontaneous intestinal inflammation. The cause of IAP desialylation after ST infection was unaccounted for but implicated as a significant trigger of pathogenesis.

**Disease onset with neuraminidase induction is TLR4-dependent and recapitated by LPS**

Neuraminidase (Neu) enzymes, also known as sialidases, hydrolyze sialic acids attached to glycan polymers and are encoded in the genomes of diverse organisms, including bacteria, mice, and humans. However, the genome of the ST isolate used in our studies does not encode a Neu (22), indicating a host source of the induced Neu activity. Four Neu genes have been identified in mammalian genomes (Neul to Neu4), with Neu1 and Neu3 enzymes expressed in multiple compartments, including the cell surface, and in the blood (23, 24). An increase in Neu activity occurred in the small intestine because of ST infection. Studies of Toll-like receptor 4 (TLR4)-null mice revealed that this induction of host Neu activity was dependent on TLR4 function (Fig. 5A). Among mammalian Neu isozymes, only NEU3 abundance and Neu3 RNA expression correlated with TLR4-dependent induction of Neu activity (Fig. 5B and C). The absence of Neu3 induction due to TLR4 deficiency resulted in normal IAP expression at the enterocyte cell surface (Fig. 5D). This was coincident with normal levels of sialic acid linkages on IAP and among apical glycoproteins of the small intestinal epithelium (fig. S5, C and D). AP activity and IAP abundance also remained normal in TLR4 deficiency, and LPS-phosphate levels did not increase significantly after ST infections (Fig. 5, E to G). The induction of inflammatory cytokines caused by recurrent ST infection was also blocked by TLR4 deficiency, and signs of disease were reduced or eliminated with the maintenance of epithelial barrier function (Fig. 5, H and I).

TLR4 is activated by binding to its LPS ligand (25, 26). The LPS bacterial endotoxin is found predominantly in the colon (27, 28), where it can initiate proinflammatory signaling to engage immune

**Fig. 3. Mechanism of IAP regulation by ST3Gal6 sialylation.** (A) AP activity. (B) IAP protein abundance. (C) LPS abundance and phosphate released from LPS of intestinal content. (D) Lectin blot of IAP from small intestine. (E to G) Pulse-chase of IAP synthesis and trafficking and IAP cell surface half-life among cultured primary enterocytes isolated from uninfected ST3Gal6-deficient mice and WT littersmates at 8 to 10 weeks of age. (H and I) In situ localization and intracellular colocalization of IAP in duodenum, depicting the percentage of IAP colocalization (yellow). Graphs are representative of 10 fields of view (n = 4 per genotype). Scale bars, 10 μm. (A to I) St3gal6-deficient mice and WT littersmates at 8 to 10 weeks of age, uninfected. (A to D) n = 8 per condition. (E to G) n = 6 per condition. Error bars represent means ± SEM. ***P < 0.001, **P < 0.01, and *P < 0.05; Student’s t test.
cells in the onset and development of disease (29, 30). We investigated whether LPS was itself sufficient for Neu induction, IAP deficiency, and concurrent elevations of inflammatory cytokines. Dose-response analyses using commercially obtained LPS were undertaken to determine minimal dosage and timing for further study (fig. S7, A and B). We found that gastric intubation of LPS induced Neu activity, NEU3 abundance, and Neu3 RNA levels with reductions in AP activity and IAP abundance, all of which were dependent on TLR4 function (Fig. 6, A to F). The induction of Neu activity by LPS resulted in TLR4-dependent reductions of sialic acid linkages with galactose exposure on isolated IAP and among apical cell surface glycoproteins of the small intestinal epithelium (fig. S7, C and D). LPS increased the internalization and colocalization of IAP with endocytic markers coincident with reduced IAP expression at the cell surface (fig. S7E). Normal IAP levels were retained after LPS administration in TLR4 deficiency with relatively low abundance of LPS-phosphate (Fig. 6G). The induction of LPS-induced inflammatory cytokines was also blocked by TLR4 deficiency with the retention of epithelial barrier function (Fig. 6, H and I). Thus, LPS/TLR4 signaling resulting from recurrent low-titer ST infection is linked to the induction of host NEU3 and increased Neu activity, resulting in IAP desialylation and its subsequent
Fig. 5. Host Neu is induced by TLR4 during recurrent ST infection. WT and Tlr4-deficient mice were analyzed after recurrent ST infections (arrows). (A) Neu activity (n = 30 per condition). (B) NEU1 to NEU4 protein abundance in the small intestine. (C) Neu3 mRNA expression in small intestine. (D) In situ localization of NEU3 and IAP in duodenum. Images are representative of 10 fields of view (n = 4 per condition). Scale bars, 50 μm. (E) AP activity (n = 24 per condition). (F) IAP protein abundance. (G) LPS abundance and phosphate released from LPS of intestinal content. (H) Inflammatory cytokine RNA abundance (n = 16 per condition). (I) Intestinal epithelial barrier function. (B, C, F, G, and I) n = 6 per condition. (B, C, F, G, and I) Animals were 20 weeks of age before the fourth infection. Error bars represent means ± SEM. ***P < 0.001, **P < 0.01, and *P < 0.05; one-way ANOVA with Tukey’s multiple comparisons test (A to I).

Fig. 6. Host Neu is induced by TLR4 and LPS. (A) Neu activity in mice at 8 weeks of age before repeated LPS administrations (arrows). (B and C) Neu protein abundance and Neu3 RNA expression in small intestine. (D) In situ localization of NEU3 and IAP in duodenum sections, representative of 10 fields of view (n = 4 per condition). Scale bars, 50 μm. (E) AP activity before repeated LPS administrations (arrows). (F) IAP protein abundance. (G to I) Phosphate released from LPS of intestinal content, inflammatory cytokine RNA abundance, and intestinal epithelial barrier function. (A and E) n = 24 per condition. (B, C, F, G, and I) n = 6 per condition. (H) n = 16 per condition. (B, C, F, G, and I) Mice on day 6 after LPS administration. Error bars represent means ± SEM. ***P < 0.001, **P < 0.01, and *P < 0.05; one-way ANOVA with Tukey’s multiple comparisons test (A to I).
Fig. 7. Effects of the Neu inhibitor zanamivir on intestinal inflammation. WT mice were analyzed at indicated ages before ST reinfection (arrows) in the absence or presence of zanamivir (Zana) (0.5 mg/ml) provided in drinking water immediately after first infection. (A) Neu activity. (B) Neu1 to Neu4 protein abundance in small intestine. (C) AP activity. (D) IAP protein abundance. (E) LPS abundance and phosphate released from LPS of intestinal content. (F) Lectin blotting of IAP protein from small intestine. (G) In situ localization of IAP in duodenum, representative of 10 fields of view (n = 4 per condition). Scale bars, 20 μm. (H) Body weight (n = 10 per condition), colon length (n = 8 per condition), diarrhea (n = 10 per condition) and fecal blood (n = 10 per condition) at 32 weeks of age, and intestinal epithelial barrier function (n = 8 per condition) at 20 weeks of age. (I) Inflammatory cytokine RNA abundance. (J) H&E-stained colon sections at 32 weeks of age. Graphs are representative of 10 fields of view (n = 4 mice per condition). Scale bars, 100 μm. (D to G) Mice at 20 weeks of age. (A and C) n = 32 per condition. (B and I) n = 30 per condition. (D to F) n = 6 per condition. Error bars represent means ± SEM. ***P < 0.001, **P < 0.01, and *P < 0.05; one-way ANOVA with Tukey’s multiple comparisons test (A to J).
Nevertheless, subsequent recurrent infections poisoned, the host rapidly cleared the pathogen. Infections of the Gram-negative pathogen ST.

Discussion

An increasingly severe colitis developed from recurrent low-titer nonlethal transient gastric infections of the Gram-negative pathogen ST. In this mouse model of repeated human food poisoning, the host rapidly cleared the pathogen. Nevertheless, subsequent recurrent infections progressively disabled a mechanism in the host that normally protects against spontaneous intestinal inflammation. This anti-inflammatory mechanism operates primarily in the colon but is dependent on IAP production and release from duodenal enterocytes of the small intestine. ST infection targeted this protective mechanism by activating host TLR4 function in the duodenum, inducing host Neu activity with elevated Neu3 expression at the luminal surface of the enterocyte. Neu induction accelerated the rate of nascent IAP aging by desialylation on the enterocyte cell surface, reducing IAP half-life, inducing IAP internalization and degradation, and resulting in a downstream IAP deficiency in the colon. IAP deficiency was linked to the deficient dephosphorylation of LPS molecules produced by commensal microbiota. This TLR4-dependent disease manifested primarily in the colon with IBD-like symptoms closer to UC than Crohn’s disease, and was linked to increases in the proinflammatory TLR4 ligand LPS-phosphate. Similarly, the genetic disruption of ST3GAl6-dependent sialic acid linkage formation during IAP synthesis caused IAP deficiency, resulting in a spontaneous colitis, which increased in severity with age and was exacerbated by recurrent ST infections. In both cases, diminished glycoprotein sialylation among enterocytes resulted in reduced IAP half-life, leading to IAP deficiency with markedly elevated LPS-phosphate abundance in the colon. Consistent with a TLR4-dependent mechanism, LPS administration alone recapitulated Neu3 induction and IAP deficiency, bypassing the requirement for IAP deficiency to increase colonic LPS-phosphate levels in provoking inflammation.

Intestinal inflammation failed to resolve after the discontinuation of periodic recurrent infections and persisted for months afterward as a lasting outcome. The degree of this persistence may be determined in part by initial infection titers and the time between recurrent infections and may result from multiple mechanisms. One possibility is the generation of epigenetic modifications to inflammatory gene promoters regulated by TLR4 function that result in the persistence of inflammatory cytokine expression, and may explain observations of the slow resolution of inflammatory processes (36, 37). The enterocyte Neu3 allele is perhaps regulated in this way because its expression remained induced long after periodic ST infections were discontinued. It is also possible that the escalating inflammation resulting from increased recruitment and activation of innate and adaptive immune cells reaches a point wherein the degree of immunological activation and damage to the epithelium is not easily reversed or attenuated. Disease persistence was also coincident with microbiota alterations that we found emerged concurrently with signs of disease and endured after the discontinuation of recurrent infections. In this regard, acute enteric infections with Yersinia can trigger gut microbiota dysbiosis and chronic inflammation after pathogen clearance in Tlr1-deficient mice (38). In addition, the microbiota alterations that we observed predominantly involved Enterobacteriaceae, which are frequently imbalanced in studies of intestinal inflammatory disease (21, 38).

Neu enzymes function in a variety of processes, including pathogen virulence, glycocalyx modulation, and biological signaling (39–41) and may differ among isoforms and origins, such as indicated from cecal sources in contributing to DSS-induced colitis (34). Mammalian Neu3 includes presumptive binding sites for transcriptional factors STAT3, RREB1, MYOD, Ik2, PAX2, Amila, HoxA9, and MEIS1 and may use alternate promoters controlled by Sp1/Sp3 transcription factors (42). Indeed, STAT3 and Sp1 transcription factors are...
activated by LPS (43, 44). The transcriptional activation of Neu3 may further underlie Neu3 induction in cancers of colon, renal, and prostate tissues, whereas mice lacking Neu3 exhibited fewer colitis-associated colonic tumors (45–47).

Our studies of an ST isolate lacking an annotated and demonstrable Neu gene have further linked Neu3 RNA induction with the elevation of host Neu activity, supporting the view that host Neu3 is involved in the desialylation of the IAP glycoprotein, which results in IAP internalization and deficiency. However, it remains possible that the Neu activity that we have measured comes from an unannotated Neu enzyme or the endogenous microbiota, neither of which can be resolved until Neu3-deficient mice are further studied. Although Neu3 activity is primarily active toward gangliosides, studies have shown significant but lesser activity toward glycoprotein substrates (46). It is also possible that Neu3 may act indirectly via the desialylation of its canonical ganglioside substrates. Nevertheless, Neu3 has been reported to desialylate the epidermal growth factor receptor glycoprotein (49). Moreover, data similar to our present findings have further implicated Neu3 in the desialylation of circulating glycoproteins in the blood linked to a mechanism that determines the various half-lives of plasma proteins (24).

The regulation of enterocyte IAP trafficking by sialylation extends this recently discovered mechanism of secreted protein aging and turnover to include the determination of protein half-lives at the cell surface. Although ST infection resulted in the desialylation and internalization of multiple enterocyte glycoproteins expressed at the cell surface, the disease phenotype was largely due to IAP deficiency. This represents an example of a specific glycan linkage that is commonly found on secreted and cell surface proteins having a biological purpose more restrictively associated with one or a few such glycoproteins (30–32). This can be explained, in part, by the presence of multiple sialyltransferases operating in the intestinal tract, which are responsible for the sialylation of different subsets of bioactive glycoproteins and which function in different biological processes. For example, the present findings involving ST3Gal4 deficiency should be compared with other studies of ST3Gal4 deficiency (34). Additional factors that may influence IAP expression and disease onset include mutations of glycan acceptor sites of IAP and transcriptional or mutagenic modifications to relevant glycosyltransferase and glycosidase genes, each of which can contribute to glycoprotein function (53). The control of IAP half-life via the increased internalization and degradation of desialylated IAP implies the possible presence of a sialic acid–binding lectin analogous to the mammalian siglecs of leukocytes (54). Such lectins may normally bind nascent sialylated IAP on the enterocyte surface to inhibit premature IAP endocytosis, enabling the subsequent release of IAP into the lumen. Alternatively, the exposure of underlying galactose may unmask cryptic ligands for galactose-binding lectins, such as the galectins, which modulate glycoprotein endocytosis (50, 55).

Diminished AP activity has been described in patients with colitis and celiac disease, and oral AP supplementation is under investigation for the treatment of inflammatory diseases, including the IBDs (56–59). Moreover, Neu3 protein abundance and activity is reportedly increased in human IBD patients (60). In animal studies, IAP deficiency contributes to colitis and allows increased bacterial transit from the intestinal lumen to the mesenteric lymph nodes (61, 35, 56, 62). We have found that IAP is highly regulated and that ST infection disables IAP function in host protection while progressively eroding microbial barriers by successive rounds of what might otherwise be considered unproductive infections. Environmental Gram-negative pathogens that access and infect the small intestine may have a similar strategy toward misappropriating host TLR4 function to diminish IAP activity and increase colonic AP-phosphate levels, provoking intestinal inflammation. This further emphasizes the dual nature of host TLR4 function, which may be either advantageous or disadvantageous perhaps depending on the context of exposure and the severity of infection. Although the link that we identified between host TLR4 function and Neu3 induction appears to favor the pathogen, advantageous features of TLR4 signaling may become evident with high Salmonella titers that engender extended pathogen colonization of the intestinal tract. Nevertheless, IAP augmentation and Neu inhibition represent candidate therapies for preventing the onset and progression of an escalating colitis that originates from recurrent low-titer Salmonella infections, as may occur in common cases of human food poisoning.

Materials and methods

Laboratory animals

Inbred C57BL/6J mice were used (Jackson Laboratory). ST3Gal4-deficient mice (89) were backcrossed six or more generations into the C57BL/6J background prior to study. TLR4+/− mice (B6Cg-Tlr4tm1Zck/J) were purchased from the Jackson Laboratory. Littermates bearing normal alleles were used as controls. All mice analyzed were provided sterile pellet food and water ad libitum.

Histology

Mouse tissues were fixed in 10% buffered formalin (Sigma-Aldrich), transferred to 30% sucrose/PBS, and embedded in TissueTek OCT compound (Sakura Finetek). Three-micron frozen serial sections were stained with hematoxylin and eosin (H & E, Sigma-Aldrich) or incubated with 1 μg/ml of antibodies to one or more molecules including CD3e (M-20, Santa Cruz Biotechnology), Gr-1 (M-66, Santa Cruz Biotechnology), F4/80 (M-300, Santa Cruz Biotechnology), TNF (M-18, Santa Cruz Biotechnology), EEA1 (E-15, Santa Cruz Biotechnology), LAMP2 (C-20, Santa Cruz Biotechnology), γ-adaptin (I-19, Santa Cruz Biotechnology), Calnuc (V-18, Santa Cruz Biotechnology), protein disulfide isomerase PDI (G-20, Santa Cruz Biotechnology), Neu3 (M-50, Santa Cruz Biotechnology), sucrase-isomaltase (A-17, Santa Cruz Biotechnology), dipetidyl peptidase-4 (H-270, Santa Cruz Biotechnology), lactase (T-14, Santa Cruz Biotechnology), or 1:1000 dilution of IAP specific antisemur kindly provided by Dr. Jose Millan (64), or 5 μg/ml of biotinylated lectins including Erythrina cristagalli (ECA), Ricinus Communis Agglutinin-1 (RCA), Peanut Agglutinin (PNA), Maackia amurensis-II (MAL-II), or Sambucus nigra (SNA) (Vector Laboratories). CD3e, TNFα, sucrase-isomaltase, and lactase were visualized with 0.4 μg/ml of FITC-conjugated rabbit anti-goat IgG (Santa Cruz Biotechnology); Gr-1, F4/80, IAP, Neu3, and dipetidyl peptidase-4 were visualized with FITC-conjugated goat 0.4 μg/ml of anti-rabbit IgG (Santa Cruz Biotechnology); EEA1, LAMP2, γ-adaptin, Calnuc, and PDI were visualized with 0.4 μg/ml of Texas Red-conjugated rabbit anti-goat IgG (Santa Cruz Biotechnology) and biotinylated lectins were visualized with 1 μg/ml of FITC-conjugated streptavidin (Vector Laboratories). These primary antibody or lectin incubations were performed at 4°C overnight and secondary antibody or streptavidin incubations were performed at room temperature for 1 h. All microscopic images were obtained with a Zeiss AxiosScope Z1 microscope equipped with an AxioCam HRc camera, and Lumen Dynamics X-Cite XLED1 illumination. All microscopic images were obtained and histopathological studies including quantification of cells, cell boundaries, and marker expression were performed with TissueFAXS (Version 3.5), TissueQuest (Version 4.0), and HistoQuest software (Version 4.0) (TissueGnostics USA Ltd.). Co-localization was further quantified by MetaMorph software (Version 7.0) (Universal Imaging Corporation) (65). Yellow signals in merged images of IAP (green) and intracellular compartments (red) indicate colocalization, and the threshold intensity value of 12 (intensity range, 0–255) and size of 0.026 μm² were used as the cut-off.

mRNA preparation and quantification by real-time PCR

Total RNA was isolated from tissues using Trizol (Invitrogen) and subjected to reverse transcription (RT) using SuperScript III (Invitrogen). Quantitative real-time PCR was performed using Brilliant SYBR Green Reagents with the Mx3000P
**Immunoprecipitation, immunoblotting, and lectin blotting**

Tissue samples were homogenized in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) supplemented with complete protease inhibitor cocktail per instructions (Roche) and incubated overnight at 4°C on a rotating wheel with 1:100 dilution of IAP-specific antiserum (Yang et al., Cell 68, 337–348, 1992) or 1:100 dilution of antibodies to sucrase-isomaltase, dipeptidyl peptidase 4, or lactase (EY Laboratories). Signals detected by chemiluminescence were visualized with Coomassie brilliant blue G250 staining (Bio-Rad). Parallel protein samples were weighed, diluted ten-fold weight to volume before incubation with serial dilutions of mouse tissue extracts that were biotinylated using 1 mg/ml of N-hydroxysuccinimide-biotin (Pierce). Antibodies were detected after the following: 1) 1000 dilution of HRP-streptavidin (BD Biosciences) and 3,3′,5,5′-tetramethylbenzidine (TMB, Sigma-Aldrich). Lectin binding was determined in parallel by addition of HRP-conjugated ECA (0.5 μg/ml), RCA (0.1 μg/ml), PNA (1 μg/ml), MAL-II (0.2 μg/ml), or SNA (0.1 μg/ml) (EY Laboratories), followed by TMB, and changes in glycogen linkages were detected by comparing lectin binding among identical amounts of biotinylated IAP, sucrose-isomaltilase, dipeptidyl peptidase 4, or lactase antigen calculated as described (66). Alkaline phosphatase activity was measured using the p-nitrophenyl phosphate substrate (pNPP, Sigma-Aldrich) as previously described (61).

**LPS phosphorylation**

To determine LPS content in the intestinal contents, LPS was isolated as previously described (67) by the hot phenol–water method with minor modifications. Briefly, the intestinal contents were weighed, diluted ten-fold weight to volume in Tris-buffered saline (TBS), then incubated with DMEM depleted of methionine (Gibco) with 10% fetal calf serum for 2 h at 37°C. Pulse labeling was performed with 400 μCi/ml [35S]methionine for 10 min at 37°C, and cells were then washed twice in ice-cold HBSS. Cells were lysed or returned to new media of above culture conditions containing 2 mM methionine for 15, 30, 45, 60, or 90 min. Media used in chase samples were harvested at each time point. Cells used in chase samples were washed twice with ice-cold PBS and incubated with 1 mg/ml of sulfo-NHS-LC-biotin ( Pierce Chemical) at 4°C for 30 min. Biotinylation was stopped by three washes with 15 mM glycine in ice-cold PBS. Cells were homogenized in RIPA buffer, and biotinylated proteins were purified using immobilized monomeric avidin gel ( Pierce). Eluates isolated in the presence of D-biotin ( Pierce) or media samples for chase were incubated with IAP-specific antiserum. Immunoprecipitates were subjected to SDS-PAGE, and gels were fixed before drying and autoradiography at –70°C for 3–7 days. For cell-surface half-life analysis, primary enterocytes were washed twice with ice-cold PBS and biotinylated with sulfo-NHS-LC-biotin as described above. Cells were then cultured at the indicated times, and then homogenized in RIPA buffer, followed by immunoprecipitation using the IAP-specific antiserum. IAP immunoprecipitates were subjected to SDS-PAGE, transferred to nitrocellulose membranes, and incubated with 2% BSA in Tris-buffered saline (TBS). They were then analyzed by immunoblotting using either 1 μg/ml of antibodies to Neut (H-270, Santa Cruz Biotechnology), Neut2 (M-13, Santa Cruz Biotechnology), Neut3 (M-50, Santa Cruz Biotechnology), Neut4 (N-14, Santa Cruz Biotechnology), sucrase-isomaltilase (A-17, Santa Cruz Biotechnology), dipeptidyl peptidase 4 (H-270, Santa Cruz Biotechnology), or lactase (T-14, Santa Cruz Biotechnology), or 1:1000 dilution of IAP antiserum, and blocked with BSA before incubation with serial dilutions of mouse tissue extracts that were biotinylated using 1 mg/ml of N-hydroxysuccinimide-biotin (Pierce). Antibodies were detected after the following: 1) 1000 dilution of HRP-streptavidin (BD Biosciences) and 3,3′,5,5′-tetramethylbenzidine (TMB, Sigma-Aldrich). Lectin binding was determined in parallel by addition of HRP-conjugated ECA (0.5 μg/ml), RCA (0.1 μg/ml), PNA (1 μg/ml), MAL-II (0.2 μg/ml), or SNA (0.1 μg/ml) (EY Laboratories), followed by TMB, and changes in glycogen linkages were detected by comparing lectin binding among identical amounts of biotinylated IAP, sucrose-isomaltilase, dipeptidyl peptidase 4, or lactase antigen calculated as described (66). Alkaline phosphatase activity was measured using the p-nitrophenyl phosphate substrate (pNPP, Sigma-Aldrich) as previously described (61).
using immobilized monomeric avdin gel and incubated on ELISA plates (Nunc) coated with IAP anti serum. Lectin binding was determined in parallel by the addition of HRP-conjugated ECA, RCA, PNA, MAL-II or SNA, followed by TMB, and changes in gray scale linkages were detected by comparing lectin binding among identical amounts of biotinylated IAP antiserum as calculated as described (86).

### Calf intestinal alkaline phosphatase (cIAP) treatment and LPS administration

cIAP (20 U/ml; Lee Biosolutions) was administered via oral gavage (600 mg/kg). In vivo intestinal barrier function was calculated as described (9).

### In vivo intestinal barrier function

Dextran-FITC (Sigma) was administered via oral gavage on day 14. For other experiments in acute colitis, 8-week-old mice were orally administered LPS (0.5 mg/ml; Sigma-Aldrich) was provided in the drinking water immediately following the administration of DSS and continued for the duration of the study as indicated. Body weight, stool consistence, and the presence of occult blood were determined as follows: 0, no blood as tested (Beckman Coulter); 1, positive hemoccult; 2, blood traces in stool visible; 3, gross rectal bleeding as previously described (77).

### Statistical analysis

All data were analyzed as mean ± SEM unless otherwise indicated. Student’s unpaired t test, one-way ANOVA with Tukey’s multiple comparisons test, log-rank test, or Kruskal-Wallis test with Dunn’s multiple comparisons test was used. GraphPad Prism software (Version 7.0) was used to determine statistical significance among multiple studies. P values of less than 0.05 were considered significant. Statistical significance was denoted by *P < 0.05, **P < 0.01, or ***P < 0.001.

### References and Notes


### Comparitive studies of intestinal microbiota

DNA from intestinal content was isolated with a QIAamp DNA Mini Kit according to the manufacturer’s instructions (Qiagen). Commercial microbiota was analyzed by quantitative real-time PCR using Brilliant SYBR Green Reagents with the Mx3000P QPCR System (Stratagene) with specific primers for bacteria (70) (Total-F-5′-GTCGAC-CGCGCGCGGTTA-3′, Total-R-5′-GACTACAGG-GTATCCTAAT-3′, Clostridiaceae-F-5′-TTAACAACAA-TAATGWAACCTACCCG-3′, Clostridiaceae-R-5′-ACCTTTCGCCTTGTTGCAAC-3′, Lactobacillaceae-F-5′-AGCAGTGGAAGACTTCCTC-3′, Lactobacillaceae-R-5′-GGCGGCTGTGTTCCTTACATA-3′, Bacteroidaceae-F-5′-CAATGTGGGGGACCTTC-3′, Bacteroidaceae-F-5′-AAGAGTGACTACGACCTTC-3′, Enterobacteriaceae-F-5′-CATGAGGTCAGGCGCAGAAGC-3′, Enterobacteriaceae-F-5′-CTTACGGACTACAGCCTTG-3′). Relative bacterial DNA levels were related to the amount of total isolated DNA from intestinal content.

### DSS-induced acute and chronic colitis

For survival studies in acute colitis, 12-week-old mice were administered drinking water containing 4% dextran sodium sulfate (DSS; molecular weight, 40,000 to 50,000; USB Corp.) ad libitum for 5 days and then returned to normal drinking water without DSS until the end of the experiment (day 14). For other experiments in acute colitis, mice were administered 2% DSS solution in drinking water for 5 days. DSS-induced chronic colitis was induced as previously described (35). Briefly, 12-week-old mice were administered 2% DSS solution in drinking water with four cycles of DSS given ad libitum for 7 days followed by a 7-day water phase. Intraperitoneal injection of cyclophosphamide (0.5 mg DMSO; Sigma-Aldrich) was provided in the drinking water immediately following the administration of DSS and continued for the duration of study as indicated. Body weight, stool consistence, and the presence of occult blood were determined as follows: 0, no blood as tested (Beckman Coulter); 1, positive hemoccult; 2, blood traces in stool visible; 3, gross rectal bleeding as previously described (77).

### Statistical analysis

All data were analyzed as mean ± SEM unless otherwise indicated. Student’s unpaired t test, one-way ANOVA with Tukey’s multiple comparisons test, log-rank test, or Kruskal-Wallis test with Dunn’s multiple comparisons test was used. GraphPad Prism software (Version 7.0) was used to determine statistical significance among multiple studies. P values of less than 0.05 were considered significant. Statistical significance was denoted by *P < 0.05, **P < 0.01, or ***P < 0.001.


Zika virus causes testicular atrophy

Ryuta Uraki, Jesse Hwang, Kellie Ann Jurado, Sarah Householder, Laura J. Yockey, Andrew K. Hastings, Robert J. Homer, Akiko Iwasaki, Erol Fikrig

Zika virus (ZIKV) is an emerging mosquito-borne flavivirus that has recently been found to cause fetal infection and neonatal abnormalities, including microcephaly and neurological dysfunction. ZIKV persists in the semen months after the acute viremic phase in humans. To further understand the consequences of ZIKV persistence in males, we infected Ifnar1−/− mice via subcutaneous injection of a pathogenic but nonlethal ZIKV strain. ZIKV replication persists within the testes even after clearance from the blood, with interstitial, testosterone-producing Leydig cells supporting virus replication. We found high levels of viral RNA and antigen within the epididymal lumen, where sperm is stored, and within surrounding epithelial cells. Unexpectedly, at 21 days post-infection, the testes of the ZIKV-infected mice were significantly smaller compared to those of mock-infected mice, indicating progressive testicular atrophy. ZIKV infection caused a reduction in serum testosterone, suggesting that male fertility can be affected. Our findings have important implications for nonvector-borne vertical transmission, as well as long-term potential reproductive deficiencies, in ZIKV-infected males.

INTRODUCTION

Zika virus (ZIKV), a positive-stranded RNA virus belonging to the Flavivirus family, has recently been associated with several unexpected viral characteristics (1, 2). Although the main mode of ZIKV transmission is thought to be mosquito-mediated, as in other flaviviruses (3, 4), ZIKV has additionally been found to use both maternal-fetal (5) and sexual transmission (6) as a means of virus spread. Upon systemic infection, virus replication can be detected in most organs; it has also been found within several immune-privileged sites, including the brain (7–18), placenta (19, 20), eyes (21), ovaries (22), and testes (23, 24). In humans, persistent shedding of infectious virus has been found in vaginal secretions (25) and semen (26–33) at times well past the acute viremic and symptomatic stage of virus infection.

RESULTS

To understand the pathology associated with persistence of ZIKV shedding within the testes, we used a murine infection model that leads to high levels of systemic virus replication but does not ultimately result in death (Ifnar1−/− mice challenged with ZIKV25450). ZIKV25450-infected Ifnar1−/− mice demonstrated relatively mild body weight loss (Fig. 1A) and developed peak viremia at 5 days post-infection (dpi) (Fig. 1B). We were surprised to find that the ZIKV25450 strain, which has not yet been published in mouse models, did not result in the lethal pathology of previous strains (22, 24). This allowed us to monitor the effects of ZIKV infection of a pandemic strain at later time points of infection. Consistent with these findings, mice sacrificed at 5 dpi were found to have high levels of viral RNA (Fig. 1C) and infectious virus (Fig. 1D) within the brain, testes, and blood. At 9 dpi, ZIKV was essentially cleared from the bloodstream and was undetectable at 21 dpi (Fig. 1B).

To decipher the cellular tropism within the testes, we performed immunohistochemistry (IHC) using a mouse monoclonal antibody recognizing ZIKV NS1 antigen on testes and epididymis of ZIKV25450-infected Ifnar1−/− mice at 5 dpi. As shown in Fig. 2A, viral antigens were mainly detected within the interstitial cells of the testes. These regions are mainly composed of Leydig cells, a testicular cell type that supports sperm production through the generation of testosterone (34). We did not detect ZIKV within the seminiferous tubules in ZIKV25450-infected Ifnar1−/− mice at 5 dpi (Fig. 2A, a and b). Because the epididymis is a highly convoluted tube that stores and carries sperm (35), we also examined epididymal tissue of ZIKV25450-infected Ifnar1−/− mice for ZIKV antigen and found an overwhelming amount of antigen associated with the sperm mass within the epididymal duct lumen (Fig. 2A, d). In addition, we also observed several epithelial cells lining the lumen that were positive for viral antigen (red arrows in Fig. 2A, d). In further support of ZIKV infection of testes, RNA analysis of several cytokines indicated that an innate immune response was elicited at 5 dpi (Fig. 2B).

Because IHC analysis suggested that Leydig cells, which are responsible for the supply of testosterone in testes, are targets for ZIKV infection, we next determined the expression level of several genes related to testosterone synthesis (36) in ZIKV25450-infected Ifnar1−/− mice tests and found a consistent reduction among all genes tested as compared in testes of ZIKV25450-infected Ifnar1−/− mice (Fig. 2C). To confirm the susceptibility of Leydig cells to ZIKV, we infected isolated WT or Ifnar1−/− Leydig cells with ZIKV25450 in vitro. As shown in Fig. 2D, Leydig cells were able to productively support ZIKV replication, indicating that Leydig cells could serve as a target and a reservoir cell type for ZIKV within the testes.

Strikingly and rather unexpectedly, at 21 dpi, the testes of ZIKV25450-infected Ifnar1−/− mice were significantly reduced in size as compared to those of uninfected mice (Fig. 3A), as determined by both weight (Fig. 3B) and length (Fig. 3C). These findings suggest that persistent ZIKV infection may lead to hypofertility. Because IHC, in vitro infection, and the decreased expression of genes related to testosterone synthesis indicated Leydig cells as putative targets for ZIKV infection within the testes, we next set to determine whether atrophy could occur as the result of decreased testosterone levels in ZIKV-infected mice. We examined levels of testosterone within serum from ZIKV25450-infected Ifnar1−/− mice at 5 and 21 dpi as compared with mock-infected Ifnar1−/− mice and found a significant decrease in testosterone at 5 dpi with a consistent reduction at 21 dpi (Fig. 3D). Notably, the testosterone levels

1Section of Infectious Diseases, Department of Internal Medicine, Yale University School of Medicine, New Haven, CT 06520, USA. 2Department of Immunobiology, Yale University School of Medicine, New Haven, CT 06520, USA. 3Department of Pathology, Yale University School of Medicine, New Haven, CT 06520, USA. 4U.S. Department of Veterans Affairs Connecticut Healthcare System Pathology and Laboratory Medicine Service, West Haven, CT 06516, USA. 5Howard Hughes Medical Institute, Chevy Chase, MD 20815, USA.

*Corresponding author. Email: erol.fikrig@yale.edu

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at 5 dpi (six of seven mice) and at 21 dpi (two of seven mice) were lower than the assay detection limit. These findings support other data documenting ZIKV infection of Leydig cells (Fig. 2, A and D) and suggest a potential mechanism for ZIKV-induced testicular atrophy. Viral RNA was noticeably higher in the epididymis compared to testes (Fig. 3E), consistent with the copious amount of viral antigen detected by IHC within the epididymal lumen (Fig. 2A). It remains conceivable that multiple cell types within the reproductive tract produce virus, and the cell-free virus becomes stored and concentrated in the epididymis before physical expulsion upon sexual activity.

DISCUSSION

Because humans with a fully functioning immune system also demonstrate persistent ZIKV infection of the testes, these findings have major implications for the fertility of men who have been exposed to ZIKV. It will be important to monitor the fertility of men who have been infected with ZIKV to better understand the impact in humans. We hypothesize that active infection of Leydig cells within the interstitial regions of the testes could cause decreased testosterone production, which ultimately results in testicular atrophy. There is precedence for deleterious effects on the production of testosterone and spermatogenesis caused by infection with mumps virus, which also targets Leydig cells as a site of replication within the testes (37–39). Alternatively, or in conjunction, because inflammation is known to also lead to testicular atrophy, the phenotype may be caused indirectly, or enhanced by, the immune response to detection of ZIKV. Because all experiments were carried out in Ifnar1−/− mice, our data rule out the possibility of type I interferon signaling in testicular atrophy caused by ZIKV.

RNA analysis of testes samples of ZIKVEXP-infected Ifnar1−/− mice found ZIKV to be present within the testes and epididymis at 5 dpi in all mice and at 21 dpi in most mice. Because there is no detectable ZIKV RNA within the blood of ZIKVEXP-infected Ifnar1−/− mice at 21 dpi (Fig. 1B), this observation recapitulates the human presentation of persistent ZIKV replication within the testes. For ZIKVEXP-infected Ifnar1−/− mice sacrificed at 35 dpi, when the epididymis was measured to be ZIKV-negative, some of the testes were still reduced in size (15 to 45% shorter in length) as compared to testes of mock-infected mice. Determination of the possibility of recovery from testicular atrophy over greater time periods is warranted. Recently, two other groups also reported that ZIKV can cause long-term testicular damage; however, the infected cell types found in these studies (spermatogonia, primary spermatocytes, Sertoli cells, and/or peritubular myoid cells) differ from those reported here, indicating that further studies are needed.
studies are required to examine the temporal and molecular details of ZIKV tropism within the reproductive tract (40, 41). In addition, further work is necessary to assess the relative contributions of the innate immune response and/or testosterone reduction in ZIKV-induced testicular atrophy. Regardless, the hypofertility implications of testicular atrophy call for an urgent global need to develop vaccines and antiviral therapeutics.

MATERIALS AND METHODS

Ethics statement

This study was performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by the Institutional Animal Care and Use Committee at the Yale University School of Medicine.

Virus

An American-derived ZIKV (MEX2-81 strain, referred to as ZIKV<sup>ME</sup>) isolated in 2016 was used in this study (19, 42). C6/36 cells were infected with ZIKV<sup>ME</sup> and maintained up to 10 days. Cell-free supernatants were collected and stored at −80°C.
Plaque assays
Vero cells were seeded in 12-well plates 24 hours before infection. Serial-diluted ZIKV was incubated for 1 hour at 37°C in 5% CO2. Then, cells were overlaid with 2% agarose and 2× medium. At 3 to 4 dpi, cells were fixed by 10% formalin and stained with 0.005% amido black, and PFU were counted.

Mouse experiments
Four- to six-week-old Ifnar1−/− (C57BL/6 background) and WT C57BL/6 mice were analyzed in this study. Mice were bred in a specific pathogen-free facility at Yale University or purchased from The Jackson Laboratory (WT C57BL/6). Mice were inoculated with ZIKV via subcutaneous injection (footpad; a volume of 50 μl) with 105 PFU of ZIKV. Survivals and weights were monitored every day up to 15 dpi. Mice exhibiting a weight loss of >20% of initial body weight or neurological disease were euthanized. To examine the viremia, we collected blood samples at 1, 3, 5, 7, 9, and 21 dpi and performed real-time PCR. To assess virus growth or host responses in organs, we euthanized three mice at 5 or 21 dpi, and their organs were collected, homogenized with DMEM with 10% FCS or TRIzol, and titrated using plaque assays or real-time PCR.

RNA extraction, real-time PCR, and PCR
The homogenized organ and blood samples from ZIKV-infected mice were transferred to a fresh 1.5-ml tube to which chloroform was added. The tubes were vortexed well and centrifuged for 10 min at 14,000 rpm at 4°C. The aqueous layers were mixed with 100% ethanol and then subjected to RNA extraction using RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. Extracted RNA was reverse-transcribed using iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer’s protocol. Gene expressions of samples were queried using iQ SYBR Green Supermix. ZIKV RNA or target gene mRNA levels were normalized to mouse β-actin RNA levels according to the 2−ΔΔCt calculations. The qRT-PCR primer sequences are available upon request.

Detection of testosterone in serum
The concentration of testosterone in serum of ZIKV-infected Ifnar1−/− mice was performed by using the Mouse Testosterone ELISA Kit (Abcam) according to the manufacturer’s instructions.

Isolation of Leydig cells
Leydig cells were isolated from WT and Ifnar1−/− mice according to previous studies (36, 43). In brief, the testes of two mice were decapsulated and incubated with collagenase (0.5 mg/ml) in F12/DMEM (Life Technologies) supplemented with 10% FCS at room temperature for 15 min with gentle inverting. The suspensions were filtered through 70-μm mesh to separate the interstitial cells from seminiferous tubules. The interstitial cells were cultured in F12/DMEM supplemented with FCS. After 24 hours, Leydig cells were detached by 0.125% trypsin treatment for 5 min and resuspended to dish.

Fig. 3. Testicular abnormalities after ZIKV infection. The testes of Ifnar1−/− mice infected with 105 PFU of ZIKV by subcutaneous route and of mock-infected mice were harvested at 5 and 21 dpi. (A) The pictures compare the testes of infected mice. Scale bar, 1 cm. (B and C) Weight and length of whole testes from infected animals were measured (n=6 testes comprising three mice; **P<0.01). Same color indicates the same mice. (D) The levels of testosterone in blood of infected mice were investigated by enzyme-linked immunosorbent assay (*P<0.05). The detection limit is 0.3 ng/ml. (E) The relative viral RNA levels in testes and epididymis at 5 and 21 dpi were examined by qRT-PCR. Data are normalized to mouse β-actin.
Histopathological analysis
The testes of mice were preserved in 4% paraformaldehyde/phosphate-buffered saline for pathological examination. The fixed samples were processed for paraffin embedding. Sections were processed for immunohistologic staining with an anti-ZIKV NS1 antibody.

Data analysis
Data analysis was performed using GraphPad Prism and Microsoft Excel. One-tailed Student’s *t* test was used to determine the significance of qRT-PCR values for host responses. One-way analysis of variance (ANOVA) was used to compare the weight and length of testes and testosterone levels.

REFERENCES AND NOTES


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Walls talk: Microbial biogeography of homes spanning urbanization

Jean F. Ruiz-Calderon,1,* Humberto Cavallin,2,* Se Jin Song,3* Atila Novoselac,4 Luis R. Pericchi,5 Jean N. Hernandez,1 Rafael Rios,6 Oralee H. Branch,7 Henrique Pereira,8 Luciana C. Paulino,9 Martin J. Blaser,10 Rob Knight,11 Maria G. Dominguez-Bello1,10†

Westernization has propelled changes in urbanization and architecture, altering our exposure to the outdoor environment from that experienced by human ancestors. These changes might affect the developmental and environmental exposures to bacteria, immune development, and human microbiome diversity. Contemporary urban humans spend most of their time indoors, and little is known about the microbes associated with different urban environments, with re-occurring indoor space isolation by walls. Microbes from house walls and floors segregate by location, and urban indoor walls contain human bacterial markers of space use. Urbanized spaces uniquely increase the content of human-associated microbes—which could increase transmission of potential pathogens—and decrease exposure to the environmental microbes with which humans have coevolved.

INTRODUCTION

Urbanization of traditional villages—the villages developing in more urban form, and historical villagers migrating to towns and cities—is occurring concurrently with a global convergence toward a more Westernized urban plan and life-style (1). This process occurs as human societies integrate into hunter-gatherers into first rural and then urban life-styles. Urbanization also involves more people spending most of their time indoors (2, 3).

A large proportion of the microbes found in the built environment are shed by humans (4–7) or animals (8), and with natural ventilation, microorganisms can also be transported from outdoors (5, 6, 9). Understanding the consequences of architectural changes on environmental exposures, including microbial exposures, is therefore important in improving home design and ultimately human health. Here, we determine the changes in architectural design and the resulting microbial communities of houses spanning a range of modernization within the Amazon River basin. We measured community demographics and architectural parameters, and characterized the microbial communities of 10 houses and their inhabitants from each of four locations: a traditional jungle village of Checherta (1A) that is 7 to 20 h−1 in the rural village, 4 to 17 h−1 in the town, and 0.8 to 15 h−1 in the city. The jungle village homes of Checherta are open huts made of wood and reeds, and are generally single open-plan spaces composed of two functional areas (Fig. 1, A and B, and fig. S2): a dormitory containing the two family members, and a fire area for cooking and socializing. Up to six core families, among extended family members, share a home. As urbanization increases, a progressive separation of the indoor environment from the outdoor occurs first, followed by internal division of home spaces and the use of a wider variety of building materials (table S4). In the rural village, a toilet appears as an external latrine, which in the town and city becomes a piped indoor bathroom. Town and city houses typically have additional spaces differentiated by functional purpose (living room, kitchen, and bathroom) and segregated by walls (Fig. 1B).

RESULTS

The jungle village of Checherta is a 21-house Achuar community of hunter-gatherers (tables S1 and S2, and fig. S1, A and B). Houses are organized around a central area, including a communal building. This community design is retained in the 25-house rural village of Puerto Almendras, with the homes surrounding a soccer field (fig. SIC and table S1). Iquitos has 0.4 million inhabitants and is the largest urban population in the world not accessible by roads (fig. SIC and tables S1 and S2). Manaus, the capital of Amazonas State in Brazil, is a contemporary Western city with 1.8 million inhabitants (fig. S1D and tables S1 and S2).

Although no significant environmental differences were found across the urbanization gradient, large architectural changes were observed (Fig. 1). No significant differences were found across the studied locations in outdoor temperature (mean variation, <2°C; table S3) or relative humidity, and all locations had high ventilation rates (air exchange rates of 25 to 100 h−1 in the jungle village, 7 to 20 h−1 in the rural village, 4 to 17 h−1 in the town, and 0.8 to 15 h−1 in the city). The jungle village homes of Checherta are open huts made of wood and reeds, and are generally single open-plan spaces composed of two functional areas (Fig. 1, A and B, and fig. S2): a dormitory containing one platform bed per family, and a fire area for cooking and socializing. Up to six core families, among extended family members, share a home. As urbanization increases, a progressive separation of the indoor environment from the outdoor occurs first, followed by internal division of home spaces and the use of a wider variety of building materials (table S4). In the rural village, a toilet appears as an external latrine, which in the town and city becomes a piped indoor bathroom. Town and city houses typically have additional spaces differentiated by functional purpose (living room, kitchen, and bathroom) and segregated by walls (Fig. 1B).

Houses in the most urbanized conditions are more variable in design, but in general, there is an urbanization-associated increase in the number of rooms per person (privacy index) (Fig. 1C, fig. S3, and table S4), house area, and its variance (P < 0.005; Fig. 1C and table S4). The average

*These authors contributed equally to this work.
†Corresponding author. E-mail: maria.dominguez-bello@nyumc.org

1Department of Biology, University of Puerto Rico, Rio Piedras Campus, San Juan 00931, Puerto Rico. 2School of Architecture, University of Puerto Rico, Rio Piedras Campus, San Juan 00931, Puerto Rico. 3Department of Ecology and Evolutionary Biology, University of Colorado at Boulder, Boulder, CO 80309, USA. 4Department of Civil, Architectural and Environmental Engineering, University of Texas at Austin, Austin, TX 78712, USA. 5Department of Mathematics, University of Puerto Rico, Rio Piedras Campus, San Juan 00931, Puerto Rico. 6Department of Environmental Sciences, University of Puerto Rico, Rio Piedras Campus, San Juan 00931, Puerto Rico. 7Department of Microbiology, New York University Langone Medical Center, New York, NY 10016, USA. 8Federal University of Amazonas, Manaus, AM 66077-000, Brazil. 9Center for Natural Sciences and Humanities, Federal University of ABC, Santo André, SP 09210, Brazil. 10Department of Medicine, New York University Langone Medical Center, New York, NY 10016, USA. 11Departments of Pediatrics and Computer Science & Engineering, University of California at San Diego, La Jolla, CA 92093, USA.

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Fig. 1. Architecture and space use covary with key structural features such as house partitioning, area, and occupant density across the four locations; differences in space use are reflected in the microbial communities of the walls, but not floors, which contribute to the microbial signatures of the homes. (A) Photos of the typical structures found across the four communities along this urbanization gradient (Checherta (jungle), Puerto Almendras (rural), Iquitos (town), and Manaus (city)). (B) Typical floor plans of houses in Checherta and Manaus (left and right, respectively). (C) Distribution of house area (left) and mean privacy index *(privacy index = number of rooms/number of people) according to occupant density (occupant density = number of people/square meters) (right) by location. (D) Classification probability of correct assignment to a sample’s true location using a random forest classifier. The probability of being able to predict a functional space using the microbial community of the walls increases with increased partitioning of spaces by use in the urban areas (for example, bathrooms and kitchens in separate walled-off spaces). Floor microbial communities, on the other hand, are not as discriminatory among rooms. (E) Classification probability of correct assignment of a given sample to the correct house.
Fig. 2. Microbial community structure in houses differs significantly across the urbanization gradient. Seven sites that were common to all houses (living room, bedroom, kitchen floors, beds, chair handles, countertops, and living room walls) were collapsed into one sample to obtain a total measure of diversity for each home. (A) Principal coordinates analysis (PCoA) of the seven collapsed samples for each home shows tight clustering of the samples by community (P < 0.01, analysis of similarities). Point size shows the α diversity level, measured as phylogenetic diversity (PD) (smallest, <150; largest, >250). (B) PCoA plot of unweighted UniFrac distances of wall and floor bacterial communities by village. Floor samples are clustered very tightly in the jungle community, but not wall samples. This indicates that floor microbial communities resemble more to each other than wall samples. This clustering of floor samples decreases with urbanization, and microbial communities of walls and floors merge in urban locations, meaning that urban locations have similar microbes on the walls and floors, whereas in rural locations, floors have very different microbial communities. (C) Top 20 feature taxa of high relative abundance (>0.1%) that allowed for correct prediction of a sample's source community; these include taxa commonly associated with humans (for example, Streptococcaceae, Lactobacillaceae, and Pseudomonadaceae) (shown in red hues) and taxa commonly associated with the environment (for example, Intrasporangiaceae and Rhodobacteraceae) (shown in blue hues). Taxa shown in the literature to be associated with both the environment and the human body are shown in green hues. (D) Distribution of the collective α diversity (PD) of each home, colored by the number of human inhabitants residing in the home. Numbers inside the points indicate the number of different material types that are represented by the seven samples, and the size corresponds to the total number of pets in the home (dog, cat, monkey, chicken, turtle, or parrot).
House occupancy (persons per square meter) decreases with urbanization ($P < 0.005$; Fig. 1C and table S4), which is consistent with higher area and smaller families.

Remarkably, classification of house functional spaces using microbes was possible (Fig. 1D and fig. S4), and the probability of correct assignment given the wall bacterial composition increased with urbanization (Fig. 1E). We tested for differences in the types and diversity of household bacteria across locations. Microbial richness ($\alpha$ diversity) did not change with urbanization (Fig. 2 and figs. S5 and S6), but bacterial composition was markedly different (Fig. 2, A and B, and figs. S7 and S8) with houses becoming more microbiologically distinct along the gradient. Bacterial community structure in samples from floor and walls converged with urbanization (Fig. 2B). At the jungle end of the gradient, floors were made of dirt and people walked barefoot, and walls were wood columns; at the city end, floors and walls were made of synthetic materials, and people walked with shoes (in all but one house). Moreover, wall microbes better differentiated the kitchen and bathroom functional spaces in urban than in rural houses (Fig. 1D and fig. S9). The 10 most important operational taxonomic units (OTUs) that help discriminate among rooms in Manaus comprise several taxa normally associated with the human oral cavity, including Streptococcus, Neisseria, Actinomyces, and Veillonella dispar, as well as taxa normally associated with the human gut such as Enterobacteriaceae.

Despite lower occupant density in urban houses, “humanization” of the houses occurred with increased urbanization (Fig. 2D), associated with home enclosure—isolation from the outdoor environment—especially in dwellings sealed for air conditioning. Human bacteria were enriched in the town and city houses, with Prevotella, Verrucomicrobia, and Serratia on the walls (figs. S7 and S10), and skin taxa on the floors, consistent with human shedding (7, 10–12) and with the isolation of homes from bacterial sources from outdoor environments. Environmental bacteria were proportionally higher in the jungle and rural village house floors and included soil bacteria [for example, Mesorhizobium and Luteimonas from water sources and Rickettsiella from arthropods (figs. S7 and S10)]. The environmental bacterium found in walls included Acidobacteriales, Bradyrhizobium, Dactylosporangium, Actinomycetospora, Actinoalloteichus, Saccharopolyspora, Pedomonobium, and Rickettsiella (figs. S7 and S10). As we move from the rural to the urban locations, there is a shift within Actinobacteria,
from *Brachybacterium* and *Brevibacterium* commonly found in the environment to *Corynebacterium*, common in human skin (Fig. 2B).

A Bayesian approach called SourceTracker allowed the estimation of proportion of each community (that is, sample) that are likely to originate from each of a specified set of source environments (9). This analysis further confirmed the presence of a partially oral-like community on the urban bathroom walls (Fig. 3); these traces of human oral microbes from bathrooms and traces of water-associated microbes on kitchen countertops and walls likely contribute to the increased ability to identify both the houses and the indoor functional spaces.

We found no systematic association between the bacterial communities and many other parameters measured in the study including the structural materials in the households, number of people living in the house, number of pets (Fig. 2C), temperature variations, light incidence, frequency of cleaning, number of outsiders at sampling time, date of last rain, and time of day samples were collected (P > 0.05 in all cases). In particular, consistent with recent studies (11, 13), we find that samples within a house with different materials are more similar to one another than samples from the same material across different houses and that, in all communities, the inhabitants of each house are a major source of bacteria (Fig. 3).

**DISCUSSION**

Our findings indicate that the bacteria from the surfaces of house walls are informative of level of urbanization based on architectural design. Floors are the most informative of the commonalities found in individual houses across urbanization levels, whereas walls, less perturbed reservoirs of microbes accumulated through room usage, provide an indicator of room function.

Ventilation, described as a key factor for microbial community composition in urban settings (14–16), was very high in all of the houses of our study and does not explain differences in home microbial composition with urbanization. Instead, we propose that the presence of walls dividing functional spaces acquires function-dependent microbes, mostly of human origin.

The current study is limited to one geographical region of the world and is a small pilot study, and thus, results may not be generalizable. Further research should identify mechanistic explanations for these phenomena. Insights into the chemical signals that bacteria provide in different sites within the home are also needed. These remarkable changes in house microbial content across urbanization might translate into differences in microbial exposure that may have developmental health implications for humans (17), according to several related hypotheses [the “hygiene” hypothesis (18), the “Old Friends” hypothesis (19), and the “Disappearing microbiota” hypothesis (20)], suggesting that the reduced pattern of microbial exposure leads to immune and metabolic disorders that have become the new disease paradigm in the industrialized world.

**MATERIALS AND METHODS**

**Design of the study**

We selected four communities at the same latitude in the Amazon Basin, with different degrees of urbanization (fig. S1): an isolated jungle village, a rural community, an urban town, and an urban city.

The specific locations were selected to represent four significantly different urbanization levels with similar climate. Ten houses from each location were sampled in four sites to characterize architectural and microbiological profiles of house walls and floors. The sample size of n = 40 per location was based on estimations using a two-sided test, for significant differences in the microbial composition, with a Cohen’s d = 0.63, power of 80%, and α = 0.05.

**Communities’ description**

Four human settings were studied in this work, spanning urbanization. Three of them were in Peru, and one in Brazil.

The Peruvian rural community of Checherta is a traditional, native, hunter-gatherer, Amerindian village in the border between Peru and Ecuador (fig. S1). It is inhabited by approximately 300 inhabitants, living in open huts, with the exception of one house that was enclosed from the outside by walls (fig. S11), made of natural materials (Fig. 1A and fig. S2). It has a recently made school consisting of one classroom and three adjacent latrines, which remain unused by the locals. Checherta has no electricity or potable water services; water is obtained from the nearest river, and the village is highly inaccessible, requiring travel by a plane that can land on an improvised landing field in Nuevo Andoas and then taking a 2-day trip on small river boats (table S1).

The second Peruvian community, Puerto Almendras, is a rural setting located at ~1-hour drive (12 km) west from Iquitos. It has ~250 inhabitants that live in houses with external walls, made out of both natural and industrial materials. Most of the houses were not internally subdivided, and those spaces that were remained connected with adjacent areas because the walls did not reach the roof. Puerto Almendras has a water reservoir (however, no potable water service), electricity service, a school, and a health care center within walking distance. Houses are distributed around a soccer field.

The third Peruvian community was the town of Iquitos, the world’s biggest populated center that is inaccessible by road—it is accessible only by plane or boat (table S1). This town has 371,000 inhabitants, an international airport, paved roads, municipally treated piped water, and electricity. All houses are enclosed in external walls that separate them from the outdoor environment and are made of industrial materials. Walls that divide the inner house do not always reach the roof.

The fourth location was Manaus in Brazil, the biggest city in the Amazon region, with a population of 1.8 million, accessible by roads, boats, and planes. Sampled houses were completely separated from the environment and internally divided by walls. Unlike the Peruvian communities, this city has enormous social differences, and we sampled homes from middle class families.

**Architectural determinations**

Sketches of the houses were created with measurements collected in the field, with photographs of each household (Fig. 1A and figs. S2 and S11), that provided the basis for estimations of floor area/surface, volume, openness (proportion of apertures, location in floor plans, and orientation), human density (number of people per square meter), and privacy index for each household. Additionally, this information sets the basis for modeling using a building modeling program (Autodesk Revit) to produce three-dimensional representations of each sampled house.

Environmental variables of temperature and relative humidity were collected. A HOBO Micro Station Data Logger (H21-002) was used to record 2-min interval data of temperature and relative humidity.
Analysis of qualitative data from both architecture and environmental variables was made using the SPSS version 20 program to compare variations in architecture and environment between locations.

**Bacterial community structure determinations**

Microbial samples were collected (using sterile swabs) from floors and walls of living rooms, kitchens, bedrooms, and bathrooms—or equivalent functional spaces in jungle houses—of each household. Metadata information from each sample was recorded, including surface material, sample height (walls), cleaning frequency, presence of pets in the home, light, surface temperature, and whether people wore shoes. Shoes were worn in 36% of family members in Checherta, 75% in Puerto Almendras, and 100% in Iquitos and Manaus. Cryovial-containing samples were frozen in a dry shipper and stored at –80°C until DNA was extracted using the MoBio Power Soil Kit (following the manufacturer’s instructions). The V3-V4 regions of the 16S rRNA gene were sequenced using the HiSeq Illumina platform. Sequences were analyzed using the Qime pipeline. Sequences were trimmed at 100 base pairs, and open-reference OTU picking (21) was performed at a 97% identity to assign taxonomy using Greengenes version 13.8 (22) and to characterize novel taxa. Diversity was estimated using PD whole tree (23) on rarefied tables at 10,000 sequences per sample for floors and 2500 sequences per sample for walls. Diversity was measured using unweighted UniFrac (24) on the rarefied tables. Finally, the Bayesian approach SourceTracker was used to identify possible sources of contamination (9).

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/2/2/e1501061/DC1.

**REFERENCES AND NOTES**


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Single-cell RNA-seq and computational analysis using temporal mixture modeling resolves $T_{H1}/T_{FH}$ fate bifurcation in malaria


Differentiation of naïve CD4+ T cells into functionally distinct T helper ($T_h$) subsets is crucial for the orchestration of immune responses. Because of extensive heterogeneity and multiple overlapping transcriptional programs in differentiating T cell populations, this process has remained a challenge for systematic dissection in vivo. By using single-cell transcriptomics and computational analysis with a temporal mixtures of Gaussian processes model, termed GPfates, we reconstructed the developmental trajectories of $T_{H1}$ and $T_{FH}$ (T follicular helper) cells during blood-stage Plasmodium infection in mice. By tracking clonality using endogenous T cell receptor sequences, we first demonstrated that $T_{H1}/T_{FH}$ bifurcation had occurred at both population and single-clone levels. Next, we identified genes whose expression was associated with $T_{H1}$ or $T_{FH}$ fates and demonstrated a T cell–intrinsic role for Galectin-1 in supporting $T_{H1}$ differentiation. We also revealed the close molecular relationship between $T_{H1}$ and interleukin-10–producing $T_{RI}$ cells in this infection. $T_{H1}$ and $T_{FH}$ fates emerged from a highly proliferative precursor that up-regulated aerobic glycolysis and accelerated cell cycling as cytokine expression began. Dynamic gene expression of chemokine receptors around bifurcation predicted roles for cell-cell interaction in driving $T_{H1}/T_{FH}$ fates. In particular, we found that precursor $T_{H1}$ cells were coached toward a $T_{H1}$ but not a $T_{FH}$ fate by inflammatory cytokines. Thus, by integrating genomic and computational approaches, our study has provided two unique resources: a database, www.PlasmoTH.org, which facilitates discovery of novel factors controlling $T_{H1}/T_{FH}$ fate commitment, and, more generally, GFates, a modeling framework for characterizing cell differentiation toward multiple fates.

INTRODUCTION

CD4+ T cells are key instructors of the immune system. They display extensive phenotypic and functional diversity by differentiating into a range of T helper ($T_h$) subsets, including $T_{H1}$, $T_{H2}$, $T_{H17}$, $T_{FH}$ (T follicular helper), $T_{H22}$, $T_{reg}$ (T regulatory), and $T_{H17}$ cells, that are distinguished mainly by cytokine and transcription factor expression profiles. Because of $T_{H1}$ cells can control infections and drive immune-mediated diseases, there remains tremendous interest in the molecular mechanisms that mediate their in vivo differentiation.

Malaria, caused by the protozoan parasite Plasmodium, afflicted 212 million humans in 2015 (1). Both $T_{H1}$ responses (2) and $T_{H17}$-dependent antibody responses (3) can independently protect against malaria and are elicited simultaneously in malaria-infected individuals (4), as well as in mice challenged with rodent-infective strains, such as Plasmodium chabaudi chabaudi (PcAS) (5). However, the molecular relationships between $T_{H1}$ and $T_{FH}$ cells remain unclear during Plasmodium infection and, more generally, during any immune challenge. A recent study has demonstrated that the unique T cell receptor (TCR) of a naïve CD4+ T cell imparted a strong preference for either a $T_{H1}$ or a $T_{FH}$ fate (6). Nevertheless, for many clones, both fates could still emerge, implying that other mechanisms, such as internal stochasticity and cell-extrinsic factors, also govern fate choices in vivo. Transcription factors including T-bet, Gata3, RORγT, and Bcl6 have been reported to drive and stabilize $T_{H1}$ fates, leading to their characterization as “lineage-defining” molecules. This has tended to present $T_{H1}$ differentiation as a choice between mutually exclusive linear pathways. However, transient coexpression of these transcription factors (for example, of Bcl6/T-bet and Foxp3/RORγT) suggests that overlapping intermediate $T_{H1}$ states also exist in vivo. Moreover, substantial heterogeneity occurs in the kinetics of CD4+ T cell responses, resulting in a complex mixture of intermediate states during differentiation, which is not easily resolved via assessment of a small number of molecules.

Conventional dendritic cells (cDCs) are the dominant initial source of antigenic signaling to naïve CD4+ T cells in secondary lymphoid tissues, for example, in the spleens of Plasmodium-infected mice (7). In other models, it was shown that cDCs made long-lasting stable contacts with naïve CD4+ T cells to initiate priming (8). Once activated, CD4+ T cells regained motility, permitting further cellular interactions. Consistent with this observation, activated CD4+ T cells required further...
antigenic stimulation to optimize clonal expansion and T<sub>H</sub> differentiation (9); cDCs were considered the most likely candidates (8, 10), with other cell types remaining less explored. Studies of mice with altered monocyctic responses suggested roles for these cells in CD4<sup>+</sup> T cell priming, specifically in tissues with few cDCs (11). Other reports used cDC deficiency to illustrate that monocytes could activate naive CD4<sup>+</sup> T cells (12). However, few in vivo studies have explored roles for monocytes in T<sub>H</sub> differentiation, where cDC responses remain intact.

Here, we used single-cell RNA sequencing (scRNA-seq) to study Plasmodium-specific TCR transgenic CD4<sup>+</sup> T (PbTII) cells during blood-stage P<sub>c</sub>AS infection in mice. We then used a computational modeling strategy to reconstruct the molecular trajectories of T<sub>H1</sub> and T<sub>FH</sub> cells. Last, we investigated cell-cell interactions based on dynamic expression of chemokines and their receptors, and examined roles for inflammatory monocytes in supporting activated CD4<sup>+</sup> T cells toward a T<sub>H1</sub> fate.

**RESULTS**

scRNA-seq resolves T<sub>H1</sub> and T<sub>FH</sub> cell fates during Plasmodium infection in mice

We used scRNA-seq to elucidate the development and heterogeneity of T<sub>H1</sub> and T<sub>FH</sub> cells during P<sub>c</sub>AS infection (Fig. 1A and fig. S1). We transferred naive, proliferative dye-labeled PbTII cells into congenic wild-type mice and recovered them at days 2, 3, 4, and 7 after infection by cell-sorting those expressing the early activation marker CD69 or displaying dilution of the proliferative dye (fig. S2). Flow cytometric measurements of the canonical T<sub>H1</sub> markers T-bet (coded by Tbx21) and interferon-γ (IFNγ) and T<sub>FH</sub> markers CXCR5 and Bcl6 indicated that these subsets emerged in parallel by day 7 after infection (Fig. 1, B to D, and fig. S3) (13, 14). Notably, markers of T<sub>H2</sub>, T<sub>17</sub>, or T<sub>reg</sub> subsets were not up-regulated by PbTII cells (fig. S4).

Initially, we used principal components analysis (PCA) to explore the overall transcriptomic landscape of the PbTII cells (fig. S5A). The top principal components were strongly associated with the number of detected unique transcripts [reflective of mRNA content and proliferative status (fig. S5B)] and differentiation (figs. S5C to S7 and table S1). As expected, the variability related to previously established T<sub>H1</sub> and T<sub>FH</sub> gene expression signatures became more prominent with time, separating two subpopulations at day 7 (Fig. 1, E and F) (15). Together, these results suggested a progressive commitment to
T<sub>H</sub>1 and T<sub>FH</sub> fates, and indicated that single-cell transcriptomes could be used for estimating both proliferative states and degrees of differentiation of individual cells.

**Delineation of T<sub>H1</sub> and T<sub>FH</sub> trajectories using a Mixture of Gaussian Processes model**

The results from the PCA suggested that variation in PbTII transcriptomes could be used to reconstruct the transcriptional programs that are underlying the T<sub>H1</sub> and T<sub>FH</sub> differentiation. To more explicitly model the temporal dynamics of this differentiation process, we developed GPfates, a temporal mixture model that builds on the Gaussian Process Latent Variable Model (16) and Overlapping Mixtures of Gaussian Processes (OMGP) (17). Briefly, this approach is based on first reconstructing the differentiation trajectory from the observed data (“pseudotime,” Fig. 2, A and B), thereby establishing an order for the cells. Although our model uses the sample time as prior information, the inferred orderings did not strictly adhere to the experimental time points (Fig. S8). For example, cells from day 4 after infection were mixed with some of the cells from days 3 and 7 at either end of the day 4 pseudotime distribution. This result is consistent with the idea that bulk assessments of cells at specific time points fail to account for the heterogeneity and differential kinetics of responses made by single cells. To assess the robustness of the established ordering, we repeated this analysis without supplying the experimental sampling times to the model, finding overall consistent results (Comp. Supp. Fig. 8).

In a second step, GPfates uses the inferred temporal orders as input for a nonparametric time series mixture model [OMGP (17)]. This approach revealed two simultaneous trends emerging during pseudotime (Fig. 2, C and D), which separated from each other, indicating that a developmental bifurcation occurred.

In a third step, GPfates uses a change point model (section 4.2 of Supplementary Computational Methods), thereby facilitating to annotate pseudotime after bifurcation. The cell fate split appeared to initiate development of bifurcation occurred.

We found that differentially expressed genes between the identified trajectories agreed with known T<sub>H1</sub>/T<sub>FH</sub> signature genes (Fig. 3, A and B, and fig. S9) (15), strongly suggesting that the fitted mixture components corresponded to cells with T<sub>H1</sub> and T<sub>FH</sub> phenotypes. Notably, these bifurcation trends could not be identified by other published methods for reconstructing bifurcating single-cell trajectories (Comp. Supp. Fig. 14) (18–22). We also successfully applied GPfates to resolve bifurcation events in other published data sets (Comp. Supps. Figs. 11 and 12) (23, 24), suggesting that our approach is more generally applicable for studying cellular differentiation using scRNA-seq data.

**Lineage barcoding using endogenous TCR sequences reveals T<sub>H1</sub>/T<sub>FH</sub> bifurcation from single CD4<sup>+</sup> T cells**

Although the TCR transgenic approach used in this study minimized the influence of TCR sequence variability on cell fate determination (6), the strain was Rag-sufficient, thus retaining potential for expression of diverse endogenous TCR chains, in addition to the transgenic TCR. Sequence analysis of TCR transcripts in single PbTII cells confirmed universal expression of the PbTII Vo2 and Vβ12 chains (tables S2 and S3), as well as highly diverse, though lower, levels of expression of endogenous TCRα chains in many cells (fig. S10). Rag-sufficient PbTII cells differentiated as effectively as Rag<sup>−/−</sup> PbTII cells into both T<sub>H1</sub> and T<sub>FH</sub> cells (fig. S11), indicating that endogenous TCR sequences had not influenced T<sub>H1</sub> fate bifurcation.

Given the vast combinatorial diversity of the endogenous TCR sequences, we used these as unique molecular barcodes to identify ancestrally related PbTII clones. We identified six clones comprising multiple sibling cells. Of these, two consisted of sibling cells that mapped close to the bifurcation point. For the remaining four clones, siblings exhibited highly diverging patterns of differentiation, with three sibling pairs falling at the extremities of the T<sub>H1</sub>-T<sub>FH</sub> phenotype spectrum (Fig. 3C). These results demonstrated that T<sub>H1</sub>/T<sub>FH</sub> bifurcation had occurred at both population and single-clone levels in our system, with the progeny of a single cell populating both T<sub>H1</sub> and T<sub>FH</sub> compartments.

**Transcriptional signatures associated with bifurcation of T<sub>H1</sub> and T<sub>FH</sub> fates**

Next, we sought to identify genes whose expression differed between the T<sub>H1</sub> and T<sub>FH</sub> branches. We derived a bifurcation statistic to estimate the concordance with bifurcation for individual genes (see section 4.2 of Supplementary Computational Methods for details and Fig. 3D). Among the highest-ranking genes, the most common pattern was up-regulation along the T<sub>H1</sub> branch (Fig. 3D). This suggested that T<sub>FH</sub> cells were developmentally closer to the shared progenitor state than T<sub>H1</sub> cells, because the T<sub>H1</sub> fate involved up-regulation of numerous genes not expressed in either the progenitor or T<sub>FH</sub> states.

To validate the robustness of these gene signatures and the timing of the bifurcation, we repeated the infection and, at days 0, 4, and 7, sequenced additional PbTII cells using the Smart-seq2 protocol (Fig. 1A and fig. S12A). A nonlinear dimensionality reduction indicated that the single cells from both experiments populated similar transcriptional landscapes (fig. S12B) and that the subset characteristic coexpression patterns of the bifurcating genes identified by GPfates emerged by day 7 (fig. S12C). Notably, the day 7 cells from each mouse could be separated into distinct T<sub>H1</sub> and T<sub>FH</sub> subpopulations using the top bifurcation genes (fig. S12D). These results indicated that the bifurcation-associated gene expression patterns were reproducible across experiments and sequencing platforms.

The highest-ranking transcription factors for the bifurcation included Tcf7 for the T<sub>H1</sub> fate and Id2 for the T<sub>FH</sub> fate (Fig. 3, D and E). Tcf7 is required for T cell development and has been recently shown to be instrumental for T<sub>H1</sub> differentiation (25, 26). It also represented one of the rare genes defined by a decrease in expression when moving toward the T<sub>H1</sub> fate. Id2 is an antagonist of Tcf7 and was recently identified as a key driver of T<sub>H1</sub> responses (27). As expected, the hallmark T<sub>H1</sub> transcription factor Bcl6 was also strongly associated with the T<sub>H1</sub> fate. In T<sub>H1</sub> cells, many bifurcating genes encoded immune-related receptors (Fig. 3, D and E), such as Cxcr6 (fig. S13, A and B), Ifngr1, and Slpr1, which mediate egress from secondary lymphoid organs. This was consistent with the notion that T<sub>H1</sub> cells can migrate to peripheral tissues and remain receptive to external signals. In contrast, the only bifurcating chemokine receptor associated with a T<sub>FH</sub> fate was Cxcr5, which is important for trafficking into B cell follicles (28).

Many of the bifurcating genes had no known role in T<sub>H1</sub> differentiation. For example, lgals1 (encoding Galectin-1), a molecule generally implicated in cDC (29) and T<sub>FH</sub> function (30), was unexpectedly up-regulated in PbTII cells around bifurcation and maintained along the T<sub>H1</sub> but not the T<sub>FH</sub> trajectory (fig. S14A). This observation was confirmed at the protein level (fig. S14B). Next, comparison of T<sub>H1</sub>/T<sub>FH</sub> fates in cotransferred wild-type and lgals1<sup>−/−</sup> PbTII cells during P<sub>C</sub>S infection (fig. S14C) revealed a specific role for Galectin-1 in supporting...
Fig. 2. GPfates modeling of bifurcation processes using scRNA-seq data. (A) Overview of the analysis workflow that underlies GPfates, consisting of dimensionality reduction of high-dimensional single-cell transcriptomes (left), inference of a pseudotemporal ordering of the cells (middle), and the reconstruction of trajectories using temporal mixture modeling (right). These individual steps build on models derived using the Gaussian process framework. Once fitted, GPfates enables for different downstream analyses, including cell orderings, bifurcation time point estimates, and inference of the genes that drive bifurcation events. (B) Illustration of intermediate results obtained from GPfates. Left: A low-dimensional representation, as well as a pseudotemporal ordering of the cells, is inferred using a nonlinear dimensionality reduction (Gaussian Process Latent Variable Model). Temporal trajectories and bifurcations are then reconstructed using a temporal mixture model (Overlapping Mixture of Gaussian Processes), with data-trend assignments per cell. B-GPLVM, Bayesian Gaussian Process Latent Variable Model; 3D, three-dimensional. (C) Low-dimensional representation (2D) of the complete data sets (408 single-cell transcriptomes). The blue line depicts the inferred progression of pseudotime. Text labels illustrate features typical of cells in the corresponding pseudotime region. (D) Inference of two simultaneous trends based on the pseudotime using the temporal mixture model.

T_{H1} but not T_{FH} fate (fig. S14D). Together, these data illustrate the potential for the GPfates model to enable identification of factors controlling T_{H1} and T_{FH} fates. Further examination of bifurcating genes is facilitated by an online database (www.PlasmoTH.org) accompanying this paper (Fig. 3F).

Coinciding with T_{H1}/T_{FH} differentiation, we also noted up-regulation of Il10 particularly in the T_{H1} branch (fig. S15A). Most of the Il10-expressing cells also expressed Ifng at equal or higher levels as those expressing Ifng alone (fig. S15, B and C). These data revealed the development of Tr1 cells, defined as interleukin-10 (IL-10)/IFN-γ–coexpressing CD4+ T cells. Given that Il10 expression was associated with the T_{H1} branch, this suggested that Tr1 cells were developmentally related to T_{H1} cells. Unexpectedly, we found that aside from Il10, only two genes, Trib2 and BC017643, were differentially expressed between Il10/Ifng-coexpressing Tr1 cells and Ifng-expressing T_{H1} cells (fig. S15D).

Furthermore, a comparison of gene expression frequencies between Tr1 and T_{H1} cells revealed a substantial degree of similarity across the genome (fig. S15E). Together, these data strongly suggest that Tr1 cells derive directly from T_{H1} cells during blood-stage Plasmodium infection.

Pseudotemporal relationships between cell cycling, aerobic glycolysis, and cytokine expression

Clonal expansion, increased aerobic glycolysis, and cytokine expression are hallmarks of T_{H1} cell development whose temporal relationships with each other remain to be fully resolved in vivo. We noted that PbTII cells became highly proliferative around bifurcation, as shown by the up-regulation of Mki67 (Fig. 4, A and B, and fig. S16A) and other known proliferation marker genes (fig. S16A) (31). This correlated with cell cycle activity, as inferred from the scRNA-seq data using the Cyclone tool, and confirmed by flow cytometric measurements of DNA content and cell size (Fig. 4, C and D, and fig. S16C). On day 4 after infection, the cells also increased expression of genes associated with aerobic glycolysis but not oxidative phosphorylation (Fig. 4F), an indication of increased metabolic requirements being met by glucose metabolism and increased mammalian target of rapamycin complex 1 (mTORC1) activity. Consistent with this was the observed
after infection, we noted that early Ifng-expressing cells cycled faster and expressed aerobic glycolysis genes more highly than non–cytokine–expressing counterparts (Fig. 4G). Together, our data suggest that around bifurcation, PbTII cells exhibited a highly proliferative and metabolically active state, with those cells cycling fastest and exhibiting most glycolytic activity being the first to acquire the capacity to secrete IFNγ.

**Gene dynamics identifies potential decision-making molecules**

To elucidate how PbTII cells transitioned from the proliferative precursor state to TH1 and TFH fates, we sought to resolve the hierarchy of gene expression before and during cell fate bifurcation. In addition to genes directly following the bifurcation trend, we reasoned that expression of genes encoding key decision-making molecules might also be likely to be dynamic and peak before the bifurcation. First, to identify these, we selected those genes displaying interesting nonlinear trends in their expression patterns over pseudotime by Gaussian Process regression. This was achieved via a D statistic (model likelihood ratio), where each gene’s expression pattern over pseudotime was tested for variation unexplained by random noise (32). On the basis of the D statistic (>50.0, Fig. 5C), we identified 2061 dynamic genes (Fig. 5A).

Second, we ordered these genes according to their peak expression time to provide a temporal overview (Fig. 5A) and noted that a substantial fraction of them peaked around bifurcation. These included the TH1-driving genes Tbx21, Il2ra, and Il2rb, supporting our initial hypothesis. Moreover, cells around bifurcation also transcribed the highest number of genes compared with those at all other points in pseudotime (Fig. 5B).

This model also infers the length-scale of the dynamic model, namely, the degree of fast-acting behavior over pseudotime (Fig. 5C). Using this additional feature, we noted roughly equivalent dynamics for Tbx21, Il2ra, and Il2rb. Furthermore, we noted similar dynamics, though with slightly later peak times, for the chemokine receptors Cxcr5 and Cxcr3. Closer examination of all chemokine receptor genes also revealed peak expression around bifurcation for Ccr4 but not others (Fig. S17). Given that Cxcr5 and Cxcr3 have been associated with TH1 and TH1 cells, respectively (10, 33, 34), and because they exhibited similar dynamics, we hypothesized that these were competing receptors that directly influenced elevated levels of ribosomal protein S6 phosphorylation on day 4 after infection (Fig. 4E).

By day 4 after infection, PbTII cells had gone through several rounds of cell division with differing kinetics and with some cells expressing IFNγ. By comparing Ifng-expressing and nonexpressing cells on day 4
TH1/TFH fate (Fig. 5D). Assessment of Cxcr3/Cxcr5 coexpression around bifurcation revealed a substantial portion of cells expressing both receptors (fig. S18). Thus, our examination of gene expression dynamics revealed large numbers of genes being expressed and peaking around bifurcation, including not only those associated with clonal expansion but also numerous sequentially expressed transcription factors and receptors with potential to influence TH fate.

Monocytes support activated PbTII cells toward a TH1 but not a TFH fate

Given similar dynamics and peak expression times for Cxcr3 and Cxcr5, and peak expression around bifurcation for Ccr4 (fig. S17), we reasoned that cell-cell interactions via these receptors controlled TH1/TFH fate. Hence, we considered cell types that could control TH fate, specifically around bifurcation. Because B cells supported a TH1 fate (fig. S19), we hypothesized that coordinated action by myeloid cells provided competing signals to support a TH1 fate.

To study this, we examined splenic cDCs and inflammatory monocytes before PbTII bifurcation. We sorted CD8α+ and CD11b+ cDCs and Ly6Chi monocytes from naïve and infected mice (fig. S20) and performed scRNA-seq. PCA of cDCs distinguished the two naïve cell types along PC2 (Fig. 6A and fig. S21) with an efficiency consistent with recent data (35) and further highlighted a number of expected and previously unknown cDC subset-specific genes (fig. S22). We next compared naïve cDCs with those from infection (Fig. 6A and fig. S21) and separated these along PC6 (Fig. 6A). Analysis of differential gene expression in cDCs due to infection identified 30 genes, 29 of which were up-regulated (Fig. 6B and fig. S23), including transcription factors Stat1 and Irf1 and...
CXCR3-attractant chemokines Cxcl9 and Cxcl10. Notably, gene expression patterns among individual cDCs varied according to the gene. For example, Stat1 and Irf1 were expressed by several naïve cDCs and further up-regulated during infection (Fig. 6C). This was similar for Cxcl9, which was expressed by CD8α+cDCs in naïve mice, whereas Cxcl10 was induced only upon infection (Fig. 6C). These data suggested interactions between cDCs and uncommitted CXCR3+ PbTII cells, consistent with a recent study (10). Next, PCA of Ly6C hi monocytes from naïve and infected mice distinguished them from each other along PC2 (Fig. 6D and fig. S24). Differential gene expression analysis between naïve and infected groups uncovered ~100 genes, both up-regulated and down-regulated during infection (Fig. 6E and fig. S25). A high proportion (~40%) of genes up-regulated in cDCs were also induced in Ly6C hi monocytes, including Stat1, Irf1, and Cxcl10 (Fig. 6, E and F), suggesting possible overlapping functionality. In addition, monocytes expressed other chemokines, including Cxcl2, Ccl2, and Ccl3 (Fig. 6, E and F). Furthermore, specific examination of all immune cellular interaction genes (fig. S26) revealed emerging variable expression of Tnf, Cd40, Pdl1, Ccl4, Ccl5, Cxcl16, Cxcl9, and Cxcl11 in monocytes, thus suggesting complex cell-cell interactions for Ly6C hi monocytes during infection.

Because Cxcl9, Cxcl10, Cxcl11, Ccl2, Ccl3, and Ccl5 signal through Cxcr3 or Ccr4, which were expressed by activated PbTII cells, we next hypothesized that Ly6C hi monocytes, in addition to cDCs (10), interacted with PbTII cells and influenced TH1/TFH fate. To test this, we first assessed chemokine expression at the protein level by Ly6Chi monocytes (Fig. 6G). Kinetics of CXCL9 production was similar in cDCs and Ly6C hi monocytes. Next, we used LysMCre × iDTR mice, in which Ly6Chi monocytes were depleted after PbTII cell activation but before bifurcation (Fig. 6H and fig. S27). We noted a modest reduction in CD68+ splenic macrophages using this approach (fig. S27B). Using this approach, we found that monocytes/macrophages supported a TH1 but not a TFH fate (Fig. 6H). Together, our data support a model in which activated PbTII cells are supported toward either a TFH fate by B cells (fig. S19) or a TH1 fate by chemokine-expressing myeloid cells, including Ly6C hi inflammatory monocytes.

**DISCUSSION**

By capturing single CD4+ T cell transcriptomes during an experimental malaria infection, and computationally reconstructing the course of events, we have resolved the bifurcation of naïve CD4+ T cells into T H1 and T H0 cells at a molecular level. GPfates modeling...
Fig. 6. Myeloid cells influence Th, bifurcation in uncommitted PbTII cells. (A to C) Splenic CD8α+ and CD11b+ CD8α− cDCs from a naive mouse, mixed cDCs from an infected mouse, and (D to F) Ly6C+ monocytes from naive and infected mice were analyzed by scRNA-seq, with mRNA reads filtered by minimum expression of 100 TPM in at least two cells. (A and D) PCA showing clustering of (A) cDCs or (D) monocytes. (B and E) Fold change and confidence for differentially expressed genes (19) between infected and naive (B) cDCs or (E) monocytes; genes were filtered on expression in >10 cells; genes satisfying $q < 0.05$ are colored per function. (C and F) Differentially expressed genes ($q < 0.05$) in (C) cDCs and (F) monocytes, between naive and infected mice: Cells and genes are ordered according to PC score and loading, respectively. Common genes between heat maps are annotated in (F). (G) Representative FACS histograms and proportions of splenic CD8α+ cDCs, CD8α− cDCs, and Ly6C+ monocytes expressing CXCL9 in naive and infected mice; data show individual mice with line at mean and are representative of two independent experiments ($n = 4$ mice per time point per experiment). (H) PbTII cells were transferred into LysMCre × iDTR mice 1 day before infection. At 3 days after infection, mice were treated with diphtheria toxin (DT) or saline. Proportions of Th1 (T-bet+ IFNγ+) and Th17 (CXCR5+) PbTII cells at 7 days after infection; data pooled from three independent experiments ($n = 5$ to 6 per experiment). ****P < 0.0001, Mann-Whitney U test; NS, not significant. (I) Summary model proposes that chemokine interactions between nonbifurcated PbTII cells and myeloid cells support a Th1 fate, whereas B cells support a Th17 fate.
of scRNA-seq data is not limited to immune cells or single bifurcation events. This model can also be combined with existing computational workflows, including alternative methods to estimate pseudotemporal dynamics (see section 6.2 of Supplementary Computational Methods) (19, 36). The GPfates approach permits analysis of cellular differentiation toward two fates (Comp. Supp. Fig. 11) and, in principle, toward multiple fates (Comp. Supp. Fig. 12). However, GPfates exhibits some limitations. Most notably, the ability to identify and pinpoint bifurcation events is linked to changes in the transcriptome that reflect these cellular decisions. In particular, because scRNA-seq profiles are subject to high levels of noise, this means that changes will only be detectable with some lag time (Supplementary Computational Methods). The processed expression data and the GPfates model presented in this study can be accessed at www.plasmoTH.org, where users can visualize their genes of interest.

Our data provide the framework for revealing molecular insights into the early stages of Th1 cell differentiation and describe the sequence of transcriptional events before and after the bifurcation of Th1 and Th17 fates. Transcriptomic profiling previously suggested developmental similarities between Th1 and Th17 cells (37). However, highly immunogenic viral or bacterial infections induced CD4+ T cells to segregate into Bcl6+ (TFH) or Blimp-1+ (Th1) subpopulations within 2 days (38, 39). In our parasitic model, single CD4+ T cell transcriptomes remained remarkably similar until 4 days of infection. Although it is difficult to directly compare infection models, we speculate that Plasmodium infection in mice may not drive Th1 bifurcation as early as observed with highly immunogenic viruses or bacteria, particularly given evidence of immunosuppression (40).

IL-10–producing Tr1 cells can suppress immune responses, which could aid the treatment of immune-mediated disorders (41) or be detrimental for chronic infections (42). Despite this, their relationship to Th1 cells is not clear (43). In our model, Tr1 cells emerged from the Th1 trajectory. This observation, coupled with similar transcriptomes for Th1 and Th1 cells, provides evidence that Tr1 cells are highly related to, and derive directly from, Th1 cells in this model. Thus, our modeling of scRNA-seq data revealed molecular relationships between Th1, Th17, and Tr1 cells and showed that a single naive CD4+ T cell can simultaneously give rise to more than one cell fate during experimental malaria.

Activated CD4+ T cells may experience different microenvironments within secondary lymphoid tissue. The observation that bifurcation toward Th1 and Th17 fates was preceded by up-regulation of chemokine receptors prompted us to investigate possible cell–cell interactions with chemokine–expressing myeloid cells. Previous studies have highlighted the potential for cDCs in lymph nodes to produce Th17-associated chemokines (10). Our study, which focused on the spleen, further implicated inflammatory monocytes in Th17 support. However, because our transgenic approach for depleting monocytes also removed a small portion of splenic red pulp macrophages, we cannot fully discount the possibility that they may partly contribute to a Th17 fate. Nevertheless, we propose that splenic monocytes/macrophages influence bifurcation by supporting a Th17 fate during Plasmodium infection. Our studies emphasize that although cDCs are key for initiating CD4+ T cell activation in the spleen, other myeloid cells can also promote a Th17 fate in the presence of cDCs. In contrast, given that CXCR5 was the only chemokine receptor notably associated with bifurcation toward a Th17 fate, cellular interaction with B cell follicles may be the primary mechanism for supporting a Th17 fate. Our model proposes that activated, uncommitted CD4+ T cells become receptive to competing chemokine signals from different zones of the spleen, and suggests intercellular communication as a major driver of bifurcation. However, upstream of these processes, internal stochasticity in uncommitted CD4+ T cells may control the balance of chemokine receptor expression. Future experiments combining our integrated single-cell genomics and computational modeling with in vivo positional and trafficking data may reveal molecular relationships between internal stochasticity, migratory behavior, and Th1 cell fate.

**MATERIALS AND METHODS**

**Study design**

The goal of this study was to use scRNA-seq to capture the transcriptomes of individual splenic PbTII cells at various time points during the first week of a blood-stage P. chabaudi AS infection. Multiple mice were used for most time points to test for possible batch effects, with an independent experimental repeat performed on a different scRNA-seq platform. scRNA-seq data were modeled using Gaussian processes, with statistical testing for significance of both genes and cells associated with the Gaussian processes.

**Experimental mice and infections**

Wild-type and transgenic inbred mouse strains were housed and used in blood-stage *Plasmodium* infections, as described in Supplementary Materials and Methods.

**Flow cytometry**

Splenocytes were isolated and assessed by flow cytometry as described in Supplementary Materials and Methods.

**Single-cell mRNA sequencing**

Single-cell capture and processing, as well as quality control analysis of scRNA-seq data, were performed as described in Supplementary Materials and Methods.

**Statistics**

Statistical analyses were conducted using R, Python, or GraphPad Prism. The types of statistical tests and significance levels are described in respective figure legends.

**SUPPLEMENTARY MATERIALS**

immunology.sciencemag.org/cgi/content/full/2/9/eaal2192/DC1

Materials and Methods

Fig. S1. Enrichment of PbTII cells for adoptive transfer.

Fig. S2. Sorting strategy for PbTII cells.

Fig. S3. Flow cytometric assessment of Th1/Th17 responses during PbAS infection.

Fig. S4. Expression of subset-specific marker genes in PbTII cells.

Fig. S5. Heterogeneity of activated PbTII cells and variability associated with cell size and differentiation.

Fig. S6. Heterogeneity of Th17 signature gene expression in activated PbTII cells.

Fig. S7. Heterogeneity of the entire PbTII time series and the contribution of Th1 and Th17 signature genes to the overall variability.

Fig. S8. The relationship of pseudotime with time points and with the Th1 assignment probability.

Fig. S9. Correlation of GPfates trends with Th1 and Th17 signature genes.

Fig. S10. Expression of transgenic and endogenous TCRs.

Fig. S11. Expression of endogenous TCRs does not influence PbTII cell Th1/Th17 differentiation.

Fig. S12. Robustness of top bifurcating genes across experiments.

Fig. S13. Flow cytometric validation of CXCR6 expression in PbTII cells before and after bifurcation.

Fig. S14. T cell–intrinsic Galectin-1 supports Th1 fate commitment.

Fig. S15. IL-10– and IFN-γ–coproducing Tr1 cells derive from Th1 cells.

Fig. S16. Proliferative burst of activated PbTII cells.

Fig. S17. Kinetics of chemokine receptor expression during PbAS infection according to the GPfates model.
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Type I interferons instigate fetal demise after Zika virus infection

Laura J. Yockey,1 Kellie A. Jurado,1 Nitin Arora,2 Alon Millet,1 Tasfia Rakib,1 Kristin M. Milano,3 Andrew K. Hastings,4 Erol Fikrig,4,5 Yong Kong,6 Tamas L. Horvath,7 Scott Weatherbee,8 Harvey J. Kliman,3 Carolyn B. Coyne,2,9 Akiko Iwasaki1,5*  

Zika virus (ZIKV) infection during pregnancy is associated with adverse fetal outcomes, including microcephaly, growth restriction, and fetal demise. Type I interferons (IFNs) are essential for host resistance against ZIKV, and IFN-α/β receptor (IFNAR)–deficient mice are highly susceptible to ZIKV infection. Severe fetal growth restriction with placental damage and fetal resorption is observed after ZIKV infection of type I IFN receptor knockout (Ifnar1−/−) dams mated with wild-type sires, resulting in fetuses with functional type I IFN signaling. The role of type I IFNs in limiting or mediating ZIKV disease within this congenital infection model remains unknown. In this study, we challenged Ifnar1−/− dams mated with Ifnar1+/+ sires with ZIKV. This breeding scheme enabled us to examine pregnant dams that carry a mixture of fetuses that express (Ifnar1−/−) or do not express IFNAR (Ifnar1+/+) within the same uterus. Virus replicated to a higher titer in the placenta of Ifnar1−/− than within the Ifnar1+/+ concepti. Yet, rather unexpectedly, we found that only Ifnar1+/− fetuses were resorbed after ZIKV infection during early pregnancy, whereas their Ifnar1−/− littersmates continue to develop. Analyses of the fetus and placenta revealed that, after ZIKV infection, IFNAR signaling in the conceptus inhibits development of the placental labyrinth, resulting in abnormal architecture of the maternal-fetal barrier. Exposure of midgestation humanchorionic villous explants to type I IFN, but not type III IFNs, altered placental morphology and induced cytoskeletal rearrangements within the villous core. Our results implicate type I IFNs as a possible mediator of pregnancy complications, including spontaneous abortions and growth restriction, in the context of congenital viral infections.

INTRODUCTION

Zika virus (ZIKV), an emerging mosquito-borne flavivirus, infected more than 500,000 individuals in 2015 and 2016 as it spread across the Americas and is now present in 62 countries across the world (1, 2). Symptoms in healthy individuals are mostly mild, including fever, rash, and conjunctivitis, with most infections remaining asymptomatic. However, the recent outbreak has led to a worldwide concern over the ability of the virus to cause birth defects, including microcephaly, in infected pregnant women. In addition to microcephaly, ZIKV causes a range of other pregnancy complications, including intrauterine growth restriction (IUGR), spontaneous abortion, and stillbirth (3). Although ZIKV is primarily transmitted through the mosquito Aedes aegypti, increasing evidence supports sexual transmission as a route of infection: ZIKV RNA persists in semen for up to 6 months after infection, and there are a number of reports of ZIKV transmission among sexual partners in areas where mosquito transmission has not been reported (4, 5). However, it is unknown whether the mechanisms of ZIKV-induced fetal pathology after mosquito-borne and sexually transmitted infection are similar.

The type I interferons (IFNs), including IFN-α and multiple subtypes of IFN-α, are key antiviral factors that mount a rapid and potent innate defense against viruses (6). Production of type I IFN is initiated through recognition of pathogen-associated molecular patterns, generated during viral infection (7, 8). Type I IFNs bind to their receptor, IFNAR (IFN-α/β receptor), to induce an antiviral state through transcription of IFN-stimulated genes (ISGs), which restrict viral replication through a broad range of antiviral mechanisms (6, 9). In addition to inducing cell-intrinsic antiviral effects, IFNs have an extensive range of biological activities, including activating adaptive immune responses (10), blocking cell proliferation, and inducing apoptosis (11). Thus, in addition to restricting viral infection, IFNs also have the potential to contribute to pathogenesis.

Multiple mouse models of ZIKV infection have demonstrated adverse pregnancy outcomes (12–16). Because ZIKV is unable to suppress the mouse IFNAR signaling as successfully as it suppresses human IFNAR signaling (17, 18), many mouse models of ZIKV infection require blockade of IFNAR using an antibody, use of Ifnar1−/− mice, or the use of high levels of virus to induce pathology (12, 14, 19). Pregnancy studies of ZIKV have used a model in which Ifnar1−/− females are crossed to wild-type (WT) males, creating Ifnar1−/− fetuses with an intact IFN response (13, 14, 20). In these models, fetuses develop severe growth restriction when pregnant dams are infected subcutaneously or intravaginally after embryonic day 7.5 (E7.5). If mice are infected earlier, between E4.5 and E6.5, most fetuses are resorbed. ZIKV infection in this model induces severe pathology of the placenta and abnormal placental architecture (13).

The placenta supports fetal development by facilitating exchange of nutrients and gases between the maternal and fetal blood. In addition, it serves as a barrier by preventing transfer of pathogens from the mother to the fetus (3, 21). It is known that inflammation and infection can disrupt the function and development of the placenta, leading to IUGR, preeclampsia, preterm birth, and fetal demise, as demonstrated in humans and animal models (22, 23). Others have
also suggested that the immune response to ZIKV at the maternal-fetal interface may be responsible for ZIKV-associated birth defects (24). In addition, type I IFNs have been shown to mediate preterm birth in mouse models (25). On the other hand, IFNs are known to have important roles in supporting normal pregnancy and protecting the fetus from viral infections (26). For example, ISGs are up-regulated during implantation in mice and humans (27), and human syncytiotrophoblasts constitutively express type III IFNs (IFN-αs), making them resistant to infection by viruses (28, 29). In addition, type III IFNs play a role in restricting ZIKV vertical transmission in mice (20).

In this study, we interrogate the effect of type I IFN signaling on fetal development using a mouse model of ZIKV infection. Vaginal or subcutaneous ZIKV infection of Ifnar1−/− dams crossed with Ifnar1+/− sires enabled us to investigate the role of IFNAR signaling in antiviral protection and disease. Paradoxically, our results revealed a detrimental role of fetal IFNAR signaling in mediating UGR and fetal resorption by causing abnormal placental development.

RESULTS

Fetal IFNAR signaling instigates fetal demise despite controlling ZIKV replication

In our previous studies, we observed that vaginal infection of mice lacking the transcription factors upstream of type I IFN, Irf3−/− Irf7−/− dams crossed to Irf3−/− Irf7−/− sires, had higher levels of ZIKV in the placenta but had minimal growth restriction compared with fetuses from Ifnar−/− dams crossed to WT sires (14). Although the Irf3−/− Irf7−/− mice are capable of responding to IFN, we hypothesize that the lack of pathology in this model, despite high levels of virus, is due to the lack of IFN induction in the first place. Consistent with this, we observed that the ISGs were induced in Ifnar−/− fetuses and placenta, but they were absent in the Irf3−/− Irf7−/− matings, correlating with the level of pathology (fig. S1, A to F). These findings led us to hypothesize that IFN signaling, rather than the levels of virus, mediates fetal pathology after ZIKV infection.

To directly test the role of type I IFNs in antiviral defense and fetal development after ZIKV infection, we crossed Ifnar1+/+ females with Ifnar1−/− males, producing a mixture of Ifnar1−/− and Ifnar1+/− fetuses in the same litter (Fig. 1A). Pregnant dams were infected intravaginally with 1.5 × 10^7 plaque-forming units (PFU) of Cambodian strain of ZIKV on either E5.5 or E8.5, corresponding to the mid and late first trimester in humans (30), respectively. Fetuses were harvested on E17.5, close to term. We observed resorption of all fetuses of the Ifnar1−/− genotype when dams were infected with ZIKV on E5.5 (Fig. 1, B and C). However, all Ifnar1−/− littersmates continued to develop after infection on E5.5, indicating that a functional copy of Ifnar1 is required to mediate fetal demise after early ZIKV infection of pregnant dams (Fig. 1, B and C). When dams were challenged with ZIKV on E8.5, Ifnar1−/+ fetuses were not resorbed but showed more severe growth restriction compared with their Ifnar1−/− littersmates (Fig. 1, B and C). Analysis of viral RNA showed that, after E8.5 infection, there was more viral RNA detected in the placentas of the Ifnar1−/− littersmates compared with their Ifnar1−/+ littersmates on E17.5 [9 days postinfection (dpi)] (Fig. 1D). Plaque assays revealed 1000-fold higher levels of infectious virus in the Ifnar1−/+ placentas compared with Ifnar1−/+ placentas (fig. S2A). After infection at E5.5, there were comparable levels of virus in the Ifnar1−/− placenta and the resorbed Ifnar1−/− conceptus, and these levels were lower than what was observed for the E8.5 Ifnar1−/− placentas on E17.5 (12 dpi) (Fig. 1D). In the fetus, viral RNA was present but at low levels in both genotypes and at both time points of infection (Fig. 1E), and infectious virus was below the limit of detection for most fetuses after infection at E8.5 (fig. S2B). These results indicated that IFNAR signaling in the conceptus, despite controlling ZIKV replication in the placenta, leads to fetal demise after congenital infection.

To understand how and when development is blocked in the Ifnar1−/− conceptus, we harvested fetuses at various days after infection of dams on E5.5. Gross examination of the Ifnar1−/+ fetuses revealed no overt differences with Ifnar1−/− fetuses or with uninfected counterparts on E9.5 (Fig. 2, A and B). On E10.5, there was a modest reduction in size of the Ifnar1−/− fetuses, and in 1 of 6 litters, all Ifnar1−/+ fetuses were resorbed (3 of 17), but fetuses otherwise appeared grossly normal (Fig. 2, A and B). However, by E11.5, the majority of Ifnar1−/+ fetuses (7 of 11) were dead, and by E12.5, all Ifnar1−/+ fetuses were resorbed (Fig. 2, A and B). Thus, IFNAR-dependent fetal demise after early ZIKV infection occurs between E10.5 and E12.5.

Placental labyrinth architecture is abnormal in IFNAR-competent concepti

Postimplantation death between E5.5 and E12.5 is typically caused by defects in the fetal red blood cells (RBCs), vasculature, heart, or the placenta (31). However, we focused on analysis of the placenta, which develops between E8.5 and E10.5 (32, 33). Global transcriptional analysis by RNA sequencing (RNA-seq) of placentas at E10.5 demonstrated evidence of active IFNAR signaling in the Ifnar1−/− placenta, with robust induction of hundreds of ISGs (fig. S3 and table S2). To determine which cells are infected by ZIKV, we stained the E10.5 placenta and decidua for ZIKV antigen. The decidua is the maternally derived endometrial lining of the uterus, and the fetus-derived placenta is composed of the junctional zone and labyrinth zone, where nutrient exchange occurs between the maternal and fetal blood (Fig. 3A) (21, 32). We detected rare ZIKV-infected leukocytes (CD45+ cells) in both the Irf3−/− and Irf7−/− decidua but not in the underlying placenta (fig. S4). These cells likely represent decidual macrophages or dendritic cells, and this is consistent with a report showing ZIKV RNA in leukocytes of maternal tissue surrounding the placenta in an infected patient (34, 35). These results suggest that ZIKV infects maternal cells in the decidua, which may be a source of type I IFNs that act on fetal cells of the underlying junctional zone or labyrinth layer to induce robust ISGs.

Next, we performed histological analysis of the developing placenta and decidua to determine the impact of ISG expression. At E9.5, the placenta and decidua were grossly normal in all groups: They showed normal decidualization, a layer of trophoblast giant cells, and a labyrinth with both maternal blood spaces, containing anucleated maternal RBCs, and fetal blood spaces, containing nucleated fetal RBCs (fig. S5). By E10.5, we observed marked abnormality in the labyrinth of the ZIKV-infected Ifnar1−/+ but not Ifnar1−/− placentas (Fig. 3B). Specifically, the labyrinth appeared to have denser cellularity, with decreased vascular spaces and minimal fetal cell content (Fig. 3B, arrows). In addition, there were abnormal spheroid structures (Fig. 3B, asterisk), likely composed of trophoblasts. The decidua and trophoblast giant cells were comparable between all groups. Although the labyrinth of the infected Ifnar1−/+ placentas looked more disorganized with increased cellularity compared with the uninfected controls, there were still abundant fetal RBCs within the fetal blood space (Fig. 3B). By E11.5, when most ZIKV-infected Ifnar1−/+ fetuses were resorbed, the labyrinth appeared disorganized with reduced fetal blood vessels and abundant spheroid structures, and by E12.5, Ifnar1−/+ placentas show almost no maternal or fetal blood vessels (fig. S5). In contrast, the labyrinths of Ifnar1−/+ placentas on E11.5 and E12.5 were

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indistinguishable from those of the uninfected controls with abundant adjacent fetal and maternal blood spaces (fig. S5). These results suggested that type I IFN induced in response to ZIKV interferes with development of fetal vasculature in the placenta labyrinth.

To examine this possibility, we stained tissue sections of E10.5 placentas from dams infected on E5.5 with cytokeratin (CK) to label trophoblasts and CD31 to label blood vessels. We observed a network of closely associated CD31+ (endothelial cell demarcating fetal blood space) and CK-positive (trophoblast demarcating maternal blood space) structures in both uninfected and ZIKV-infected Ifnar1−/− placentas (Fig. 3C). In contrast, CD31 staining in the ZIKV-infected Ifnar1−/− placentas was markedly reduced, wherein the limited CD31+ cells were found on the edge of the placental structure devoid of luminal spaces (Fig. 3C). Immunohistochemistry staining of the placentas with anti-CD31 antibody confirmed the collapsed vasculature and reduced number of the fetal endothelial cells (fig. S6A). Anti–E-cadherin antibody, which stains the trophoblasts, revealed that many of the densely packed cells in the E10.5 placenta were Ecad+ trophoblasts (fig. S6B). These results indicate that defective development of fetal vasculature and abnormal trophoblasts in the labyrinth immediately precede the death of the IFNAR-intact fetus after ZIKV infection of pregnant dams.

IFNAR signaling leads to increased apoptosis in the placental labyrinth, an abnormal maternal-fetal barrier, and fetal hypoxia

On the basis of the known functions of type I IFNs, we hypothesized that IFNAR signaling in the fetus inhibits placental development through three possible mechanisms: inducing immune cell recruitment and invasion of the labyrinth, blocking cellular proliferation, or inducing cell death (10, 11). At E10.5, CD45+ leukocytes were restricted to the decidua in all groups and did not infiltrate the CK-positive junctional zone or the labyrinth (fig. S6C), ruling out inflammatory leukocyte infiltration as the mechanism of fetal demise. Next, we examined cell proliferation in the labyrinth by Ki67 staining. All placenta showed abundant Ki67+ cells at E10.5 (Fig. 4A), excluding the role of IFNAR signaling in blocking cell proliferation as the mechanism of fetal demise. To examine whether IFNAR signaling is inducing cell death, we stained for activated (cleaved) caspase-3 (Casp3) as a marker of apoptotic cells. There was no Casp3-positive staining in the labyrinth of the uninfected placentas or infected Ifnar1−/− placentas at E10.5 (Fig. 4B). In contrast, in the infected Ifnar1−/− placenta, we detected Casp3 staining in the labyrinth in a pattern consistent with the endothelial cells, fetal blood cells, or adjacent trophoblasts surrounding the spheroid structure (Fig. 4B). These results are consistent with a previous report (13) and suggest a role for IFNAR in mediating apoptosis of fetal
endothelial cells and trophoblasts as an underlying mechanism of placental dysfunction.

To examine the abnormal placenta architecture at a cellular level, we performed electron microscopy to analyze the maternal-fetal interface on E10.5. In the uninfected placentas and infected Ifnar1−/− placentas, the expected trilaminar interhemal barrier was seen, with anucleated maternal blood (mrbc) and nucleated fetal blood (frbc) being separated by four layers of cells: the sinusoidal trophoblast giant cell (stgc) that directly contacts the maternal blood, two continuous syncytial layers of trophoblast that are closely connected (ST-I and ST-II), and a layer of endothelial cells (ec) that directly contact the fetal blood (Fig. 4C) (32, 36). ST-I and ST-II were tightly adhered to one another (Fig. 4C, arrows). There was no mixing between maternal and fetal blood in the uninfected placenta. In the infected Ifnar1+/− placentas, there were multiple instances of mixing between the maternal and fetal blood in the labyrinth (Fig. 4C). When separated maternal and fetal circulations were found, the barrier between the two was highly abnormal, with no evidence of the normal four-cell layer barrier and breakdown between cells making the maternal-fetal barrier (Fig. 4C). Thus, IFNAR signaling in the placenta leads to an abnormal maternal-fetal blood barrier with local breakdown.

On the basis of the findings of an abnormal vasculature in the placental labyrinth and abnormal maternal-fetal barrier, we examined the transcriptional changes in the fetus to examine whether lack of adequate gas exchange between mother and fetus may be contributing to fetal demise. We found that hypoxia response genes, including Vegfa, Adm, Bnip3, Glut1, and Pfkfb3, were all significantly upregulated in the ZIKV-infected Ifnar1+/− fetuses relative to uninfected controls and to their infected Ifnar1−/− littermates (Fig. 5, A to E) (37, 38). Thus, IFNAR signaling in the placenta leads to an abnormal maternal-fetal blood barrier with local breakdown.

IFNAR signaling mediates fetal death after subcutaneous ZIKV infection and poly(I:C) treatment
To determine whether IFNAR signaling mediates fetal resorption after other routes of ZIKV infection and with other strains
of ZIKV, we challenged Ifnar1−/− females crossed with Ifnar1+/− males subcutaneously with the Brazilian strain of ZIKV on E6.5 (13). After challenging with a high dose (3.4 × 10⁵ PFU) of ZIKV, most Ifnar1+/− fetuses were resorbed by E12.5, but Ifnar1−/− fetuses were grossly normal (fig. S7, C and D). Thus, IFNAR-dependent fetal resorption occurs after subcutaneous and intravaginal ZIKV challenge, with both Brazilian and Cambodian ZIKV strains.

To examine whether IFNAR signaling is sufficient to induce fetal resorption independent of ZIKV infection, we challenged mice with polyinosinic/polycytidylic acid [poly(I:C)], a double-stranded RNA viral mimic capable of eliciting robust type I IFN responses (39). After intraperitoneal injection of 200 μg of poly(I:C) at E7.5, all fetuses of WT females mated with WT males were resorbed by E9.5, and we could not recover any fetal material in five of six injected females by E10.5, consistent with previous reports (Fig. 6, A and C) (40). When we challenged Ifnar1−/− females crossed with Ifnar1+/− males with poly(I:C) on E7.5, the majority of both Ifnar1−/− and Ifnar1+/− fetuses continued to develop as examined on E10.5 and E12.5 (Fig. 6, B and C). Thus, maternal IFNAR signaling was necessary for mediating poly(I:C)-induced fetal resorption.

Type I IFN (but not type III IFN) treatment of human midgestation villous explants leads to deformation

To determine the impact of type I IFNs on the human placenta, we treated midgestation (19 to 23 weeks) human chorionic villous explants with a type I IFN, recombinant IFN-β, or a type III IFN, recombinant IFN-λ3. Isolated villi were treated with recombinant IFN within hours after their isolation, when their structure and morphology remained completely intact, as characterized by a continuous layer of CK-positive trophoblasts covering the surfaces of the isolated villi [Fig 7, A (left) and B (top)]. After ~16 to 20 hours of treatment, the architecture of IFN-β–treated villi became markedly abnormal, with areas of aggregated nuclei formed at the distal end of villi treated with IFN-β (either 100 or 1000 U) [Fig. 7, A (arrows) to C]. In contrast, treatment of the villous explants with IFN-λ3 showed no gross impact (Fig. 7, A and C). The abnormal villous structures resembled syncytial knots, which are associated with pathological states of pregnancy; sprouts, which represent overproliferation of the syncytiotrophoblast layer; or apoptotic shedding of IFNAR, we challenged Ifnar1−/− females with a sublethal dose of ZIKV (1 × 10⁵ PFU) subcutaneously at E6.5 and harvested the fetuses at E17.5. Again, similar to the vaginal ZIKV infection, all Ifnar1−/− fetuses were resorbed, but Ifnar1+/− fetuses were grossly normal (fig. S7, C and D). Thus, IFNAR-dependent fetal resorption occurs after subcutaneous and intravaginal ZIKV challenge, with both Brazilian and Cambodian ZIKV strains.

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(41, 42). In addition to the formation of syncytial knot– or sprout-like structures, we noted that IFN-β–treated villi also exhibited alterations in the actin cytoskeleton within the core of the villi as characterized by actin filament disassembly, suggesting widespread damage to the villi (Fig. 7D, bottom). To examine the global transcriptional changes in response to IFN-β and IFN-λ3 treatment, we performed whole-genome RNA-seq on villi treated with IFN-β or IFN-λ3 isolated from three different placental preparations. We found that both IFN-β and IFN-λ3 treatment induced significant transcriptional changes (273 and 101 total genes, respectively; \( P < 0.05 \)) as illustrated by MA plots (Fig. 8A). However, we found that there was little overlap between the genes induced by IFN-β and IFN-λ exposure—whereas IFN-β treatment induced the up-regulation of the majority of transcripts (260 of 273), IFN-λ treatment correlated with the down-regulation of the majority of transcripts (89 of 101) (Fig. 8B). Consistent with this differential expression pattern, only 12 transcripts were differentially expressed by both IFN-β and IFN-λ treatment (Fig. 8C). We found that whereas IFN-β treatment induced many known ISGs, IFN-λ treatment had little impact on ISG expression (Fig. 8, D to F). In addition, consistent with the significant morphologic alterations of villous architecture induced by IFN-β treatment, IFN-β–treated villi exhibited suppression (by ~4-fold, \( P = 0.04 \)) in the expression of the β chain of human chorionic gonadotropin, which is exclusively produced by syncytiotrophoblasts and is associated with placentation function. Collectively, these data suggest that type I IFN is sufficient to induce morphological alterations and also possibly adversely affect placental function in the human developing placental villi.

**DISCUSSION**

Our findings highlight the detrimental impact of type I IFNs on the developing placenta and fetus by demonstrating that only the fetuses with a functional copy of IFNAR are resorbed after ZIKV infection. IFNAR signaling in the conceptus leads to abnormal placenta labyrinth development with apoptosis in the labyrinth, impaired fetal endothelial development, and disrupted maternal-fetal blood barrier. IFNAR signaling was important in controlling viral replication in the placenta. Despite this, IFNAR-mediated pathology outweighed the benefit of IFNAR-dependent control of viral replication. How exactly IFNAR signaling leads to the observed labyrinth pathology is unknown. The hypoxic state of the IFNAR-sufficient fetus, likely resulting from the fetal endothelial disruption, suggests an impaired delivery of oxygen and possibly nutrients being the underlying cause of fetal demise. Our results do not rule out a role for the direct action of ZIKV in mediating certain aspects of pathology, such as microcephaly, ocular defects, or other neurological abnormalities, which we do not address in this study. Despite having a less severe phenotype than their Ifnar1−/− littersmates, Ifnar−/− mice did exhibit growth restriction compared with their uninfected counterparts, consistent with previous reports (43). This growth restriction may be due to poor maternal health, but it could also be indicative of IFNAR-independent causes of birth defects, including direct pathogenic effects of the virus infection or immune response unrelated to type I IFNs. One limitation to our study is that the host-pathogen interactions, including the suppression of host IFNAR signaling by ZIKV NS5 protein, are not preserved in mice. Thus, to what extent the mouse model recapitulates ZIKV infection and disease in humans is unknown. An additional limitation is that the structure and development of the
mouse placenta are significantly different from those of the human placenta, making it difficult to directly compare the pathological changes seen in the mouse placenta with those of humans.

To extend our findings to humans, we examined the impact of recombinant IFN-β on second-trimester villous explants. This experimental system allowed us to examine the impact of type I IFNs in the pre-existing syncytium and in a model that fully retains the architecture and multicellular composition of the human placenta. IFN-β exposure induced morphological changes in the human placenta, which correlated with alterations in syncytial and actin cytoskeletal architecture. Altered syncytial morphology resembled syncytial knots, a feature commonly associated with placentas of adverse pregnancy outcomes, including preeclampsia (42). Although common in full-term placentas, syncytial knots are rare in normal midgestation pregnancies (44). Given the short time scale over which they developed, they could also be apoptotic shedding of damaged cells. Our study required high levels (1000 U) of IFN-β to produce these effects, although we did note the appearance of syncytial knot–like structures at lower levels (100 U). It is difficult to assess whether this level of IFN may be present locally in congenital infections and what effects IFN-β may have over a longer period and during earlier stages of development, which were not possible to assess in this model. We found that treatment of villi with recombinant type III IFN, IFN-λ3, was not associated with the altered villous morphology or a strong ISG induction. These are consistent with previous reports that the syncytium constitutively produces IFN-λs (28) and may suggest that type III IFNs primarily function in an autocrine and paracrine manner to defend the developing fetus against viruses, as has been shown to occur in mice (20). Our data also showed that type I IFN, but not type III

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**Fig. 5.** ZIKV-infected *Ifnar1*−/− fetuses show up-regulated hypoxia response genes just before demise. *Ifnar1*−/− females mated to *Ifnar1*+/− males were infected intravaginally with 1.5 × 10^5 PFU Cambodian ZIKV at E5.5 and harvested at E10.5. RNA was extracted from fetuses, and expression of previously reported hypoxia-response genes *Vegfa* (A), *Adm* (B), *Bnip3* (C), *Pfkfb3* (D), and *Glut1* (E) analyzed by reverse transcription qPCR. Data represent *n* = 9 fetuses per genotype from three litters from ZIKV-infected litters and *n* = 3 *Ifnar1*−/− and *n* = 5 *Ifnar1*+/− fetuses from two uninfected litters. Data were normalized to *Hprt* and represented as fold change over *Ifnar1*−/− uninfected placentas.

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**Fig. 6.** Poly(I:C) injection of pregnant dams leads to fetal resorption in a maternal-IFNAR–dependent fashion. WT females mated to WT males (A) or *Ifnar1*−/− females mated to *Ifnar1*+/− males (B) were injected with 200-µg HMW poly(I:C) (PIC) at E7.5. Representative images from mice harvested between E9.5 and E12.5 are shown. Scale bars, 1 mm. (C) Crown-rump length was measured using ImageJ. Mean with SD and individual data points are shown. Data points represent the following: WT x WT litters untreated E9.5 (*n* = 17 from two litters), PIC E9.5 (*n* = 33 from four litters), untreated E10.5 (*n* = 13 from two litters), and PIC E10.5 (*n* = 8 from one litter); additional five injected litters showed no fetal remnants at time of harvest at E10.5. For *Ifnar1*−/−x*Ifnar1*+/− litters, untreated E10.5 (*n* = 15 *Ifnar1*−/− and *n* = 5 *Ifnar1*−/− from three litters), PIC E10.5 (*n* = 5 *Ifnar1*−/− and *n* = 11 *Ifnar1*+/− from two litters), untreated E12.5 (*n* = 11 *Ifnar1*−/− and *n* = 11 *Ifnar1*−/− from three litters), and PIC E12.5 (*n* = 13 *Ifnar1*−/− and *n* = 7 *Ifnar1*−/− from two litters). Uninfected measurements for *Ifnar1*−/−x*Ifnar1*+/− litters are the same as those shown in Fig. 2B.
IFN, treatment led to robust ISG induction in the villous explants. The mechanistic basis for this differential signaling remains unknown but may reflect the constitutive expression of type III IFN already secreted by the syncytium during midgestation, which may affect receptor binding by the recombinant protein or perhaps reflect some level of receptor desensitization (29). Alternatively, it could also reflect the more restricted tissue expression of IFN-λ receptor, which is limited to the epithelium (45). However, midgestation explants express high basal levels of many ISGs, suggesting that the tissue is likely responsive to type III IFNs (29). Consistent with this, even in the placenta of mice that lack IFNAR and harbor high levels of virus in the placenta, the virus was mostly restricted from the fetus, indicating that other structures and pathways are capable of restricting ZIKV from the fetus.

How might type I IFNs trigger fetal death? We did not observe any differences in leukocyte infiltration into the placenta or block in proliferation of cells in the placenta. However, we did observe apoptosis of cells in the labyrinth, which likely represented endothelial cells or adjacent trophoblasts. Consistent with this, human explants treated with IFN-β exhibited significant alterations in actin cytoskeletal structure, consistent with cellular damage. These results are consistent with a previous report showing similar placenta damage after ZIKV infection (13). In addition to these possibilities, type I IFN is also known to inhibit angiogenesis and blood vessel development (46) and consistent with the abnormal and reduced fetal blood vessels we observed in the labyrinth. Another possibility is that IFN may impair trophoblast fusion or differentiation. A previous report showed that IFN-β suppresses syncitin-1 expression (47). Consistent with this hypothesis, the placental histology and timing of death of the Ifnar1+/- fetuses resemble those of mice lacking syncitin-A, which have a defect in trophoblast fusion (36). We speculate that type I IFNs may serve as a quality control system to eliminate the developing embryo if coincident viral infection is detected and the levels of circulating IFNs reach a certain threshold. Many mammals carry their fetus for a prolonged period, to upward of ~650 days in elephants. This costly investment by the mothers may justify high levels of scrutiny of the health of the fetus at every level but particularly early in pregnancy when the embryo is vulnerable to various stressors (48, 49). Type I IFNs may report on the viral infection status of the mother or the fetus within the local milieu, sending abortive signals to terminate pregnancy.

The effects of IFN on the developing placenta likely have implications for pregnancy complications beyond ZIKV, and it could be a...
mechanism for adverse pregnancy outcomes, such as IUGR or early spontaneous abortions, which often occur without a known underlying etiology. Consistent with Type I IFN being an underlying cause of pregnancy complications, many congenital or “TORCH” (Toxoplasmosis, Other, Rubella, Cytomegalovirus, and Herpes) infections have a common presentation of microcephaly, cerebral calcifications, and IUGR (3). Beyond viral infections, our study may be relevant to diseases in which Type I IFNs are overproduced, collectively known as interferonopathies (50). Interferonopathies may be induced by monogenic mutations, such as those found in Aicardi-Goutières syndrome, or polygenic diseases, including systemic lupus erythematosus (SLE). Fetuses with Aicardi-Goutières syndrome can present with developmental defects similar to classic TORCH infection with fetal growth restriction, microcephaly, and intracerebral calcifications (50). SLE is associated with pregnancy complications, including fetal death in utero, preeclampsia, and preterm birth; and elevated serum IFN is one of the key factors that closely correlate with poor pregnancy outcomes (51). Our study implicates Type I IFNs as a possible common culprit for virus-associated pregnancy complications and suggests blockade of Type I IFNs as a possible intervention to prevent pregnancy complications in the settings of nonviral interferonopathies.

MATERIALS AND METHODS

Study design

To test the effect that fetal IFNAR signaling has on development after ZIKV infection, Ifnar1−/− females were crossed to Ifnar1+/− males to allow for direct comparison between littermates of different genotypes. Fetuses and placentas were harvested at various time points after infection and appearance, histology, and transcriptional changes were analyzed. To test how human placentas were affected by IFN signaling, midgestation chorionic villous explants were treated with IFN-β or IFN-λ, and villi were analyzed by immunofluorescent imaging or transcriptional changes were analyzed. For mouse studies, analysis was performed on litters containing both genotypes, and genotyping of litters was performed after initial analysis of fetal weight and appearance. No other formal randomization or blinding method was used. Subjects were assigned a litter and fetus number to allow unbiased selection for sample processing (RNA and imaging). A minimum of three infected litters were analyzed per time point. Exact n is indicated in figure legends. No formal statistical tool was used to determine power.

Mice

Ifnar1−/−, Ifnar1+/−, C57BL/6, and Ifγ−/− mice were bred and maintained at Yale University. All pregnant dams were between 9 and

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**Fig. 8.** ISGs are induced in IFN-β–treated villous explants. Human midgestation (19 to 23 weeks) chorionic villi were isolated, placed in culture, and treated with 1000 U of recombinant human IFN-β or 1000 U of IFN-λ for ~24 hours, and RNA was extracted. (A) MA plots generated in R after DESeq2 analysis demonstrating the differential expression between IFN-β–treated (left) or IFN-λ–treated (middle) villi relative to mock-treated controls and between IFN-β– and IFN-λ–treated villi (right). Data are plotted as log2 fold changes (y axis) and mean expression (x axis). Red symbols denote transcripts whose expression was differentially expressed at P < 0.05. (B) Graph demonstrating the number of transcripts up-regulated (in green) or down-regulated (in red) after IFN-β or IFN-λ treatment of villi. (C) Venn diagram denoting the overlap of transcripts (12 in total) between villi treated with IFN-β and those treated with IFN-λ. (D) Heat map (based on log reads per kilobase of transcript per million mapped reads values) of known ISGs from mock-, IFN-β–, or IFN-λ–treated villi. (E and F) Volcano plots of villi treated with IFN-β (E) or IFN-λ (F) denoting specific ISGs differentially expressed by treatment. Red circles denote ISGs and purple circles denote non-ISG transcripts. For RNA-seq experiments, data represent villi isolated from three independent placental preparations.
20 weeks of age. Littermates were randomly assigned to infected or uninfected groups. Ifnar1+/− male breeders are the F1 generation from cross between C57BL/6 (B6) and Ifnar1−/− parents. Matings were timed by checking for the presence of a vaginal plug, indicating gestational age E0.5. About 11% of infected concepti were analyzed between E9.5 and E12.5 and 17% of uninfected concepti developed into abnormal spheroid shapes (distinct from infection-induced resorbed fetuses), without the presence of a fetus or yolk sac. These were excluded from analysis because of inability to obtain adequate fetus-derived tissue for genotyping analysis and because it was present in equal frequencies for infected and uninfected groups. All animal procedures were performed in compliance with Yale Institutional Animal Care and Use Committee protocols.

**Midgestation chorionic villous explants**

Human fetal placental tissue from 19 to 23 weeks' gestation that resulted from elective terminations was obtained from the University of Pittsburgh Health Sciences Tissue Bank through an honest broker system after approval from the University of Pittsburgh Institutional Review Board and in accordance with the University of Pittsburgh anatomical tissue procurement guidelines. Chorionic villi (about 1 cm x 1 cm in size) were dissected and cultured in Dulbecco’s modified Eagle’s medium (DMEM)/F12 (1:1) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin, and amphotericin B. Immediately after isolation, isolated villi were treated with the indicated dose of recombinant IFN-β or IFN-α for 4 hours, and then tissue was fixed and processed for imaging. For imaging studies, villi were fixed in 4% paraformaldehyde (PFA) followed by permeabilization of 0.25% Triton X-100 for 30 min at room temperature with gentle agitation, washed in phosphate-buffered saline (PBS), incubated with primary antibody, washed again in PBS, and then incubated with Alexa Fluor-conjugated secondary antibodies. Alexa Fluor–conjugated phalloidin was purchased from Invitrogen (A12379 or A12381). Rabbit anti-CK19 (ab52625) was purchased from Abcam. After staining, villi were mounted with VECTASHIELD (Vector Laboratories) and 3,3′-diamidino-2-phenylindole (DAPI), and images were captured with an Olympus FV1000 confocal or Zeiss LSM 710 confocal microscope. Images were adjusted for brightness/contrast using Adobe Photoshop (Adobe), and syncytial knot size was calculated using Imaris.

**Viruses and in vivo infections**

ZIKV Cambodian FSS10325 stain, obtained from the World Reference Center for Emerging Viruses and Arboviruses at University of Texas Medical Branch, Galveston, was used for intravaginal infection studies. Stocks were propagated in Vero cells and titrated by plaque assay as previously described (14). ZIKV Brazilian PE243, used for subcutaneous infections, was recovered from a 19-year-old female in Brazil from 2015 (52). Vero cells were obtained from the American Type Culture Collection. Cell lines were verified by morphology and were tested for mycoplasma contamination every 1 to 2 years.

Intravaginal infection was performed as previously described: At E5.5 or E8.5, a calginate swab (Fischer Scientific) was used to remove mucus from the vaginal lumen, and 1.5 × 10⁶ PFU of ZIKV was inoculated into the vagina using a pipette (14). Subcutaneous infection was performed by injecting 100 μl of virus stock diluted in PBS (3.4 × 10⁶ or 1 × 10⁶ PFU) into the footpad. Pregnant mice were euthanized, tissues were harvested at indicated time points, and fetuses and placentas were either collected in TRIzol (for RNA extraction), fixed in 4% PFA (for imaging), or collected in DMEM with 10% FBS and penicillin/streptomycin (for plaque assay). Fixed fetuses were imaged using a Zeiss Discovery V8 stereomicroscope (Zeiss). Yolk sac for E9.5 to E12.5 or tail for E17.5 fetuses was collected for each fetus and genotyped using the following primers: ATTATAAAAAGAGAAGAGGAGCGAACCTGG (forward) and AAGATATGCTGTTC-CTCTCCTCTGTCTGA (reverse), with a 150–base pair product, indicating the presence of a WT copy of IFNAR.

**Poly(I:C) challenge**

Two hundred micrograms of HMW VacciGrade Poly(I:C) (InvivoGen) was injected intraperitoneally into pregnant mice at E7.5. Mice were then harvested between E9.5 and E12.5 to examine fetuses.

**Quantification of ZIKV genome, ISGs, and hypoxia response genes by qRT-PCR**

Tissues were extracted using TRIzol (Thermo Fischer Scientific) and purified using an RNeasy Mini Kit (Qiagen). iScript cDNA synthesis kit (Bio-Rad) was used to synthesize cDNA. Quantitative polymerase chain reaction (qPCR), which was performed using SYBR green (Bio-Rad), was used to quantify ZIKV levels, ISGs, and hypoxia response genes and ran on a CFX Connect instrument (Bio-Rad). Primer sequences are provided in table S1. Virus and ISGs were normalized to Hprt (14).

**Histology, immunofluorescence, and immunohistochemistry staining of mouse placentas**

Placentas were fixed in 4% PFA overnight at 4°C. For immunohistochemical (IHC) and hematoxylin and eosin (H&E) staining, tissues were embedded in paraffin blocks and sectioned by the Yale Pathology Tissue Services. H&E was performed by the Yale Pathology Tissue Services. For IHC, paraffin sections were heated for 30 min at 55° to 60°C. Antigen retrieval was performed by boiling in sodium citrate (J.T. Baker) (pH 6.0) for 60 min. Blocking was performed using PBS (AmericanBio, 1% bovine serum albumin (BSA) (Sigma-Alrich), and 0.5% Tween 20 (Sigma-Alrich) adjusted to pH 7.4. Slides were stained for CD31 (goat, 1:1000, R&D AF3628), Casp3 (rabbit, 1:1000, Cell Signaling Tech 9664T), Ecad (1:500, Thermo Fischer Scientific 13-1900), and Ki67 (rabbit 1:1000, Cell Signaling Tech 12202T) at 4°C overnight. Slides were blocked with Blockxall (Vector Laboratories) and stained with rat (for Ecad), rabbit (for Casp3, Ki67), or goat (for CD31) ImmPRESS antibodies (Vector Laboratories) and 3,3′-diaminobenzidine (DAB) (Vector Laboratories) per the manufacturer’s instructions. For immunofluorescence staining, slides were embedded in optimal cutting temperature media (Tissue Tek). Five- to seven-micrometer frozen sections were cut using a cryostat, and sections were allowed to dry at room temperature. Sections were blocked with 2% normal Donkey serum (Jackson ImmunoResearch) in PBS with Tween 20 and 1% BSA (Sigma-Alrich) and stained with ZIKV-immune rat serum (53), CD45 (R&D Systems AF3628), CK (Dako Z0622), or CD31 (R&D Systems AF3628) overnight at room temperature. Sections were then stained with A488 anti-rat secondary (Thermo Fischer), A647 anti-rabbit (Thermo Fischer), Cy3 anti-rabbit (Jackson ImmunoResearch), A488 anti-goat (Thermo Fischer), or NL557 anti-goat (R&D Systems). Samples were stained with DAPI and mounted with ProLong Diamond Antifade Mountant (Molecular Probes). H&E and IHC images were captured using light microscopy (BX51; Olympus), and immunofluorescence images were captured using fluorescence microscopy (BX51; Olympus) or confocal microscopy (TCS SP2; Leica). Images were merged and brightness and contrast were adjusted using ImageJ.
Transmission electron microscopy of mouse placentas

Placentas were fixed in formaldehyde/glutaraldehyde 2.5% in phosphate buffer for at least 24 hours. Samples were washed and secondarily fixed in osmium tetroxide; negative staining was performed with uranyl acetate, treated in ascending alcohols, and finally embedded in Durcupan ACM (EMS 14040). Ultrathin sections (70 nM) were cut on a Leica ultramicrotome, collected on Formvar-coated grids, and analyzed on a Tecnai 12 FEI electron microscope.

RNA-seq and qRT-PCR analysis of migestation chorionic villous explants

RNA was isolated from the villous explants using GenElute RNA total RNA miniprep kit (Sigma-Aldrich) and treated with deoxyribonuclease (Sigma-Aldrich). For RNA-seq, as previously described (28, 54), libraries were prepared using New England Biolabs Ultra Library Preparation kit. An Illumina HiSeq2500 was used for sequencing, and CLC Genomics Workbench 9.0 (Qiagen) was used to process, normalize, and map sequence data to the human reference genome (hg19). DESeq2 in R (55) or CLC Genomics Workbench 9 was used to identify differentially expressed genes and to generate MA plots.

Statistical analysis

In all analyses except for RNA-seq, data analysis was performed using Microsoft Excel and GraphPad Prism. Exact statistical test and value of \( n \) are detailed in the figure legends. A Tukey’s multiple comparison test or Dunnnett’s multiple comparison test was used to determine significance when determining significance between multiple groups (\( >3 \)). A paired Student’s \( t \) test was used when comparing only two groups. \( t \) tests assumed a normal distribution for all samples and \( t \) tests assumed an unequal standard deviation and variance between groups.

SUPPLEMENTARY MATERIALS

immunology.sciencemag.org/cgi/content/full/3/19/eaao1680/DC1

Methods

Fig. S1. ISG expression is elevated in \( \text{Ifnar}^+/- \) placentas and fetuses but not in \( \text{Ifnar}^-/- \) after ZIKV infection.

Fig. S2. \( \text{Ifnar}^-/- \) placentas harbor more infectious ZIKV compared with \( \text{Ifnar}^+/- \) littermates.

Fig. S3. Global gene expression analysis reveals elevated ISG levels in infected \( \text{Ifnar}^-/- \) placentas.

Fig. S4. ZIKV infection of the maternal-fetal interface is restricted to the decidua.

Fig. S5. Placental architecture of \( \text{Ifnar}^-/- \) fetuses is normal at E9.5 but disrupted at E11.5 and E12.5.

Fig. S6. Labyrinth of \( \text{Ifnar}^-/- \) placenta exhibits decreased fetal endothelial cells.

Fig. S7. \( \text{Ifnar}^-/- \) but not \( \text{Ifnar}^-/- \) fetuses are resorbed after subcutaneous infection with Brazilian ZIKV.

Table S1. Primers for mouse qPCR

Table S2. Top differentially regulated genes and pathways in ZIKV-infected \( \text{Ifnar}^-/- \) versus \( \text{Ifnar}^-/- \) placentas.

Table S3. Individual values included in all graphs

REFERENCES AND NOTES

1. Pan American Health Organization/World Health Organization, Zika suspected and confirmed cases reported by countries and territories in the Americas Cumulative cases, 2015-2016 (Washington, D.C., 2016).


Research
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Resveratrol stimulates the metabolic reprogramming of human CD4+ T cells to enhance effector function

Marco Craveiro,1,2* Gaspard Cretenet,1* Cédric Mongellaz,1 Maria I. Matias,1 Olivier Caron,3,4 Maria C. Pedroso de Lima,2 Valérie S. Zimmermann,1 Eric Solary,3,4 Valérie Dardalhon,1 Vjekoslav Dulić,† Naomi Taylor1†

The polyphenol resveratrol activates the deacetylase Sirt1, resulting in various antioxidant, chemoprotectant, neuroprotective, cardioprotective, and anti-inflammatory properties. We found that at high concentrations of resveratrol, human CD4+ T cells showed defective antigen receptor signaling and arrest at the G1 stage of the cell cycle, whereas at low concentrations, cells were readily activated and exhibited enhanced Sirt1 deacetylase activity. Nevertheless, low-dose resveratrol rapidly stimulated genotoxic stress in the T cells, which resulted in engagement of a DNA damage response pathway that depended on the kinase ATR (ataxia telangiectasia–mutated (ATM) and Rad3-related), but not ATM, and subsequently in premitotic cell cycle arrest. The concomitant activation of p53 was coupled to the expression of gene products that regulate cell metabolism, leading to a metabolic reprogramming that was characterized by decreased glycolysis, increased glutamine consumption, and a shift to oxidative phosphorylation. These alterations in the bioenergetic homeostasis of CD4+ T cells resulted in enhanced effector function, with both naïve and memory CD4+ T cells secreting increased amounts of the inflammatory cytokine interferon-γ. Thus, our data highlight the wide range of metabolic adaptations that CD4+ T lymphocytes undergo in response to genomic stress.

INTRODUCTION

Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a natural polyphenolic compound that is produced by plants in response to environmental stress, providing them with protection from microbial infections (1–4). Resveratrol appears to mimic the effects of caloric restriction, increasing life span in lower organisms (5). Furthermore, this pharmacological agent has elicited much interest because of its potential to modulate a diverse array of pathological conditions, and it is associated with anticancer, antiaging, and anti-inflammatory properties (6–10). On the basis of the promising data emerging from ex vivo studies and preclinical animal models, resveratrol has been tested in more than 30 clinical trials involving more than 1000 individuals. Nevertheless, the specific pathologies in which resveratrol has substantial clinical benefits are not yet clear (11–16).

The pharmacological properties of resveratrol have been attributed, at least in part, to its activation of the nicotinamide adenine dinucleotide (NAD+)–dependent silent information regulator 2 (Sirt2) deacetylase (17) both in vitro (18, 19) and in vivo (20). Overexpression of the mammalian Sir2 homolog sirtuin-1 (Sirt1) in mice extends their life span (21, 22) and protects them from a diverse array of diseases (23–28). Conversely, knocking out Sirt1 is associated with autoimmunity (29–32). However, the effects of Sirt1 are likely to be complex. Although Sirt1 attenuates murine T cell signaling and effector function (29, 30, 33–35), it also promotes the differentiation of naive CD4+ T cells into T helper 1 (Th1) effector cells in mice (36). Furthermore, physiological modifications of Sirt1 function in human T cell subsets have thus far not been evaluated.

†GMM, CNRS, Université de Montpellier, Montpellier, France. ‡CNC–Centre for Neuroscience and Cell Biology, University of Coimbra, Coimbra, Portugal. §INSERM U1170, Gustave Roussy Cancer Center, Villejuif, France. ††Faculty of Medicine, Université Paris-Sud, Le Kremlin-Bicêtre, France.

*These authors contributed equally to this work. ††Corresponding author. Email: vjekoslav.dulic@gmm.cnrsc.fr (V.D.); taylor@gmm.cnrs.fr (N.T.)

T cell activity is of great importance in a wide range of pathophysiological conditions for which resveratrol activity is being clinically evaluated. Hence, elucidating the potential on-target and off-target effects of resveratrol on T lymphocytes is critical. T cells present a complex target because their cellular metabolism is altered after activation by a cognate antigen. The capacity of T lymphocytes to respond to stimulation by antigen depends on an extensive proliferative response, a process that requires new energetic and biosynthetic components that are supplied, at least in part, through a metabolic shift from oxidative phosphorylation (OXPHOS) toward glycolytic and glutaminolytic pathways (37–39). This shift from OXPHOS contrasts with the activity of resveratrol, a compound that generally increases mitochondrial activity and associated OXPHOS (40–42). However, note that resveratrol leads to a wide range of effects, including decreased, stabilized, and enhanced T cell effector functions (43–46).

Disparate effects of resveratrol on genomic stability have also been reported. In some studies, resveratrol contributes to genomic stability and reduces tumorigenesis by reducing the amount of reactive oxygen species (ROS), which leads to oxidative damage (47–51). However, in other studies, resveratrol mediates DNA damage, facilitating antitumor treatments (47, 52–59). One possible reason for these discrepancies could be that resveratrol has distinct effects on quiescent cells versus proliferating cells. In this regard, T lymphocytes present a challenging target. Although they are generally quiescent, exposure to foreign antigen rapidly stimulates cell cycle entry and cellular proliferation. A coordinated response to genotoxic stress is regulated by the kinases ATM (ataxia telangiectasia–mutated) and ATR (ATM and Rad3-related) (60, 61). Of interest are reports that resveratrol activates one or both of these kinases in different cellular contexts (53–55). Here, we report that resveratrol rapidly stimulates the ATR-dependent damage pathway in antigen-stimulated human CD4+ T cells, with activation of the tumor suppressor p53. This genotoxic stress response links a metabolic reprogramming to an enhanced CD4+ T cell effector function characterized by increased production of the cytokine interferon-γ (IFN-γ).
RESULTS
Low-dose resveratrol increases the activity of the NAD+-dependent deacetylase SirT1 in primary human CD4+ T cells

To gain insight into the role of resveratrol in modulating SirT1 function in human CD4+ T lymphocytes, we first examined its expression profile in response to T cell receptor (TCR) stimulation. We found that TCR engagement resulted in a substantial increase in SirT1 abundance, with augmented nuclear localization and aggregation (Fig. 1A). Low-dose resveratrol (20 μM) further increased the mean fluorescence intensity (MFI) of SirT1 staining by about twofold (Fig. 1A and fig. S1A). However, high-dose resveratrol (100 μM) attenuated the TCR-mediated increase in SirT1 abundance, and these CD4+ lymphocytes did not undergo blast formation (Fig. 1A and fig. S2A). This differed markedly from treatment with low-dose resveratrol, which augmented blast size (fig. S2A). Separating subsets of TCR-stimulated CD4+ T cells based on their forward scatter (FSC) and side scatter (SSC) profiles demonstrated that SirT1 abundance paralleled increases in cell size and granularity (Fig. 1B). As expected from these data, low-dose resveratrol substantially increased SirT1 activity in TCR-stimulated lymphocytes, as monitored by the generation of O-acetyl–adenosine diphosphate–ribose (OAADPr), a reaction product of the SirT-catalyzed, NAD+-dependent deacetylation of target proteins (Fig. 1C). Thus, TCR stimulation combined with low-dose, but not high-dose, resveratrol augments SirT1 activity in human T lymphocytes.

Low- and high-dose resveratrol stimulate distinct types of G1, and premitotic cell cycle arrest in TCR-stimulated CD4+ T cells

In light of our findings that high-dose resveratrol inhibited TCR-mediated increases in T cell size and granularity, it was of interest to determine how different doses of resveratrol affected T cell proliferation and cell cycle progression. Although cell viability was not affected by resveratrol (fig. S2A), high-dose resveratrol inhibited the entry of T lymphocytes into the G1 phase of the cell cycle, as assessed by the detection of reduced amounts of total RNA (Fig. 2A and fig. S1B). On the other hand, T cells exposed to low-dose resveratrol exhibited a cell cycle entry and progression that was equivalent to that observed in control TCR-stimulated cells, with about 40% of cells having entered into S phase by day 3 of stimulation (Fig. 2A and fig. S1B). However, note that low-dose resveratrol almost completely abrogated TCR-mediated cellular proliferation (Fig. 2B and fig. S1C), an effect that was not alleviated by the addition of exogenous interleukin-2 (IL-2) nor by extended time in culture (fig. S2, B and C).

Cell cycle entry and progression are tightly controlled processes involving the action of cyclin-dependent kinases (Cdk) and cyclins (Fig. 2C). To understand the molecular bases underlying the cell cycle arrest caused by different doses of resveratrol, we investigated the regulation of components of the cell cycle machinery. Cyclins D2, E1 and A2, and B1, as well as their cognate kinases, Cdk4/ Cdk6, Cdk2, and Cdk1, respectively, were increased in abundance upon T cell activation. Although this increased abundance was not altered by low-dose resveratrol, it was significantly attenuated by high-dose resveratrol under conditions in which high amounts of the Cdk inhibitor p27Kip1 were maintained (P < 0.05 at day 1 and P < 0.005 at day 3; Fig. 2, D and E, and fig. S1D). However, by day 3 of activation, high-dose resveratrol-treated cells showed increased cyclin D2 and cyclin E1, but these cells did not progress into S phase (Fig. 2A). Cdk2, cyclin A2, and Cdk1 were not detectable, and phosphorylation of the
Fig. 2. Low- and high-dose resveratrol block TCR-mediated cell cycle progression at distinct stages of the cell cycle. (A) Cell cycle entry after TCR stimulation at day 1 (D1; top) and day 3 (D3; bottom) in the presence or absence of resveratrol was monitored by simultaneous staining of DNA and RNA with 7-aminoactinomycin D and pyronin Y, respectively. Representative dot plots from five experiments of nonstimulated and TCR-activated CD4 T cells, in the absence or presence of resveratrol, are shown. The percentages of cells in G0-G1A phase (lower left quadrant), G1B phase (lower right quadrant), and S, G2, and M phases (upper right quadrant) are indicated. (B) T cell proliferation under the indicated conditions was monitored by carboxyfluorescein diacetate succinimidyl ester (CFSE) labeling, and dilution of the fluorescent dye was assessed at 72 hours. The number of division peaks is indicated in each histogram. Data are representative of six experiments. (C) Schematic representation of cell cycle regulators that are altered upon TCR-mediated cell cycle entry. Cell cycle progression requires the expression of cyclins and Cdk5, the F-box protein Skp2-dependent and ubiquitin-mediated degradation of the p27Kip1 Cdk inhibitor, and Cdk-mediated hyperphosphorylation of the pRB tumor suppressor and the related p130 pocket protein. Cdk1 activity and mitotic entry are regulated by the kinase Wee1 and the phosphatase of Cdc25. (D) The abundances of the cyclins-Cdks that regulate cell cycle entry, including cyclins D2, E1, A2, and B1, and Cdk4, Cdk6, Cdk2, and Cdk1 were monitored by Western blotting analysis on days 1 and 3 of activation. Data are representative of three independent experiments. The arrow indicates the hyperphosphorylated Cdk1 isoform. (E) The abundances of the Ki-67 proliferation marker, cell division inhibitors (pRB, p130, and p27), and the p27 regulator Skp2 under the indicated conditions were monitored by Western blotting analysis. Data are representative of three independent experiments. Arrows indicated hyperphosphorylated p130 and phosphorylated Skp2. Quantification of all panels is shown in fig. S1.

Low-dose resveratrol does not alter TCR-stimulated proximal and distal signaling cascades

To determine whether the cell cycle blockade mediated by low-dose resveratrol was due to defective initiation of the TCR signaling cascade, we first assessed proximal signaling intermediates. The kinase ZAP-70, which is associated with the TCRζ chain in activated lymphocytes, was phosphorylated by 1 min after TCR engagement and was not affected by either low- or high-dose resveratrol (Fig. 3A). Further downstream signaling was monitored as a function of extra-cytosolic phosphatase 1/2 (ERK1/2) and AKT phosphorylation. Neither ERK1/2 nor AKT phosphorylation was altered by low-dose resveratrol, and phosphorylation was only marginally decreased in the presence of high-dose resveratrol (Fig. 3A, B, and fig. S1F).

TCR signaling also activates mammalian target of rapamycin (mTOR) (64–66), a serine/threonine protein kinase that integrates environmental cues such as nutrients, growth factors, and stress signals into an "optimal" cellular response (67, 68). Sirt1 activity generally inhibits mTOR signaling (69–71), but in murine T cells, ectopic Sirt1 has not been shown to alter this cascade (35). mTOR complex 1 (mTORC1) activity is negatively regulated by tuberous sclerosis complex 1/2 (TSC1/2), which serves as a hub for both positive and negative cues for signaling kinases. Phosphorylation of TSC2 at T1462 by Akt leads to the activation of mTORC1, and in human CD4+ T cells, low-dose resveratrol did not affect the TCR-mediated phosphorylation of this signaling molecule.
Furthermore, neither phosphorylation of mTOR itself nor S6 ribosomal protein, a downstream mTOR substrate, was altered by low-dose resveratrol. Note that phosphorylation was significantly decreased in the presence of 100 μM resveratrol (P < 0.01; Fig. 3C and fig. S1G). Thus, mTOR signaling in TCR-stimulated T cells is attenuated by high-dose resveratrol, whereas at low doses, the activity resulting from TCR engagement is maintained.

We next assessed whether distal TCR signaling was altered by resveratrol, monitored as a function of the cell surface abundance of the CD69, CD25 (IL-2Rα subunit), and CD71 (transferrin receptor) activation markers. Surface abundance of CD69, due to the translocation of intracellular stores to the cell membrane without a requirement for protein synthesis (72, 73), was increased in most of the activated cells, irrespective of the presence of resveratrol (Fig. 3D). In marked contrast, induction of CD25 and CD71, both of which are dependent on de novo protein synthesis, was significantly attenuated by high-dose resveratrol but was unaffected by low doses of the polyphenol (P < 0.001; Fig. 3D). Thus, only high-dose resveratrol impedes mTOR and distal TCR signaling cascades.

Low-dose resveratrol stimulates a replication stress response in TCR-stimulated CD4+ T cells

The experiments performed thus far demonstrated that low-dose resveratrol inhibits CD4+ T cell division under conditions in which TCR and mTOR signaling responses are maintained. To further explore this phenomenon and to determine the origin of the cell cycle arrest, we focused on the effects of low-dose resveratrol on genomic integrity. Resveratrol has been found to both positively and negatively affect genome integrity in cancer cells (57, 74–78), but its function in primary human T cells has not been elucidated. To specifically address this point in T lymphocytes, we monitored histone H2AX phosphorylation (γH2AX). This modification identifies DNA damage foci as well as stalled replication forks that promote the concentration of repair proteins (79, 80).

TCR engagement of CD4+ T cells did not result in the augmentation of γH2AX (Fig. 2, A and D), at least at time points before entry into S phase (2 to 24 hours; Fig. 4A). However, in low-dose resveratrol, γH2AX+ cells reached significantly higher percentages by 24 hours (45%, P < 0.005; Fig. 4A); these percentages were similar to those detected in the presence of aphidicolin, an inhibitor of replication polymerases that stalls replication forks and results in a late G2-phase arrest (81). High-dose resveratrol had a distinct effect, increasing the abundance of H2AX foci in 7 to 10% of cells, irrespective of the kinetics or TCR stimulation. The lower amount of H2AX phosphorylation in cells treated with high-dose resveratrol may be due to their attenuated response to TCR stimulation (Fig. 3). Notably, stimulation with the homeostatic cytokine IL-7 significantly increased γH2AX in cells treated with high, but not low, doses of resveratrol (P < 0.005; Fig. 4B), suggesting that resveratrol effects on genomic integrity are likely to be dependent on the nature of the activation signal. Whereas it is not known how IL-7 signaling affects the potential of resveratrol to alter genomic integrity or its response to this stress, note that the addition of IL-2 did not alter resveratrol-driven H2AX phosphorylation in TCR-stimulated T cells (Fig. 4C).
**Fig. 4. Low-dose resveratrol stimulates H2AX phosphorylation in TCR-stimulated CD4+ T lymphocytes.** (A) Top: Freshly isolated quiescent CD4+ T cells were either left nonstimulated or were TCR-stimulated in the presence of resveratrol (20 and 100 μM) or aphidicolin. The amount of H2AX phosphorylation (γH2AX) was assessed at 24 hours by flow cytometry. Data are representative of four independent experiments. Bottom left: The percentages of γH2AX-positive cells were quantified after 2, 6, 12, and 24 hours of stimulation. Data are representative of four independent experiments. Bottom right: Means ± SEM of three experiments with statistical significance determined by one-way ANOVA with Tukey’s post hoc test. **P < 0.005. (B) Left: CD4+ T cells were cultured with the homeostatic cytokine IL-7 (10 ng/ml) in the absence or presence of resveratrol (20 and 100 μM). H2AX phosphorylation (γH2AX) was assessed at 24 hours by flow cytometry. Dot plots are representative of three experiments. Right: Means ± SEM of three independent experiments. Data were analyzed by one-way ANOVA with Tukey’s post hoc test. (C) CD4+ T cells were TCR-stimulated in the presence or absence of resveratrol (20 and 100 μM) and in the presence or absence of IL-2 (50 U/ml). H2AX phosphorylation was assessed by flow cytometry as described earlier, and the increase in phosphorylation relative to that in cells stimulated by TCR engagement alone is shown. Data are means ± SEM of three experiments with statistical significance determined by one-way ANOVA with Tukey’s post hoc test. **P < 0.01 and ***P < 0.005.

**Resveratrol stimulates p53 phosphorylation and ATR-mediated cell cycle arrest**

In light of the data shown earlier, it was critical to monitor activation of the p53 tumor suppressor, a protein that is phosphorylated in response to a wide range of genotoxic insults (60). At day 1 of TCR stimulation, low-dose resveratrol resulted in the accumulation and phosphorylation of p53, similar to that detected under conditions of aphidicolin-mediated stalled replication. Furthermore, by day 3, p53 phosphorylation was also increased under high-dose resveratrol conditions (P < 0.005; Fig. 5A and fig. S1H). Several hypotheses could account for the phosphorylation of p53: first, activation of the adenosine monophosphate–activated protein kinase (AMPK) pathway (82–84); second, activation of the ATM pathway in response to double-stranded DNA breaks (60, 85); and third, activation of the ATR pathway in response to single-stranded breaks and replication stress due to stalled fork progression or DNA synthesis (61). Regarding the first hypothesis, glucose deprivation and AMPK activation have been specifically shown to stimulate p53 phosphorylation (82, 83). However, an AMPK inhibitor (compound C) did not significantly alter resveratrol-stimulated p53 phosphorylation, although it decreased AMPK phosphorylation by a mean of 75% (Fig. 5B and fig. S1I). These data suggest that AMPK signaling does not drive p53 phosphorylation in resveratrol-treated CD4+ T lymphocytes.

To address the second and third hypotheses, we assessed the implication of the ATM and ATR pathways in the response of CD4+ T cells to resveratrol. Classically, ATR is stimulated by replication stress or single-stranded DNA breaks, resulting in the activation of checkpoint kinase 1 (Chk1) (86) and its downstream target, the kinase Wee1, a negative mitotic regulator (Fig. 5C) (85). Although Chk1 activation is often underestimated because of its transient phosphorylation (87), resveratrol stimulated both Chk1 and Wee1 phosphorylation (Fig. 5D and fig. S1J). Chk1 and Wee1 block Cdk1 activation by inhibiting Cdc25 phosphatase and hyperphosphorylating Cdk1, respectively (Fig. 5C). Accordingly, ATR signaling in response to low-dose resveratrol was associated with a defective dephosphorylation of Cdk1 that is required for Cdk1 activation and mitotic entry (Fig. 5D, arrow). In contrast, the ATM pathway was not activated by low-dose resveratrol because neither phosphorylation of Chk2 nor the downstream Cdk inhibitor p21Waf1/Cip1 (p21) was detected (Fig. 5D and fig. S1J). As positive controls, we assessed the capacity of bleomycin and aphidicolin to efficiently activate ATM/ATR and ATR pathway intermediates, respectively (Fig. 5, C and D).

These data suggested that the early H2AX phosphorylation in resveratrol-treated CD4+ T cells was due to a replication stress–like insult rather than an ATM signaling cascade stimulated by double-stranded DNA breaks. To test this possibility, we assessed the phosphorylation of the minichromosome maintenance (MCM) helicase complex, a key component of the prereplication complex that is specifically phosphorylated by ATR at Ser861 in response to multiple forms of DNA damage (88–90). Before the onset of S phase (24 hours), Mcm2 phosphorylation at S108 was augmented by low-dose resveratrol to a similar extent to that detected in response to aphidicolin and bleomycin (Fig. 5D and fig. S1J). Furthermore, although T cells exposed to high-dose resveratrol never progressed to S phase (Fig. 2, A and D), p53, Chk1, Wee1, and Mcm2 phosphorylation were also detected in these cells by 72 hours after stimulation (Fig. 5D). Together, these data suggest that resveratrol activates the ATR, but not ATM, signaling cascade in stimulated CD4+ T cells.

We therefore assessed whether the resveratrol-stimulated phosphorylation of p53 was directly regulated by ATR signaling in resveratrol-treated...
To this end, we tested the effects of VE-821, a potent ATR inhibitor (91, 92). As expected, VE-821 decreased Mcm2 phosphorylation (Fig. 5E and fig. S1K). Moreover, p53 phosphorylation was attenuated by a mean of 90% in the presence of VE-821, and the global amount of p53 decreased ($P < 0.001$; Fig. 5E and fig. S1K), demonstrating that the ATR stress response pathway regulates the resveratrol activation of p53 in TCR-stimulated CD4$^+$ T cells. We also found that the ATR pathway is directly implicated in the formation of $\gamma$H2AX foci (61) as VE-821 inhibited resveratrol activation of H2AX phosphorylation ($P < 0.05$; Fig. 5F). Together, these data reveal a critical role for the ATR cascade in mediating a p53-associated stress response in response to resveratrol.

CD4$^+$ T cells exhibit increased expression of p53-dependent target genes and undergo a metabolic switch in response to low-dose resveratrol

To elucidate potential molecular mechanisms associated with resveratrol treatment of CD4$^+$ T cells, we performed an array analysis of genes involved in DNA damage signaling responses. Whereas the expression
of multiple genes was altered after treatment with high-dose resveratrol or aphidicolin, only 1 of 84 assessed genes, Bbc3 [PUMA (p53 up-regulated modulator of apoptosis)], was consistently increased in expression in low-dose resveratrol–treated CD4+ T cells (4.1- to 6.4-fold, n = 3; Fig. 6A and fig. S3). Furthermore, it was even more highly expressed at high-dose resveratrol (8- to 22-fold, n = 3; fig. S3). Notably, PUMA is a proapoptotic gene whose transcription is directly regulated by p53 (93). Because the p53 pathway has also been linked to a metabolic reprogramming (94–96), at least in part through the regulation of metabolic genes, we assessed whether expression of these genes is altered by resveratrol. Notably, expression of TIGAR (TP53-induced glycolysis and apoptosis regulator), PGM (phosphoglycerate mutase), GLS2 (glutaminase 2), and SCO2 (synthesis of cytochrome c oxidase 2) was significantly altered by resveratrol (Fig. 6B). p53-mediated induction of TIGAR, a fructose-2,6-bisphosphatase, and attenuated amounts of PGM, would both be expected to decrease glycolysis (97–99). Moreover, we found that whereas TCR engagement significantly increased the cell surface abundance of the glucose transporter Glut1, the amount was significantly lower in the presence of low-dose resveratrol and was abrogated by high-dose resveratrol (P < 0.05 and P < 0.001; Fig. 6C and fig. S1L). The changes correlated directly with glucose uptake and glycolysis, as monitored by the production of lactate and extracellular acidification (P < 0.001; Fig. 6D).

Glucose metabolism was decreased in the presence of resveratrol, but lymphocyte metabolism can also be fueled by other nutrients such as glutamine. Notably, the ASC2 glutamine transporter, recently shown to be critical for T cell activation (100), was augmented in the presence of low-dose, but not high-dose, resveratrol. Moreover, the ratio of surface ASC2/Glut1 was significantly increased by low-dose resveratrol as compared to TCR activation alone (P < 0.01; Fig. 6D). The potential importance of resveratrol-linked glutaminolysis in CD4+ T cells was also suggested by the induction of the p53-dependent GLS2 gene (Fig. 6B), catalyzing the hydrolysis of glutamine to glutamate (101–103). This link was corroborated by an increased glutamine uptake in activated T cells treated with low-dose resveratrol (Fig. 6E).

The increase in glutamine entry and the enzymes involved in the first steps of glutaminolysis suggested that p53 activation might augment OXPHOS in these T lymphocytes. In tumor cells, p53 decreases glycolysis while enhancing OXPHOS (104, 105). One of the mechanisms through which p53 augments tricarboxylic acid (TCA) cycling from glutamine intermediates (anaplerosis) is through the expression of SCO2, a protein that catalyzes the transfer of reducing equivalents from cytochrome c to molecular oxygen in mitochondria (103). To assess the extent of OXPHOS in human CD4+ T cells, we directly monitored the oxygen consumption rate (OCR) and found that TCR stimulation significantly increased the basal cellular respiration of CD4+ T cells (P < 0.005; Fig. 6E). Low-dose resveratrol further enhanced respiration, but TCR-stimulated respiration was almost completely abrogated by high-dose resveratrol (P < 0.05; Fig. 6F). Increased oxygen consumption would also be expected to result in increased ROS, with mitochondria serving as the major intracellular source of ROS. Superoxide anion—the predominant ROS in mitochondria—was significantly augmented by resveratrol, as monitored by MitoSOX staining (P < 0.05; Fig. 6F). These metabolic parameters correlated directly with the bioenergetic profile of the CD4+ T cells: intracellular adenosine triphosphate (ATP) was significantly increased in low-dose resveratrol–treated cells but was attenuated in lymphocytes treated with high-dose resveratrol (P < 0.005; Fig. 6F). Thus, resveratrol significantly modulated the expression of p53 target genes, concordant with changes in the metabolic state of these lymphocytes.

**DISCUSSION**

Our study demonstrates that resveratrol modulates the potential of human CD4+ T lymphocytes to respond to antigen receptor stimulation. Although resveratrol exerts effects in both Sirt1-dependent and Sirt1-independent manners (20, 114), we found that high-dose resveratrol (100 μM) inhibited the TCR-induced expression of Sirt1 in T lymphocytes. In this condition, this was likely caused by an attenuation of mTOR and distal TCR signaling. In contrast, low-dose resveratrol (20 μM) markedly increased TCR-stimulated Sirt1 activity. Furthermore, p53—a tumor suppressor whose activity is coordinated by and coordinates that of Sirt1 (115)—was highly phosphorylated in response to low-dose resveratrol. We determined that this p53 phosphorylation was mediated by the kinase ATR, a key regulator of the genotoxic stress response pathway. Concordant with ATR and p53 signaling, T cells exposed to low-dose resveratrol underwent Chk1- and Wee1-mediated premitotic cell cycle arrest and induced expression of p53-dependent metabolic target genes, resulting in a metabolic shift with increased OXPHOS. Note that these conditions,
which promoted an enhanced bioenergetic profile, endowed CD4+ T cells with a substantially enhanced cytokine secretion potential (Fig. 7B).

The role of resveratrol in protecting against carcinogenesis has been the subject of intense study, and multiple reports showed that resveratrol functions by preventing DNA damage formation as well as by improving DNA damage repair (47, 50, 116). Resveratrol affects multiple aspects of DNA metabolism, including DNA replication, recombination, repair, and telomere maintenance, as well as the redox state, thereby promoting the integrity of genomic DNA. However, in vitro, resveratrol mediates DNA cleavage in a process requiring DNA-bound copper [Cu(II)] ions (49, 57, 59, 117–119). On the basis of diverse studies, it is nearly impossible to draw clear-cut conclusions about the effects of sirtuins or resveratrol on genomic stability. Both reduction of DNA breaks and an inhibition of replicative senescence (57, 74–78), as well as the generation
Phosphorylation in T cells exposed to the homeostatic cytokine IL-7. In TCR-stimulated cells, whereas high-dose resveratrol resulted in H2AX phosphorylation in the T cell. Low-dose resveratrol was associated with H2AX phosphorylation (\(^{76}\)) of DNA breaks with associated senescence (\(^{57, 76, 77}\)), have been reported (\(^{120}\)). Here, we found that low-dose resveratrol triggered a marked DNA damage response in TCR-stimulated T cells, as shown by the presence of \(\gamma\)H2AX in 20 to 50% of cells. Furthermore, DNA damage, or more precisely the response to genotoxic stress, was linked to the activation state of the T cell. Low-dose resveratrol was associated with \(\gamma\)H2AX phosphorylation in TCR-stimulated cells, whereas high-dose resveratrol resulted in \(\gamma\)H2AX phosphorylation in T cells exposed to the homeostatic cytokine IL-7.

The ATR pathway is activated under conditions of single-stranded DNA breaks or instability of replication forks (\(^{85}\)). We find that low-dose resveratrol activated the ATR pathway within 24 hours of the treatment of stimulated CD4\(^+\) T cells, well before S-phase entry. Under these conditions, ATR rapidly phosphorylated histone H2AX and Mcm2 at Ser\(^{68}\), with the latter potentially stabilizing prereplication complexes in response to DNA damage (\(^{121}\)). In addition, at later time points, we found that resveratrol stimulated the ATR-mediated phosphorylation of Chk1 and Wee1, blocking Cdc25-mediated Cdk1 activation and mitotic entry (Fig. 7B). Although both ATR and ATM phosphorylate Ser\(^{15}\) of p53 (\(^{60}\)), we found no evidence that resveratrol activated an ATM-Chk2-p53-p21 pathway, a pathway that is generally activated in response to double-stranded DNA breaks. Thus, our data suggest that resveratrol triggers a replication stress-like response rather than classical DNA damage. In this state, p53 appears to serve as a node between upstream stress signaling cascades and downstream DNA repair pathways (Fig. 7B) (\(^{122}\)).

In CD4\(^+\) T cells, both low- and high-dose resveratrol induced transcription of the p53 proapoptotic target, PUMA. This was the only gene in an 84-gene DNA damage signaling pathway array to exhibit increased expression in response to low-dose resveratrol (fig. S3), suggesting that many of the effects of low-dose resveratrol occur at a posttranscriptional level. However, low-dose resveratrol altered the expression of all p53-directed metabolic gene targets that we assessed. Although other transcription factors, such as c-Myc and HIF-1\(\alpha\), also regulate cell metabolism and are produced in TCR-stimulated human CD4\(^+\) T cells, their abundance was not substantially modulated by resveratrol (fig. S5), suggesting that many of the effects of low-dose resveratrol do not occur at a posttranscriptional level. However, low-dose resveratrol altered the expression of all p53-directed metabolic gene targets that we assessed. Although other transcription factors, such as c-Myc and HIF-1\(\alpha\), also regulate cell metabolism and are produced in TCR-stimulated human CD4\(^+\) T cells, their abundance was not substantially modulated by resveratrol (fig. S5), suggesting that many of the effects of low-dose resveratrol do not occur at a posttranscriptional level. However, low-dose resveratrol altered the expression of all p53-directed metabolic gene targets that we assessed. Although other transcription factors, such as c-Myc and HIF-1\(\alpha\), also regulate cell metabolism and are produced in TCR-stimulated human CD4\(^+\) T cells, their abundance was not substantially modulated by resveratrol (fig. S5), suggesting that many of the effects of low-dose resveratrol do not occur at a posttranscriptional level.
metabolic shift, adjusting the balance between glycolysis and OXPHOS (Fig. 7B). The bioenergetic profile of resveratrol-treated T cells was altered by this skewing of metabolism away from glycolysis and toward a setting characterized by an increased ASCT2-to-Glut1 ratio, with an augmented glutamine transport, substantially increased mitochondrial ROS production, and increased OXPHOS. How this would affect T cell effector function is unclear because glycolysis can increase and decrease the potential of T cells to secrete effector cytokines, such as IFN-γ and IL-17 (128–133). Note that amino acid metabolism is essential for effector T cell differentiation (100, 109, 134, 135), and memory cells rely more on OXPHOS than on glycolysis (38, 130). In both naive and memory CD4+ T cells, low-dose resveratrol markedly augmented the amount of IFN-γ secreted, but this increase was even higher for naïve cells than for memory cells (means of 10- and 2-fold, respectively). Furthermore, in both cell types, resveratrol substantially increased the number of cells that produced both IL-2 and IFN-γ. Thus, our data suggest that resveratrol is an agent that, by altering the metabolic fitness of T lymphocytes, enhances their cytokine effector potential.

Adjusting the balance between glycolysis and OXPHOS can also have substantial effects in other cell types. Decreasing OXPHOS in mice expressing a mutant p53 markedly attenuates tumorigenesis (136). Thus, generating a context that is the converse of that shaped by resveratrol, that is, inhibiting a p53-mediated shift to mitochondrial metabolism, may be beneficial for individuals with an increased risk of developing cancers, such as Li-Fraumeni syndrome patients with germline mutations in the TP53 gene. The potential use of resveratrol as a therapy for the treatment of neurological, cardiovascular, hepatic, and metabolic pathologies therefore necessitates a critical evaluation of its effect on T lymphocytes in vivo, especially in an autoimmune setting. The data shown here reveal a complex network of resveratrol-stimulated changes in cell cycle progression and metabolism, altering the potential of T lymphocytes to respond to foreign antigens.

**MATERIALS AND METHODS**

**T cell isolation and culture**

CD4+ T cells were isolated from adult peripheral blood, obtained from healthy donors after informed consent. Cells were purified using negative-selection Rosette tetramers (STEMCELL Technologies), and the purity of the cell population was monitored on a FACS Canto II (BD Biosciences). Purities were always greater than 94%. Naïve and memory CD4+ T cells were sorted on a FACSAria after staining with anti-CD4, anti-CD45RA, anti-CD45RO, CD62L, CD127, and CD25 antibodies (fig. S4A). Lymphocytes (1 × 10^6 per well in a 24-well plate) were cultured in RPMI 1640 + GlutaMAX (Gibco, Life Technologies) supplemented with 10% fetal calf serum (FCS) and 2% penicillin/streptomycin (Gibco, Life Technologies). For TCR stimulation, 24-well plates were coated with anti-CD3 (clone 2C11, 1 μg/ml; Sigma-Aldrich) and anti-CD28 (clone 93, provided by C. June) monoclonal antibodies (mAbs) at a concentration of 1 μg/ml, and recombinant IL-2 (rIL-2) (50 U/ml) was added as indicated. T cells were also cultured in the presence of rIL-7 (10 ng/ml). As indicated, resveratrol (20 or 100 μM; Sigma-Aldrich), compound C1 (1 μM; Sigma-Aldrich), VE-821 (1 and 5 μM; Euromedex), aphidicolin (1 μM), and bleomycin (1 μM) were added to T cell cultures 1 hour before TCR stimulation.

**Immunofluorescence**

Cells were collected and coated on poly-L-lysine–treated slides. Cells were fixed in a 4% paraformaldehyde (PFA) solution [phosphate-buffered sa-

**Metabolic assays**

OCRs were measured on an XF-24 Extracellular Flux Analyzer (Seahorse Bioscience). TCR-stimulated T cells with and without low-dose resveratrol (20 μM) were seeded at a concentration of 1.5 × 10^6 cells, whereas nonstimulated and high-dose resveratrol-stimulated cells were seeded at a concentration of 2.0 × 10^6 cells in XF medium (nonbuffered Dulbecco’s modified Eagle’s medium containing 2.5 mM glucose, 2 mM l-glutamine, and 1 mM sodium pyruvate). Oxygen consumption was monitored under basal conditions and in response to oligomycin (1 μM), carbonyl cyanide p-trifluoromethoxyphenylhydrazone (1.5 μM), rotenone (100 nM), and antimycin A (1 μM; Sigma-Aldrich). The basal respiration rate was calculated as the difference between basal OCR and the OCR after inhibition of mitochondrial complexes 1 and 3 with rotenone and antimycin A, respectively. ATP and l-lactate were measured according to the standard procedures of the ATPLite kit (PerkinElmer) and l-lactate kit (Eton Bioscience), respectively. Extracellular pH was measured immediately after harvesting of medium using a standard pH meter.

**Glucose and glutamine uptake assays**

Cells (2 × 10^6) were starved by incubation at 37°C in serum and glucose- or glutamine-free RPMI 1640 for 30 min. Radiolabeled 2-deoxy-d-[1-3H] glucose or glutamine-1-[3,4-3H(N)] (PerkinElmer) was added to a final concentration of 0.1 mM (2 μCi/ml). Cells were incubated for 10 min at room temperature, washed in cold serum/glucose/glutamine-free RPMI 1640, and solubilized in 500 μl of 0.1% SDS. Radioactivity was measured by liquid scintillation.
Total protein extraction and analyses
Cells were lysed in lysis buffer containing 20 mM Hepes (pH 7.6), 100 mM KCl, 0.1 mM EDTA, 1 mM NaF, 1 mM sodium orthovanadate, 1% Triton X-100, 0.5% NP-40, 0.15 mM spermine, 0.5 mM spermidine, 1 mM dithiothreitol, and a protease inhibitor cocktail. After a 30-min incubation on ice, extracts were centrifuged, and supernatants were harvested. Extracts (20 µg) were resolved on SDS–polyacrylamide gel electrophoresis gels (8.5 to 12%) and transferred electrothermally onto polyvinylidene difluoride (PVDF) membranes. PVDF membranes were probed with the SABiosciences DNA template analysis software.

Statistical analyses
Data were analyzed with GraphPad Software (GraphPad Prism), and P values were calculated by one-way ANOVA (Tukey’s post hoc test), two-way ANOVA (with Tukey’s post hoc or Bonferroni’s tests), or paired t tests, as indicated.

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BACKGROUND

Reprogramming the immunosuppressive tumor environment to promote antitumor immunity is a major objective of immunomodulatory therapies currently in clinical use or development. Cancer cells contribute to orchestrating the composition of this environment through various mechanisms. In murine squamous cell carcinoma (SCC) cells, specifically nuclear FAK enhanced the expression of the genes encoding IL-33, the chemokine CCL5, and the soluble, secreted form of the IL-33 receptor, called soluble ST2 (sST2). The abundance of IL-33 and CCL5 was increased in FAK-positive SCC cells but not in normal keratinocytes. IL-33 associated with FAK in the nucleus, and the FAK–IL-33 complex interacted with a network of chromatin modifiers and transcriptional regulators, including TAF9, WDR82, and BRD4, which promote the activity of nuclear factor κB (NF-κB) and its induction of genes encoding chemokines, including CCL5. We did not detect secretion of IL-33 from FAK-positive SCC cells; thus, we propose that the increased production and secretion of sST2 likely sequesters IL-33 secreted by other cell types within the tumor environment, thus blocking its stimulatory effects on infiltrating host immune cells. Depleting FAK, IL-33, or sST2 from SCC cells before implantation induced tumor regression in syngeneic mice, except when CD8+ T cells were co-depleted. Our data provide mechanistic insight into how FAK controls the tumor immune environment, namely, through a transcriptional regulatory network mediated by nuclear IL-33. Targeting this axis may boost antitumor immunity in patients.

RESULTS

Focal adhesion kinase (FAK) mediates tumor cell–intrinsic behaviors that promote tumor growth and metastasis. We previously showed that FAK also induces the expression of inflammatory genes that inhibit antitumor immunity in the microenvironment. We identified a crucial, previously unknown role for the dual-function cytokine interleukin-33 (IL-33) in FAK-dependent immune evasion. In murine squamous cell carcinoma (SCC) cells, specifically nuclear FAK enhanced the expression of the genes encoding IL-33, the chemokine CCL5, and the soluble, secreted form of the IL-33 receptor, called soluble ST2 (sST2). The abundance of IL-33 and CCL5 was increased in FAK-positive SCC cells but not in normal keratinocytes. IL-33 associated with FAK in the nucleus, and the FAK–IL-33 complex interacted with a network of chromatin modifiers and transcriptional regulators, including TAF9, WDR82, and BRD4, which promote the activity of nuclear factor κB (NF-κB) and its induction of genes encoding chemokines, including CCL5. We did not detect secretion of IL-33 from FAK-positive SCC cells; thus, we propose that the increased production and secretion of sST2 likely sequesters IL-33 secreted by other cell types within the tumor environment, thus blocking its stimulatory effects on infiltrating host immune cells. Depleting FAK, IL-33, or sST2 from SCC cells before implantation induced tumor regression in syngeneic mice, except when CD8+ T cells were co-depleted. Our data provide mechanistic insight into how FAK controls the tumor immune environment, namely, through a transcriptional regulatory network mediated by nuclear IL-33. Targeting this axis may boost antitumor immunity in patients.
Ccl5 expression and tumor growth. Mechanistic protein network analyses suggested that IL-33 regulates gene expression by interacting with chromatin modifiers and transcriptional regulators. ST2 was secreted by SCC cells, and it suppressed CD8+ T cell–mediated tumor clearance. Our findings reveal new insight into the molecular mechanisms by which nuclear FAK regulates chemokine expression, placing nuclear IL-33 at the heart of a complex transcriptional network that specifies the antitumor immune response.

RESULTS

Nuclear FAK regulates expression of IL-33 and its receptor ST2

We have previously reported that nuclear FAK regulates the expression of chemokines, including Ccl5, and this is important for driving infiltration of Treg cells into murine SCC tumors, enabling them to evade the antitumor immune response (9). To explore the mechanisms of FAK-dependent chemokine regulation, we analyzed Affymetrix microarray data comparing murine FAK−/− SCC cells with those re-expressing FAK-WT (wild-type) (herein referred to as FAK-WT) to identify genes that are regulated by FAK. In the set of genes that significantly down-regulated after FAK depletion, the only significantly enriched gene ontology term was “extracellular region” (P = 0.0049). Using the genes contained within this category, we generated a protein interaction network based on direct physical interactions. The largest connected network was found to contain Ccl5 and the gene encoding the cytokine IL33 (Fig. 1A). Given the link between IL-33 and the regulation of gene expression (16, 17), we investigated whether, and if so how, IL-33 contributed to FAK-dependent transcription of chemokines.

We first used quantitative real-time polymerase chain reaction (qRT-PCR) to compare IL33 expression in SCC cells expressing FAK-WT, FAK−/−, FAK-nls (a mutant that is largely excluded from the nucleus), and FAK-kd (a kinase-deficient mutant) and found that regulation of IL33 mRNA was dependent on both FAK kinase activity and its nuclear localization (Fig. 1B). Western blotting for IL-33 abundance in whole-cell lysates revealed similar findings at the protein level (Fig. 1C and fig. S1A). We have shown previously that mutation of the nuclear localization signal in FAK does not completely abolish FAK nuclear localization. We believe that this explains why we observed slightly increased abundance of IL-33 in cells expressing this mutant when compared to FAK−/− or FAK-kd SCC cells. Similarly, analysis of SCC cells expressing FAK-Y397F, an autophosphorylation-defective mutant of FAK that is kinase-deficient, showed reduced IL33 expression similar to that observed in FAK−/− SCC cells (fig. S1B). Treatment of FAK-WT cells with the FAK catalytic inhibitor VS4718 inhibited IL-33 abundance on both mRNA and protein levels (Fig. 1, D and E), even with a low (50 nM) concentration of VS4718 (fig. S1, C and D). IL-33 can function both as a nuclear cytokine and a secreted alarmin (20). Using both an anti–IL-33 enzyme-linked immunosorbent assay (ELISA; Fig. 1F) and Western blotting (fig. S1E), we could not detect IL-33 in SCC cell–conditioned media, implying that IL-33 predominantly functions as a nuclear cytokine in SCC cells.

As an extracellular cytokine, IL-33 mediates signaling by binding to the IL-33R complex, composed of ST2 (also termed IL1RL1) and IL-1RAcP (21). Furthermore, both IL-33 signaling and nuclear IL-33 have been shown to regulate ST2 expression (22, 23). The protein encoded by ST2 exists in two forms: (i) as ST2L, a membrane-anchored receptor that activates downstream signaling upon IL-33 engagement, or (ii) as sST2, a secreted soluble decoy receptor that inhibits IL-33 signaling (13). Using qRT-PCR with a primer set that would detect cumulative amounts of mRNA encoding both ST2L and sST2, we found that ST2 expression was greater in FAK-WT cells when compared to FAK−/−, FAK-nls, or FAK-kd cells (Fig. 1G). Thus, FAK’s regulation of ST2 expression is also dependent on both nuclear localization and kinase activity. Using an anti-ST2 ELISA, we found abundant secreted amounts of sST2 in FAK-WT cell–conditioned medium, and this was reduced in media conditioned by FAK−/−, FAK-nls, and FAK-kd cells (Fig. 1H). However, flow cytometry analysis did not detect the presence of ST2L on the surface of FAK-WT and FAK−/− SCC cells (fig. S1F), indicating that sST2 is the predominantly produced isoform upon ST2 induction in SCC cells. Treatment with the FAK inhibitor VS4718 reduced its expression and secretion (Fig. 1, I and J). Collectively, these results indicate that the kinase activity of FAK in the nucleus is a key regulator of the abundance of both nuclear IL-33 and sST2, thereby influencing both the nuclear and extracellular/alarmin functions of IL-33 signaling.

We next investigated the mechanism by which FAK controls IL33 expression and sST2 abundance in SCC cells. Using an experimentally derived nuclear FAK interactome from FAK-WT cells (9), we used Ingenuity Pathway Analysis (IPA) to identify direct experimentally observed relationships between nuclear FAK-interacting proteins and transcription factors that regulate the expression of IL33 and ST2 (obtained from Qiagen’s DECODE database; fig. S1G). This identified associations with several IL33- and ST2-regulatory transcription factors. We noticed that four of these associations, RUNX1, SP1, NCOA2, and NR3C1, were linked to transcription factors associated with regulating the expression of both IL33 and ST2. We know that FAK associates with SP1 (confirmed in fig. S1, H and I) and RUNX1 (24) and is involved in regulating RUNX1-containing protein complexes, posttranslation modification, and, ultimately, transcription factor function. Small interfering RNA (siRNA)–mediated depletion of both RUNX1 and SP1 suggested that these transcription factors acted together to regulate IL-33 abundance (fig. S1, J and K). The precise details of their coordinated activities require further investigation, but nonetheless, our findings identify several connections between FAK-interacting proteins and transcription factors that regulate IL33 and ST2.

IL-33 is required for FAK-dependent expression of a subset of chemokines

Nuclear IL-33 has been linked to regulation of gene expression in several model systems (16, 17). To determine whether nuclear IL-33 was required for FAK-dependent chemokine expression, we depleted IL-33 in FAK-WT SCC cells using both short hairpin RNA (shRNA; Fig. 2, A and B, and fig. S2A) and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) (fig. S2B). IL-33 has previously been linked to the regulation of Ccl5 (23), and we have shown that FAK-dependent Ccl5 expression regulates the antitumor immune response (9). We found that IL33 and Ccl5 expression was increased in SCC cells when compared to primary keratinocytes (Fig. 2, C and D), correlating with nuclear FAK in SCC cells, and that IL-33 was required for Ccl5 expression in FAK-WT SCC cells (Fig. 2E and fig. S2C). A previous study in endothelial cells (23) demonstrated that IL-33 silencing increased sST2 expression. Here, we found that IL-33 silencing reduced sST2 abundance (fig. S2D). Although we have used different readouts (mRNA versus protein), it is likely that the regulation of ST2 by IL-33 may be context-dependent. To investigate further the requirement for IL-33 in FAK-dependent
Fig. 1. Nuclear FAK regulates expression of IL-33 and its receptor ST2. (A) Gene ontology enrichment analysis (cellular component terms) on the significantly down-regulated set of genes in the FAK−/− SCC transcriptome relative to the wild-type [WT; percentage false-positives (pfp) < 0.05]. Genes annotated with the over-represented term (extracellular region; Benjamini-Hochberg-corrected hypergeometric test) were used to seed a protein interaction network based on direct physical interactions (gray lines). Color of each node (circle) is proportional to the log-transformed fold change in gene expression. The largest connected graph component is displayed. (B and C) Abundance of IL-33 at the mRNA level [(B); by qRT-PCR] and protein level [(C); by Western blot] in FAK-WT, FAK−/−, FAK-nls (a mutant that is largely excluded from the nucleus), and FAK-kd (a kinase-deficient mutant) SCC cells. (D and E) Abundance of IL33 mRNA [(D); by qRT-PCR] and IL-33 protein [(E); by Western blot] in FAK-WT SCC cells treated with control [dimethyl sulfoxide (DMSO)] or VS4718 (250 nM; for 24 hours). Western blot additionally assessed in FAK−/− SCC cells for reference. (F) Analysis of enzyme-linked immunosorbent assay (ELISA) for IL-33 in conditioned media from FAK-WT, FAK−/−, FAK-nls, and FAK-kd SCC cells. (G) qRT-PCR analysis of ST2 expression in FAK-WT, FAK−/−, FAK-nls, and FAK-kd SCC cells. (H) Analysis of ELISA for sST2 in conditioned media from FAK-WT, FAK−/−, FAK-nls, and FAK-kd SCC cells. (I) qRT-PCR analysis of ST2 expression in FAK-WT SCC cells treated with control (DMSO) or VS4718 (250 nM; for 24 hours). (J) Analysis of ELISA for sST2 in conditioned media from FAK-WT SCC cells treated with control (DMSO) or VS4718 (250 nM; for 24 hours). Data are means ± SEM. n = 3 experiments. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001 by Tukey's corrected one-way analysis of variance (ANOVA) (B, G, and H) or two-tailed unpaired t test (D, I, and J).
chemokine expression, we performed NanoString PanCancer Immune Profiling of FAK-WT/pLKO, FAK−/−, and FAK-WT IL-33 shRNA1 SCC cells. Hierarchical clustering of log-transformed fold changes, relative to control cells, identified a subset of chemokines coregulated by FAK and IL-33 (Fig. 2F), including CCL5. Furthermore, overexpression of IL-33 in FAK−/− SCC cells increased Ccl5 expression (Fig. 2G), suggesting that IL-33 is sufficient to promote Ccl5 expression. Therefore, FAK regulates IL-33, which in turn can mediate FAK-dependent chemokine expression.

IL-33/ST2 axis supports tumor growth by suppressing the immune response

IL-33 is expressed by several cell types within the tumor environment, and secreted IL-33 can have both pro-and antitumor effects (14, 15). ST2L, the IL-33 receptor, is present on various immune cells, including Treg cells, macrophages, and CD8+ T cells, and activation of ST2L signaling can alter their phenotype and/or function. For example, ST2L-positive Treg cells exhibit a more potent immunosuppressive function than ST2L-negative Treg cells (25), implying that IL-33–ST2L

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signaling could enhance the suppressive activity of T<sub>reg</sub> cells, thereby promoting tumor growth. In contrast, activation of ST2L on cytotoxic CD8<sup>+</sup> T cells enhances the cells' cytotoxic function (18), resulting in an improved antitumor immune response. Therefore, it is possible that FAK-dependent regulation of the decoy receptor sST2 could have pro- or antitumor effects.

Having established that nuclear IL-33 regulates chemokine expression, including Ccl5, we next assessed the effects of IL-33 depletion on SCC tumor growth. Therefore, 1 × 10<sup>6</sup> FAK-WT, FAK<sup>−/−</sup>, and FAK-WT/IL-33<sup>−/−</sup> mice were injected subcutaneously into Friend leukemia virus B strain (FVB) mice (the syngeneic host strain), and tumor growth was monitored. FAK-WT tumors exhibited exponential growth until they reached defined end points by which time the mice had to be sacrificed (see Materials and Methods). In contrast, FAK<sup>−/−</sup> SCC tumors grew until about day 7, after which they stalled and underwent complete regression (Fig. 3A, left graph), as we have also reported previously (9). IL-33 depletion from FAK-WT cells resulted in six of eight tumors exhibiting a period of growth followed by complete regression, in a similar manner to FAK<sup>−/−</sup> tumors, whereas two of eight tumors showed a growth delay (Fig. 3A). Similar studies using CRISPR/Cas9 to deplete IL-33 from FAK-WT cells confirmed the requirement for IL-33 in supporting SCC tumor growth (Fig. 3B). Thus, IL-33 is required to support tumor growth and permit immune evasion, likely by regulating transcription of vital chemokines, including Ccl5.

To next probe the role of sST2 in tumor growth, we generated sST2-depleted FAK-WT SCC cell lines using shRNA (Fig. 3C) and injected 1 × 10<sup>6</sup> FAK-WT, FAK-WT/pLKO (shRNA control), FAK-WT/ST2-shRNA1, and FAK-WT/ST2-shRNA2 SCC cells into syngeneic FVB mice. We found that most tumors (six of eight for shRNA1 and five of eight for shRNA2) exhibited a period of growth followed by complete regression, in a similar manner to FAK<sup>−/−</sup> tumors, albeit with different kinetics. To address how sST2 depletion might result in tumor regression, we profiled the immune cells in FAK-WT tumors to determine the quantity of ST2L on the cell surface. We found that after 12 days of growth, FAK-WT tumors have an extensive immune cell infiltrate accounting for about 60% of the viable cell population (Fig. 3, E and F). Using a range of surface and intracellular markers (table S1), we identified activated CD8<sup>+</sup> T cells, activated CD4<sup>+</sup> T cells, T<sub>reg</sub> cells, neutrophils, and macrophages as the major immune cell populations that displayed surface-bound ST2L (Fig. 3, G and H). Because IL-33 engagement with ST2L expressed on activated CD8<sup>+</sup> T cells can enhance cytotoxic function and drive increased expression of the effector cytokine interferon-γ (IFN-γ) (14), we hypothesized that tumor regression in response to depletion of sST2 from SCC cancer cells was likely CD8<sup>+</sup> T cell-dependent. To test this, we used CD8-depleting antibodies (Abs) and found that depletion of CD8<sup>+</sup> T cells was sufficient to completely rescue the growth of FAK-WT/ST2-shRNA SCC tumors (Fig. 3I), implying that sST2 from the tumor cells plays an important role in suppressing CD8<sup>+</sup> T cell–mediated antitumor immunity. Consistent with previous results (9), we also observed enhanced growth of FAK-WT tumors upon depletion of CD8<sup>+</sup> T cells, implying that even the FAK-WT cells remain under negative pressure from the CD8<sup>+</sup> T cell–mediated immune response.

Nuclear IL-33 interacts with an extensive network of transcriptional regulators

Having established an important role for nuclear IL-33 in the regulation of chemokine expression and tumor growth, we addressed the molecular mechanisms that may underpin IL-33’s regulation of chemokine transcription. Using cellular fractionation, we prepared cytoplasmic, nuclear, and chromatin extracts from FAK-WT and FAK<sup>−/−</sup> SCC cells as we have done before (9). Our results show that IL-33 is largely chromatin-associated (Fig. 4A), in agreement with previous studies that identified IL-33 as a histone 2A (H2A)/2B–binding protein (26). To investigate the functional significance of IL-33 association with chromatin, we made use of a proteomics technique called “BioID” (27). First, we generated an IL-33–protein fused to the 35-kDa Escherichia coli biotin protein ligase BirA (IL-33–BirA). Next, we expressed either IL-33–BirA or BirA alone in FAK-WT cells, from which endogenous IL-33 was deleted (FAK-WT/IL-33–CRISPR SCC cells; Fig. 4, B and C), and cultured these in the presence of biotin for 24 hours. This resulted in the biotinylation of proximal interacting proteins, enabling their purification and identification by mass spectrometry (MS). To ensure identification of robust interactions, we applied stringent criteria, including that (i) proteins must be present in all three biological replicates, (ii) proteins must have greater than threefold enrichment when compared to BirA–only control, and (iii) fold enrichment must be statistically significant (P < 0.05). With these criteria, we identified 105 proteins that associated with IL-33. Gene ontology analysis of both biological and cellular processes [Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics database] represented within the data set identified significantly enriched terms associated with chromatin organization and transcriptional regulation (fig. S3, A and B). Further analysis of the nuclear IL-33 interactome using IPA identified a connected network of proteins that were implicated in regulation of transcription, chromatin remodeling, and nucleosome disassembly (Fig. 4D). There were several members of the Baf-type complex (also known as SWI/SNF), the PTW/PP1 phosphatase complex, and the transcription factor transcription factor IIID (TFIID) complex, establishing a link between nuclear IL-33 and the core transcriptional machinery. We extracted a complete list of genes belonging to these complexes from the gene ontology database AmiGO and used IPA to reconstruct all three complexes on the basis of known physical interactions (fig. S3, C to E). We next contextualized the BioID proteomics–informed IL-33 nuclear interactome onto these networks (highlighted in pink in fig. S3, C to E) and identified potential interactions linking IL-33 to key members of these complexes. Using streptavidin pulldown after incubation of IL-33–BirA fusion protein–expressing FAK-WT/IL-33–CRISPR SCC cells in the presence of biotin, we confirmed the interactions of IL-33 with WRDR2, SMARCC1, and TAF9 (Fig. 4E). We found that IL-33 depletion in FAK-WT cells resulted in loss of WRDR2 from the chromatin fraction (Fig. 4F), implying that IL-33 is required to stabilize the association of WRDR2 with chromatin. We find this interesting because WRDR2 is a key component of complexes associated with chromatin modification, such as the PTW/PP1 phosphatase complex (28) and the Set1A/SET1B histone H3-Lys4 (H3K4) methyltransferase complex (29). Therefore, IL-33 likely plays a key role in scaffolding complexes required to modulate chromatin structure and permit transcription.

IL-33 interacts with and enhances regulators of Ccl5 expression

As mentioned above, we previously showed that CCL5 secreted by FAK-WT SCC cells drives the infiltration of immunosuppressive T<sub>reg</sub> cells into SCC tumors, shifting the CD8<sup>+</sup> T cell/T<sub>reg</sub> ratio in favor of tumor tolerance (9). Thus far, we have found that IL-33 is both necessary and sufficient to drive Ccl5 expression downstream of FAK (Fig. 2, E and G). To define how IL-33 contributes to regulation of
Fig. 3. IL-33 and sST2 support SCC tumor growth through suppressing the antitumor immune response. (A) and (B) Representative growth of FAK-WT, FAK−/−, and FAK-WT IL-33−shRNA1 (A) or FAK-WT IL-33−CRISPR (clustered regularly interspaced short palindromic repeats) (B) SCC tumors after orthotopic subcutaneous implantation. (C) qRT-PCR analysis of ST2 expression in FAK-WT pLKO, FAK-WT ST2-shRNA1, and FAK-WT ST2-shRNA2 SCC cells. (D) Growth of FAK-WT ST2-shRNA (pools 1 and 2) SCC tumors after orthotopic subcutaneous implantation. (E) CD45+ cells as a percentage of live cells isolated from FAK-WT SCC tumors 12 days after implantation. (F) Abundance of different immune cell populations as a percentage of CD45+ cells. Marker sets used to identify cell populations are listed in fig. S3. (G) Percentage ST2+ cells in different immune cell populations. (H) Mean fluorescent intensity (MFI) of ST2 expression in different immune cell populations. MFI, fluorescence minus one. (I) Representative growth of FAK-WT, FAK-WT ST2-shRNA1, and FAK-WT ST2-shRNA2 SCC tumors receiving treatment with either a CD8-depleting antibody (Ab) or isotype control (Ctrl) Ab. Statistics in (C): ***P ≤ 0.0001 by Tukey’s corrected one-way ANOVA. Data are means ± S.E.M. n = 3 for qRT-PCR, n = 6 to 8 tumors.

Ccl5 expression, we mapped the nuclear IL-33 interactome onto a network of proteins associated with predicted Ccl5 transcription factors (Fig. 4G). This revealed that IL-33 interacts with transcription factors and transcriptional regulators that affect expression of Ccl5. Notably, three factors belonging to the TFIID complex were found to interact with nuclear IL-33, including TAF9, a protein that we found previously to form complexes with FAK (9). To explore the connection between FAK, IL-33, and Ccl5 transcription, we mapped the nuclear FAK interactome onto the set of Ccl5 regulatory proteins found to interact with nuclear IL-33 (Fig. 4G). This identified proteins...
Fig. 4. Nuclear IL-33 interacts with an extensive network of transcriptional regulators. (A) Representative Western blot of IL-33 abundance in cytoplasmic (Cyto), nuclear (Nuc), and chromatin (Chr) fractions from FAK-WT and FAK−/− SCC cells. (B) Representative Western blot of IL-33 abundance in FAK-WT IL-33–CRISPR/BirA-E.V. and FAK-WT IL-33–CRISPR/IL-33–BirA SCC cells. (C) Western blot analysis of lysates from nuclear fractionations of IL-33–BirA fusion protein expression in FAK-WT IL-33–CRISPR/BirA-E.V. (empty vector) or FAK-WT IL-33–CRISPR/IL-33–BirA SCC cells. (D) Functional interaction network analysis of the IL-33 interactome. Direct physical interactions (solid gray lines) and functional association with transcription (dashed gray lines) are shown. Node style indicates association with additional relevant overrepresented functions (P < 0.0001 by Benjamini-Hochberg-corrected hypergeometric test). Components of the PTW/PP1 phosphatase, Baf-type, and TFIID complexes are highlighted. (E) Western blot analysis of key network components using streptavidin pulldowns from biotinylated lysates of SCC FAK-WT IL-33–CRISPR cells expressing either BirA empty vector or IL-33–BirA fusion protein. (F) Representative Western blot of chromatin fractions and whole cell lysates from SCC FAK-WT, FAK-WT pLKO, and FAK-WT IL-33 shRNA1 cells. (G) Interrogation of the IL-33 BioID protein interaction network to identify potential upstream regulators of mCcl5 promoter associated transcription factors (taken from Qiagen ENCODE database), PP, protein-protein interaction; E, expression; PD, protein-DNA interaction; T, transactivation; A, activation. (H) Western blot analysis of IL-33:BRD4, IL-33:FAK, and IL-33:HDAC1 associations using streptavidin pulldowns from biotinylated lysates of FAK-WT IL-33–CRISPR SCC cells expressing either BirA empty vector or IL-33–BirA fusion protein. HDAC1, histone deacetylase 1. (I) qRT-PCR analysis of Ccl5 expression in SCC FAK-WT cells treated with DMSO or JQ1 (200 nM for 48 hours). (J) qRT-PCR analysis of Ccl5 expression in FAK-WT SCC cells treated with DMSO or vorinostat (10 μM for 24 hours). Statistics in (I) and (J): ****P ≤ 0.0001, ***P ≤ 0.01 by two-tailed unpaired t test. n = 3 for all experiments.

in common between the nuclear FAK and nuclear IL-33 interactomes, suggesting that these proteins may be part of the same molecular complexes that regulate Ccl5 expression. Using streptavidin-coated beads, we isolated the IL-33 BioID fusion protein and confirmed that FAK and IL-33 exist in complex under steady-state conditions (Fig. 4H). Hence, we conclude that FAK binds to IL-33, and together, they complex with key Ccl5-regulatory transcription factors, to coregulate chemokine gene expression.
Besides TFIID, many of the IL-33–interacting partners converge on regulators of NF-xB, suggesting that NF-xB may be central to FAK and IL-33 regulation of chemokine expression. Notably, we showed that IL-33 interacts with the bromodomain protein BRD4 and the histone deacetylase HDAC1 (histone deacetylase 1; Fig. 4H). BRD4 is a member of the bromodomain and extraterminal domain (BET) family of transcriptional coactivators and elongation factors that recruit chromatin remodeling factors, including the SWI/SNF complex (30), to the promoters of genes via recognition of polyacetylated histone tails (31). Because IL-33 binds to both BRD4 and members of the Baf-type (SWI/SNF) complex, we hypothesize that IL-33 plays a role in formation of this complex at actively transcribing genes. It is known that BRD4 directly binds to acetylated p65 NF-xB, which leads to enhanced NF-xB transactivation activity (32), together suggesting a role for BRD4 in inflammatory transcriptional signaling. To support these conclusions, we treated FAK-WT SCC cells with the BET family inhibitor JQ1. This resulted in reduced Ccl5 expression (Fig. 4I), implying a role for BET family proteins in the regulation of chemokine expression. In contrast, HDAC1 has been shown to negatively regulate NF-xB transcriptional activity via a direct interaction with p65 (RelA) (33). We used the HDAC inhibitor vorinostat and identified a clear induction of Ccl5 expression upon HDAC inhibition (Fig. 4J). Collectively, our data support a model, whereby FAK binds to IL-33, which is a central component of a network of transcriptional regulators associated with the dynamic regulation of NF-xB–dependent chemokine transcription.

**DISCUSSION**

Nuclear FAK is emerging as an important regulator of gene expression in cancer cells, controlling transcriptional networks that influence multiple cellular functions. For example, FAK is reported to interact with the transcription factors p53 and GATA4, resulting in their inactivation with effects on cell survival (34). We have shown that nuclear FAK regulates expression of chemokines and cytokines, including Ccl5, likely via interactions with transcription factors and transcriptional regulators (9). Here, we show that both IL-33 and sST2 are transcriptionally regulated by nuclear FAK in a kinase-dependent manner and that FAK interacts with transcription factors and transcriptional regulators linked to control of expression of IL33 and ST2.

Depletion of IL-33 abundance in FAK-WT cells revealed that it is vital downstream of FAK in the regulation of chemokine expression, including that of Ccl5. We have previously identified a CCL5–CCR1/3/5 paracrine signaling axis between SCC FAK-WT cells and tumor-infiltrating T_reg cells and have shown that CCL5 depletion results in FAK-WT tumor regression as a result of reduced tumor-infiltrating T_reg cells (9). Our tumor growth studies here revealed that IL-33 depletion caused FAK-WT tumor regression, presumably because of IL-33–dependent regulation of Ccl5 and other chemokines. Thus, IL-33 regulates proinflammatory gene programs downstream of FAK that we have shown to play an important role in defining the tumor-immune environment, affecting SCC tumor growth and survival.

sST2 functions as a decoy receptor that is secreted into the tumor environment, leading to competitive inhibition of IL-33–ST2 autocrine and paracrine signaling (13). Although IL-33 is not secreted by SCC cells, it can be secreted by macrophages and neutrophils (35), influencing the function of immune cell populations. ST2L is also present on activated CD8+ T cells and natural killer cells, and IL-33 stimulation can increase IFN-γ expression (14) and cytotoxic activity (18). We identified ST2L on several immune cell types in the SCC tumor environment, including activated CD8+ T cells. Depletion of sST2 in FAK-WT tumors largely resulted in CD8+ T cell–dependent tumor regression, implying that sST2 contributes to inhibition of CD8+ T cell–mediated immunity.

![Fig. 5. Nuclear FAK regulates IL-33/ST2 signaling to control the antitumor immune response. Model of the mechanism. Nuclear FAK regulates IL33 expression ("1") through interaction with transcription factors (TFs) and transcriptional regulators (TRs). Nuclear FAK and IL-33 cooperate to drive expression of Ccl5 and sST2 ("2" and "3", respectively) through interaction with transcription factors and transcriptional regulators. Ccl5 and sST2 are secreted from SCC cancer cells, promoting immune evasion. We have previously reported a CCL5–CCR1, 3, and 5 paracrine signaling axis between FAK-WT SCC cells and tumor-infiltrating regulatory T (T_reg) cells that contributes to immune evasion. We propose that sST2 contributes to immune evasion through competitive inhibition of IL-33/ST2 signaling on cytotoxic CD8+ T cells ("4"), resulting in tumor tolerance.](image-url)
Studies using an ST2 knockout mouse have shown that host ST2 signaling is required for tumor regression to occur in response to overexpression of a secreted form of IL-33 (14). Therefore, we conclude that the presence of ST2 likely enables FAK-abundant tumors to benefit from nuclear IL-33 while countering the potential antitumor effects of secreted IL-33 (Fig. 5).

Nuclear IL-33 has been linked to regulation of transcription previously (16, 17), although the precise mechanisms underpinning this are unknown. We confirmed that nuclear IL-33 interacts with chromatin, supporting its role in transcriptional regulation, and BioID proteomics identified proximal interactions with components of the Baf-type (SWI/SNF), PTW/PP1 phosphatase, and TFIID complexes. This implies that nuclear IL-33 functions in chromatin remodeling and transcriptional initiation. In support of this, we confirmed binding of WDR82, SMARCC1, TAF9, BRD4, and HDAC1 and showed that WDR82 was absent from chromatin after depletion of IL-33 from FAK-WT cells, showing that IL-33 is required to stabilize the WDR82 chromatin complex. WDR82 is a component of the PTW/PP1 phosphatase complex and is involved in regulating chromatin structure (28). It is also a core component of the mammalian Set1A/Set1B histone H3K4 methyltransferase complex that is associated with regulating H3K4 trimethylation, a key step in transcriptional activation (36).

Finally, we identified BRD4 and HDAC1 positive (37) and negative (33) regulators of NF-κB activity, respectively, as previously unknown nuclear IL-33–interacting proteins. BRD4 is also required for recruitment of the SWI/SNF complex to active promoters (30), and we have shown that IL-33 binds to several components of this complex. Therefore, IL-33 likely binds to BRD4, HDAC1, and other chromatin modifiers to control the dynamic expression of NF-κB target genes, such as C2l5 and other chemokines. Collectively, our data suggest that IL-33 acts to regulate chromatin organization and FAK-dependent transcription, promoting a proinflammatory gene program that enables evasion of the antitumor immune response.

MATERIALS AND METHODS

Cell lines

Isolation and generation of the FAK SCC cell model is described in detail by Serrels et al. (12). Briefly, SCC cells were induced in K14CreER FAKflox/flox mice on an FVB background using the dimethylbenz[a]anthracene/12-O-tetradecanoylphorbol 13-acetate two-stage skin chemical carcinogenesis protocol and cells isolated. After treatment with 4-hydroxytamoxifen, a FAK-null (FAK−/−) cell clone was isolated, and retroviral transduction was used to stably reexpress FAK-WT and FAK mutant proteins. Phoenix Ecotropic cells were transfected with pWZL (Hygro) FAK using Lipofectamine 2000 (Thermo Scientific) according to the manufacturer’s instructions. Twenty-four hours after transfection, cell culture supernatant was removed, filtered through a 0.45-μm Millex-AC filter (Millipore) and mixed at a 1:1 ratio with normal SCC growth medium, supplemented with polybrene (Millipore) to a final concentration of 5 μg/ml, and added to SCC cells for 24 hours. Cells were subjected to two rounds of lentiviral infection before selection in puromycin (2 μg/ml).

shRNA-mediated ST2 and IL-33 knockdown

To generate lentiviral particles, 2 × 10^6 human embryonic kidney 293FT cells were transduced with a mixture of 2 μg of shRNA (RMM4534-EG77125 (IL-33) and RMM4534-EG17082 (ST2/IL11R); GE Healthcare), 0.5 μg of MDG2, and 1 μg of PAC2 plasmid DNA using Lipofectamine 2000 (Thermo Fisher Scientific) as per the manufacturer’s guidelines. Forty-eight hours after transfection, medium was removed and filtered through a 0.45-μm Millex-AC filter (Millipore) and mixed at a 1:1 ratio with normal SCC growth medium, supplemented with polybrene (Millipore) to a final concentration of 5 μg/ml, and added to SCC cells for 24 hours. Cells were subjected to two rounds of lentiviral infection before selection in puromycin (2 μg/ml).

siRNA-mediated knockdown of SP1 and RUNX1

To knockdown SP1 and RUNX1, FAK-WT SCC cells were transfected in 10 nmol of nontargeting siRNA SMARTpool, SP1 siRNA SMARTpool, or Runx1 siRNA SMARTpool (all from Dharmacon, siGenome), or a combination of 10 nmol of SP1 and RUNX1 siRNA SMARTpool using HyPerFect Transfection Reagent (Qiagen) as per the manufacturer’s guidelines. Transfection was performed in serum-free medium for 24 hours, after which the medium was replaced with normal growth medium. Protein lysates were collected 48 hours after transfection.

IL-33 CRISPR construct generation

Guide sequence oligonucleotides (forward: TTTCTTGGCTTTATA-TATCTTTGTG GAAAGGACGAAACACCGTGTTGCTAGAA TCCCGTGGAT and reverse: GACTAGCCTTA TTTAATTGCT- TATTTCTAGCCTAAAACATCCAGGGGTATTAGGAAC) including Eco RI restriction overhangs and a protoscaler adjacent motif (PAM) sequence targeting exon 3 within IL-33 were annealed using Phusion High-Fidelity Polymerase [New England Biolabs (NEB)] according to the manufacturer’s guidelines. Ten micromolar of each oligonucleotide was mixed with 25 mM MgCl2, 2.5 mM deoxynucleotide triphosphate, 5X Phusion High-Fidelity Buffer, and Phusion Polymerase to a final volume of 50 μl. Cycling conditions were 98°C (30 s), 30 cycles of 98°C (10 s), 55°C (30 s), and 72°C (20 s), followed by a final incubation at 72°C (20 s). The final PCR product was analyzed by agarose gel electrophoresis. Annealed oligonucleotides were ligated into the gRNA_cloning vector (pcR-Blunt II-TOPO), a gift from G. Church (Addgene plasmid no. 41824). gRNA_cloning vector DNA (1.5 μg) was digested with 1.5 units of Alu II restriction enzyme (NEB) according to the manufacturer’s guidelines and incubated for 1 hour at 37°C. Four nanograms of annealed oligonucleotides was ligated with 30 ng of linearized gRNA_cloning vector using Gibson Assembly Master Mix (NEB) according to the manufacturer’s guidelines and incubated for 1 hour at 50°C. Constructs were transformed into DH5α chemically competent cells and selected on agar plates containing kanamycin (50 μg/ml). To identify positive colonies, DNA was prepared and digested with Eco RI restriction enzyme (NEB).

IL-33 CRISPR/Cas9 transfection and single-cell clone expansion

SCC-FAK-WT cells were seeded on a 100-mm tissue culture dish and grown until ~70% confluent. Cells were cotransfected with 3 μg of hCas9 and 3 μg of gRNA_cloning vector IL-33–specific guides, using脂质体转染试剂(Lipofectamine 2000, Life Technologies) according to the manufacturer’s guidelines. Transfection was performed in serum-free medium for 24 hours, after which the medium was replaced with normal growth medium. Protein lysates were collected 48 hours after transfection.
Cells (1.5 × 10⁶) were plated in 100-mm dishes and, after 24 hours, chromatin preparation (PBS-T). Bound Ab was detected by incubation with anti-rabbit, anti-anti-HDAC1 Abs (Cell Signaling Technology; all 1:1000 in 5% BSA/PBS-T), anti-SP1 (Abcam), anti-Runx1 (Cell Signaling Technology), or anti-WDR82 (Abcam), anti-TAF9 (Abcam), anti-SMARC C1 dehydrogenase (GAPDH; Cell Signaling Technology), anti-phospho-FAK (Y397; Cell Signaling Technology), anti-FAK (Cell Signaling Technology), anti-WDR82 (Abcam), anti-TAF9 (Abcam), anti-SMARC C1 (Abcam), anti-BRD4 (Abcam), anti-HPI α/β (Cell Signaling Technology), anti-Sp1 (Abcam), anti-Runx1 (Cell Signaling Technology), or anti-HDAC1 Abs (Cell Signaling Technology; all 1:1000 in 5% BSA/PBS-T). Bound Ab was detected by incubation with anti-rabbit, anti-mouse, or streptavidin-conjugated horseradish peroxidase secondary Ab (Cell Signaling Technology) and visualized using the Bio-Rad ChemiDoc MP Imaging System.

Immunoblotting

Cells were washed twice in ice-cold phosphate-buffered saline (PBS) and lysed in radiomunonprecipitation assay (RIPA) lysis buffer (50 mM tris-HCL at pH 7.4, 150 mM sodium chloride, 5 mM EGTA, 0.1% SDS, 1% NP-40, and 1% deoxycholate) supplemented with a protease and phosphatase inhibitor cocktail (mini Complete ULTRA Protease tablet and phosSTOP tablet from Roche). Lysates were clarified by high-speed centrifugation (16,000×g for 15 min at 4°C). Protein concentration was measured using a Micro BCA Protein Assay (Thermo Scientific), and 10 to 20 μg of total protein were supplemented with 2X SDS sample buffer (tris (pH 6.8), 20% glycerol, 5% SDS, β-mercaptoethanol, and bromophenol blue) and boiled at 95°C for 5 min. Samples were separated by polyacrylamide gel electrophoresis using 4 to 15% Mini-PROTEAN TGX gels (Bio-Rad), proteins transferred to nitrocellulose, blocked [5% bovine serum albumin (BSA) in PBS], and probed with either anti-IL-33 (R&D Biosystems), anti-γ-aminobutyrate 3-phosphate dehydrogenase (GAPDH; Cell Signaling Technology), anti-phospho-FAK (Y397; Cell Signaling Technology), anti-FAK (Cell Signaling Technology), anti-WDR82 (Abcam), anti-TAF9 (Abcam), anti-SMARC C1 (Abcam), anti-BRD4 (Abcam), anti-HPI α/β (Cell Signaling Technology), anti-Sp1 (Abcam), anti-Runx1 (Cell Signaling Technology), or anti-HDAC1 Abs (Cell Signaling Technology; all 1:1000 in 5% BSA/PBS-T). Bound Ab was detected by incubation with anti-rabbit, anti-mouse, or streptavidin-conjugated horseradish peroxidase secondary Ab (Cell Signaling Technology) and visualized using the Bio-Rad ChemiDoc MP Imaging System.

Chromatin preparation

Cells (1.5 × 10⁶) were plated in 100-mm dishes and, after 24 hours, washed twice in cold PBS. Cells were lysed in 400 μl of extraction buffer [10 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 0.34 M sucrose, 10% glycerol, and 0.2% NP-40 substitute] containing protease/phosphatase inhibitors (mini Complete ULTRA Protease tablet and phosSTOP tablet from Roche). Lysates were cleared at 6500×g for 5 min at 4°C. The resulting nuclear pellet was washed in extraction buffer without NP-40 and centrifuged at 6500×g for 5 min at 4°C. The pellet was resuspended in 400 μl of no-salt buffer [10 mM Hepes (pH 7.9), 3 mM EDTA, and 0.2 mM EGTA], incubated at 4°C for 30 min with agitation, and centrifuged at 6500×g for 5 min at 4°C. The pellet was resuspended in 160 μl of high-salt solubilization buffer [50 mM tris-HCL (pH 8.0), 2.5 M NaCl, and 0.05% NP-40] vortexed briefly, incubated at 4°C for 30 min with agitation, and centrifuged at 16,000×g for 10 min at 4°C. The supernatant containing chromatin fraction was collected, and trichloroacetic acid (TCA) precipitation was performed. TCA was added to a final volume of 10%, and samples were incubated for 15 min on ice. After centrifugation at 21,000×g for 15 min, the resulting pellet was washed twice in 500 μl of cold acetone and then allowed to air-dry. The pellet was then resuspended in 20 μl of 2X SDS sample buffer [tris (pH 6.8), 20% glycerol, 5% SDS, β-mercaptoethanol, and bromophenol blue] and boiled at 75°C for 10 min. Samples were separated by polyacrylamide gel electrophoresis on a 12% Mini-PROTEAN TGX gel (Bio-Rad), transferred onto nitrocellulose membrane, blocked (5% BSA in BSA/PBS-T), and then incubated with primary and secondary Abs as above.

sST2 and IL-33 ELISA

Cells (2 × 10⁶) were plated in a 100-mm tissue culture dish and left to adhere overnight. Medium was replaced with 4 ml of fresh complete growth medium and conditioned for 24 hours, collected, and spun at 1000 revolutions per minute (rpm) for 5 min to remove debris. Medium samples were analyzed for sST2 levels using a mouse ST2 Quantikine ELISA kit or mouse IL-33 Quantikine Elisa kit (R&D Systems). Cells adhered to the tissue culture dish were washed two times in ice-cold PBS and lysed in RIPA lysis buffer as above. Protein concentration was measured using Micro BCA Protein Assay, and protein quantities were used for ELISA values.

qRT-PCR and NanoString

RNA was prepared from cells using the Qiagen RNeasy mini kit as per the manufacturer’s instructions, inclusive of deoxyribonuclease (DNase) digestion. Final concentration of RNA was measured using a NanoDrop (Thermo Scientific). For qRT-PCR, 5 μg of total RNA was converted to complementary DNA (cDNA) using the SuperScript II cDNA Synthesis Kit (Thermo Fisher Scientific). For qRT-PCR, 62.5 ng of cDNA was added to SYBR Green (Applied Biosystems) and supplemented with 0.25 μl of 10 μM qPCR primers to a final reaction volume of 10 μl. IL-33 primers used were forward: GGATCCGATTTTCGAGAGCTTAAATGAT and reverse: GCGGGCGATGAGACCTAGAATGAAGT. Ccl5 primers used were forward: GTTGGGAATGGAAGCAACTA and reverse: AAGCAAGCTGAACAGGCAAT. Ccl5 primers used were forward: GCCCTACCATGACCTAGAATGAAGT. CT2 primers were used forward: GCGGAGAATGGAACGCAACTA and reverse: AACGAGAATGGAACGCAACTA. Ct2 primers were used forward: TCCCTACCATGACCTAGAATGAAGT. NanoString analysis was carried out by the Newcastle NanoString nCounter analysis service. Analysis was performed using nSolver analysis software (NanoString).

Generation of IL-33–BirA–expressing SCC cell line

IL-33 cDNA was amplified by PCR using gene-specific primers (forward: GCAGCAGCGCGGCGCATGAGACTGATGAAATTCACCAC and reverse: TGGCTGCGATTCGATTTTCGGAGACCTAGAATGAAGT) and subcloned into the NotI BamHI sites of pQXIN-BirA-Myc. Specifically, 20 ng of pcDNA-3.1 IL-33 DNA template was mixed with 10 μM of each primer in PfuUltra Hotstart master mix (Stratagene) and subjected to PCR with cycle conditions as follows: 98°C (30 s; 30 cycles), 98°C (10 s), 60°C (30 s), and 72°C (1 min), followed by a final incubation at 72°C (10 min). After PCR, samples were gel-purified (Qiagen Gel Purification Kit) and eluted in 30 μl of nuclease-free water (NFW). The purified PCR product was incubated with 1 μl of BamHI restriction enzyme, 1 μl of NotI restriction enzyme, 5 μl of Buffer 3.1 (all from NEB) to a total of 20 μl, and incubated at 37°C.
for 2 hours. One microgram of pQCXIN-BirA-Myc was incubated with 1 μl of BamH1 restriction enzyme, 1 μl of Not1 restriction enzyme, and 5 μl of Buffer 3.1 to a total of 20 μl and incubated at 37°C for 1 hour. After digestion, both the PCR product and digested vector were gel-purified and eluted in 30 μl of NFW. To ligate the digested IL-33 PCR product into the BamH1/Not1 sites of pQCXIN-BirA-Myc, a 1:4 ratio of digested vector to PCR product was used and incubated with 0.5 μl of T4 DNA ligase (NEB), along with DNA ligase buffer in a final reaction volume of 10 μl, and incubated for 2 hours at room temperature (RT). DH5α chemically competent cells (50 μl) (Life Technologies) were transformed with 5 μl of ligation reaction according to the manufacturer’s guidelines and plated in agar plates containing ampicillin (100 μg/ml). Resulting colonies were checked for successful ligation using a diagnostic digest with BamH1 and Not1 restriction enzymes and, if positive, sequenced. Phoenix Ecotropic cells were transfected with empty vector–BirA or IL-33–BirA using Lipofectamine 2000 (Thermo Scientific) according to the manufacturer’s guidelines. Forty-eight hours after transfection, cell culture supernatant was removed, filtered through a 0.45-μm Millex-HA filter (Millipore), diluted at a 1:1 ratio in normal SCC cell culture medium, supplemented with polybrene (5 μg/ml), and added to SCC FA-K WT IL-33–CRISPR cells for 24 hours. A total of two rounds of infection were performed to generate each cell line before selection in G418 (400 μg/ml).

**Proteomic analysis of the IL-33 interactorome using BioID**

Cells were incubated with 50 μM biotin (Sigma-Aldrich) in complete cell culture medium at 37°C for 24 hours, washed twice in ice-cold PBS, and lysed in RIPA lysis buffer as above. Cell lysates were sonicated using a Bioruptor (Diagenode) (30-s pulses with 30-s intervals over 5 min) and cleared by high-speed centrifugation (16,000g for 15 min at 4°C). Protein concentration was measured using Micro BCA Protein Assay (Thermo Scientific), and 1 mg of total cell lysate was incubated with 50 μl of streptavidin-C1 Dynabeads (Life Technologies) overnight at 4°C with agitation. Beads were washed using a magnetic tube rack three times with ice-cold RIPA buffer and two times with ice-cold PBS. Captured proteins (from experiments performed in biological triplicate) were subjected to on-bead proteolytic digestion, desalting, and liquid chromatography–tandem MS as described previously (38). Mean label-free MS intensities were calculated for each biological replicate. Peptide and protein false discovery rates were set to 1%. Proteins enriched from SCC-FAK-WT IL-33–CRISPR–IL-33–BirA cells by at least threefold when compared to SCC-FAK-WT IL-33–CRISPR–E.V.-BirA (P < 0.05) were considered specific. Proteomics analysis of nuclear FAK protein complexes is described in (9). All other protein interaction network analysis was performed using IPA (Qiagen). The IL-33 MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRoteomics IDEntifications (PRIDE) database partner repository (39) with the data set identifier PXD007698.

**Interaction network analysis**

Genes differentially expressed in FAK lowers SCC cells compared to FAK WT SCC cells were extracted from microarray data (9) using rank product analysis. Significantly differentially expressed genes [P < 0.0005, percentage false-positives (pfp) < 0.05] were subjected to gene ontology enrichment analysis using DAVID Bioinformatics Resources (version 6.8) (40). Genes annotated with overrepresented top-level cellular component terms as determined by Benjamini-Hochberg–corrected hypergeometric test (P < 0.05) were used to seed a protein interaction network based on direct physical interactions constructed using the GeneMANIA plugin (version 3.4.1; mouse interactions) in Cytoscape (version 3.3.0) (41). The largest connected graph component was clustered using the Allegro Spring–Electric force–directed algorithm (Allegro Viva).

**Hierarchical cluster analysis**

Unsupervised agglomerative hierarchical clustering was performed on the basis of Euclidean distance or Pearson correlation computed with an average-linkage or complete-linkage matrix using R or Cluster 3.0 (C Clustering Library, version 1.50) (42). Clustering results were visualized using R or Java TreeView (version 1.1.6) (43) and MultiExperiment Viewer (version 4.8.1) (44).

**Inhibitor treatment**

FAK-WT SCC cells were treated with doses ranging from 50 to 250 nM VS4718 for 24 hours, 10 μM vorinostat for 24 hours, or 200 nM IQ1 for 48 hours, after which RNA or protein lysates were collected for qRT-PCR or Western blot analysis as described above. All inhibitors were obtained from Selleckchem.

**Subcutaneous tumor growth**

All experiments involving animals were carried out in accordance with the UK Coordinating Committee on Cancer Research guidelines by approved protocol (Home Office Project License no. 60/4248). SCC cells (1 × 10⁶; defined earlier) were injected into both flanks of FVB mice, and tumor growth was measured twice weekly using calipers. Animals were sacrificed by cervical dislocation when tumors reached maximum allowed size (1.5 cm in diameter) or when signs of ulceration were evident. Group sizes ranged from three to five mice, each bearing two tumors, and tumor volume was calculated in Excel (Microsoft) using the formula \( V = \frac{4}{3}\pi r^3 \). Statistics and graphs were calculated using Prism (GraphPad).

**CD8 T cell depletion**

Anti-mouse CD8 (clone 53-6.7) and appropriate isotype control Abs (rat immunoglobulin G 2a) were purchased from BioXcel. T cell depletion was achieved after intraperitoneal injection of 150 μg of depleting Ab (same for all Abs) into female age-matched FVB mice for three consecutive days and maintained by further intraperitoneal injection at 7-day intervals until the study was terminated. SCC FAK-WT (1 × 10⁶) or FAK−/− cells were injected subcutaneously into both flanks 6 days after initial Ab treatment, and tumor growth was measured twice weekly as described above.

**Fluorescence-activated cell sorting analysis of immune cell populations**

Tumors established after injection of 1 × 10⁶ SCC cells into both flanks of FVB mice were removed at day 12 into RPMI (Sigma-Aldrich) supplemented with 10% FBS (Life Technologies). Tumor tissue was mashed into a pulp using a scalpel and resuspended in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich) supplemented with collagenase D (2 mg/ml) and DNase1 (200 U/ml; Roche). Samples were incubated for 30 min at 37°C with agitation, pelleted by centrifugation at 1600 rpm for 5 min at 4°C, resuspended in 5 ml of red blood cell lysis buffer (PharmLysing Buffer; Becton Dickinson) for 10 min at 37°C, pelleted by centrifugation at 1600 rpm for 5 min at 4°C, resuspended in PBS, and mashed through a 70-μm cell strainer. The resulting single-cell suspension pellet was pelleted by centrifugation at 1600 rpm for 5 min at 4°C and resuspended in PBS. This step was repeated a further time, and the
resulting cell pellets were resuspended in 100 μl of PBS containing e506 fixable viability dye (1:1000 dilution) and transferred into the well of a round-bottom 96-well plate. Samples were incubated at 4°C for 30 min. Cells were then pelleted by centrifugation at 1600 rpm for 5 min at 4°C and resuspended in fluorescence-activated cell sorting (FACS) buffer (PBS containing 1% FBS and 0.1% sodium azide). This step was repeated for a total of three times. Cell pellets were resuspended in 100 μl of Fc block [1:200 dilution of Fc anti-Fc (eBioscience) in FACS buffer] and incubated for 15 min at RT. One hundred microliters of Ab mixture [1:200 dilution of Abs except anti-FoxP3, which was used at 1:100 (listed and incubated for 15 min at RT). One hundred microliters of Ab mixture was added to each well, and the samples were incubated for 30 min in the dark at 4°C. The plate was then centrifuged at 1600 rpm for 5 min at 4°C, and the cells were resuspended in FACS buffer. This step was repeated three times. Samples were analyzed using a BD Fortessa FACS Analyzer. Data analysis was performed using FlowJo software. All Abs were from eBioscience. Statistics and graphs were calculated using Prism (GraphPad).

SUPPLEMENTARY MATERIALS
www.sciencesignaling.org/cgi/content/full/10/508/eaan8355/DC1
Fig. S1. Identification of common upstream regulators of IL33 and ST2 promoter–associated transcription factors.
Fig. S2. CRISPR knock out of IL33 reduces CD8 expression.
Fig. S3. Gene ontology enrichment analysis.
Table S1. Immune cell population markers.
Table S2. Primer sequences.

REFERENCES AND NOTES

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A phenotypically and functionally distinct human T_{H}2 cell subpopulation is associated with allergic disorders

Erik Wambre,1,8 Veronique Bajzik,1 Jonathan H. DeLong,1 Kimberly O’Brien,1 Quynh-Anh Nguyen,1 Cate Speake,1 Vivian H. Gersuk,1 Hannah A. Deberg,1 Elizabeth Whalen,1 Chester Ni,2 Mary Farrington,2 David Jeong,2 David Robinson,2 Peter S. Linsley,1 Brian P. Vickery,3 William W. Kwok1,4

Allergen-specific type 2 helper T (T_{H}2) cells play a central role in initiating and orchestrating the allergic and asthmatic inflammatory response pathways. One major factor limiting the use of such atopic disease–causing T cells as both therapeutic targets and clinically useful biomarkers is the lack of an accepted methodology to identify and differentiate these cells from overall nonpathogenic T_{H}2 cell types. We have described a subset of human memory T_{H}2 cells confined to atopic individuals that includes all allergen-specific T_{H}2 cells. These cells are terminally differentiated CD4+ T cells (CD27− and CD45RBlow) characterized by coexpression of CRT_{H}2, CD49d, and CD161 and exhibit numerous functional attributes distinct from conventional T_{H}2 cells. Hence, we have denoted these cells with this stable allergic disease–related phenotype as the T_{H}2A cell subset. Transcriptome analysis further revealed a distinct pathway in the initiation of pathogenic responses to allergen, and elimination of these cells is indicative of clinical responses induced by immunotherapy. Together, these findings identify a human T_{H}2 cell signature in allergic diseases that could be used for response-monitoring and designing appropriate immunomodulatory strategies.

INTRODUCTION

As part of their specialization, CD4+ effector T cells acquire functional and phenotypic characteristics to specifically respond against pathogens. Within different T helper (T_{H}) cell subsets, the T_{H}2 cell subset is characterized by the production of interleukin-4 (IL-4), IL-5, IL-9, and IL-13 cytokines, which promote both immunoglobulin E (IgE)– and eosinophil–mediated immune responses (1). Although T_{H}2 cells were initially considered to be a homogeneous subset, their functional heterogeneity is now appreciated, as is the fact that additional T_{H}2 subpopulations may determine T_{H}2-driven pathology (2–4). For example, a recent study revealed a subpopulation of human memory T_{H}2 cells that produces IL-17 along with cardinal T_{H}2 cytokines (5). Remarkably, the proportion of these circulating T_{H}17/T_{H}2 cells was extremely low in nonallergic individuals compared to patients with chronic severe asthma, suggesting a possible role in the pathogenesis and severity of the disease. Another source of heterogeneity among CD4+ T cell subsets is at the level of T cell surface marker expression that determines their differentiation states, effector functions, and migratory capacity. With respect to the T_{H}2 cell subset, our group recently demonstrated that pathogenic allergen-specific T cells are highly matured effector T_{H}2 cells characterized by the lack of expression of CD27, a tumor necrosis factor receptor superfamily member of costimulatory molecules (6, 7). Similarly, distinct subpopulations of T_{H}2 cells with enhanced function have been described in a murine model of allergic inflammation based on differential expression of CXCXR3 and CD62L (8) or CCR8 (9) and in human allergic eosinophilic inflammatory diseases, according to the expression of the hematopoietic prostaglandin D synthase (hPGDS) (10) or IL-17RB (11). In these studies, the authors suggested that heterogeneity within T_{H}2-mediated immune responses plays differential roles in immunopathology. Hence, we surmise that allergic individuals have specific subpopulations of T_{H}2 cells associated with global atopic inflammatory disorders.

Until now, there has been no biological measurement to accurately reflect and quantify an underlying allergic disease process and ideally provide accurate surrogate end points to assess immunotherapy efficacy. A major impediment to the use of allergic disease–causing T cells as a therapeutic target and clinically useful biomarker is the lack of an accepted method to both identify these cells and differentiate them from the overall T_{H}2 cell types. Recent progress in peptide–major histocompatibility complex (MHC) class II (pMHCII) tetramer staining has allowed direct ex vivo visualization of allergen–specific CD4+ T cells and enabled quantification and characterization of these cells in a setting closer to their natural physiological state (7, 12). Description of a set of T cell surface markers that are differentially expressed in allergen–specific T_{H}2 cells as compared to classical T_{H}2 cells would allow this issue to be addressed.

Here, we describe an allergic T cell signature characterized by the coexpression of the chemoattractant receptor CRT_{H}2, the natural killer cell marker CD161, and the homing receptor CD49d in human terminally differentiated (CD45RBlow CD27−) CD4+ T cells. The vast majority of allergen–specific T cells in allergic individuals with either food, pollen, pet’s dander, mold, or house dust mite allergy fall into this subset and were preferentially deleted during allergen–specific immunotherapy (AIT). Hence, we have denoted this proallergic subpopulation of T_{H}2 cells, confined to atopic individuals, as the T_{H}2A cell subset. Transcript analysis further highlights key functional differences between T_{H}2A cells and conventional T_{H}2 cells, providing molecular signatures that suggest specific contribution of the T_{H}2A cell subset to allergic disease. Together, these findings identify a pathogenic T_{H}2 cell signature unique to allergic individuals that could potentially be used as a clinically relevant biomarker and therapeutic target in atopic disorders.

RESULTS

Allergic disease–related phenotypic differences exist in the T_{H}2 cell subset

For many years, chemokine receptors and surface markers have been instrumental in the characterization of memory T cell subsets

1Benaroya Research Institute at Virginia Mason, Seattle, WA 98101, USA. 2Virginia Mason Medical Center, Seattle, WA 98101, USA. 3Aimmune Therapeutics, Brisbane, CA 94005, USA. 4Department of Immunology, University of Washington, Seattle, WA 98195, USA.

*Corresponding author. Email: ewambre@benaroyaresearch.org
with distinct migratory capacity and effector functions. To determine whether a set of T cell surface markers can be differentially expressed in allergen-specific T\(_{H}2\) cells, we undertook a detailed ex vivo phenotypic profiling of total CD4\(^+\) T cells, conventional T\(_{H}12\) cells, and allergen-specific CD4\(^+\) T cells. Using alder pollen allergy as a model, freshly isolated peripheral blood mononuclear cells (PBMCs) from DR07:01- or DR15:01-restricted allergic individuals were stained with fluorescently labeled pMHCII tetramers, followed by magnetic column enrichment process to directly examine allergen-specific CD4\(^+\) T cell phenotypic profiles. Among T\(_{H}12\)-associated surface markers, CRTH2, the prostaglandin D2 receptor chemoattractant receptor–homologous molecule expressed on T\(_{H}12\) cells, is reported as the most reliable marker to identify human T\(_{H}12\) cells (13). As a control, we examined the ex vivo phenotypic profile of total CRTH2\(^+\) CD4\(^+\) memory T cells to compare with the ex vivo enriched allergen-specific CD4\(^+\) T cells. During these flow cytometric screen analyses, fluorochrome-conjugated antibodies directed against cell surface marker antigens were selected to elucidate the differentiation, maturation, activation, and homing properties of each group (fig. S1 and table S1). Variation in surface marker expression between groups is shown in fig. S2 (A and B). As expected, ex vivo enriched allergen-specific CD4\(^+\) T cells from allergic individuals share numerous memory T\(_{H}12\) cell features with the conventional T\(_{H}12\) cell group featuring the expression of CD45RO, CCR4, CD200R, CD58, CD29, and CRTH2. However, we identified an allergic T cell signature that includes two up-regulated (CD161 and CD49d) and four down-regulated (CD27, CD45RB, CCR7, and CD7) T cell surface markers with significant differential expression (greater than 20% change; \(P < 0.001\)) between groups (fig. 1A). The CD27\(^{low}\), CCR7\(^{low}\), CD7\(^{low}\), and CD45RB\(^{low}\) phenotypes, which are associated with terminally differentiated memory CD4\(^+\) T cells, likely reflect recurrent natural allergen exposure (14, 15). This is consistent with previous findings by our group demonstrating a strong relationship between pathogenicity of allergen-specific CD4\(^+\) T cells and the maturation stage of the cells (7, 16). Although loss of CD27 expression within CD4\(^+\) memory T cells is consistently associated with cells lacking CCR7 and CD7, we observed that CD27\(^{low}\) CD4\(^+\) T cell subset can be subdivided into two groups by CD45RB expression (fig. S3). Thus, to define a smaller set of surface markers, we chose CD27 and CD45RB as convenient down-regulated markers reflecting allergic features.

Another striking finding from this T cell profiling was the overexpression of the C-type lectin-like receptor CD161 (2,2-fold difference, \(P < 0.001\)) as part of the signature characterizing allergen-specific T\(_{H}12\) cells. Expression of CD161 on CD4\(^+\) T cells is typically associated with T\(_{H}17\) responses (17, 18), and like the conventional T\(_{H}12\) cell subset (CRT12\(^+\) CD4\(^+\)), allergen-specific T\(_{H}12\) cells do not express the T\(_{H}17\)-associated chemokine receptor CCR6 (fig. 1B). We next performed quantitative polymerase chain reaction (PCR) analysis on sorted cells from allergic donors and confirmed the higher expression of CD161 mRNA in CRTH2\(^+\)-expressing allergen-specific T cells compared to conventional T\(_{H}12\) cells (Fig. 1C). However, although allergen-specific T\(_{H}12\) cells express similar levels of CD161 as the T\(_{H}17\) cell subset (CCR6\(^+\) CXC3\(^+\) CD4\(^+\)), these cells did not exhibit mRNA expression of T\(_{H}17\) phenotypic markers such as CCR6, IL23R, and the transcription factor RORC. Together, these data indicate that allergic disease–related phenotypic differences (not related to a type 17 phenotype) occur in the T\(_{H}12\) cell subset.

To demonstrate that our data were not restricted to tree pollen allergy, we next performed our ex vivo pMHCII tetramer approach to characterize allergen-specific CD4\(^+\) T cells in patients with either food allergy (peanut), perennial allergy (cat and house dust mite), mold allergy (Aspergillus and Alternaria), or seasonal pollen allergy (alder and timothy grass). We also used nonallergic individuals as controls. Whatever the allergen tested in this study, IgE-mediated allergic diseases were characterized by high frequencies of allergen-specific CRTH2\(^+\) CD4\(^+\) T cells, which were strictly absent in nonallergic subjects, suggesting that the presence of these CD4\(^+\) effector T cells is necessary for allergy pathogenesis (Fig. 2A). In all allergic individuals tested, the vast majority of pMHCII tetramer–positive T cells were also characterized by the lack of CD27 expression along with expression of CD161 (Fig. 2B). Remarkably, CRTH2\(^+\) expression on

**Fig. 1.** Allergic disease–related phenotypic differences emerged in the T\(_{H}12\) cell subset. (A) Fluorescence-activated cell sorting (FACS)–based T cell surface expression screening revealed up-regulated and down-regulated T cell surface markers in ex vivo magnetically enriched allergen-specific CD4\(^+\) T cells compared to total CRTH2\(^+\) CD4\(^+\) T cells. Average expression levels for each T cell surface marker in the allergen-specific CD4\(^+\) T cell group and in total CRTH2\(^+\) CD4\(^+\) T cell group are plotted against each other. Data are means from four allergic subjects per group. The gray field depicted less than 20% expression variation between groups. Differences between groups were analyzed using the Mann-Whitney U test. (B) Examples of intensity distributions of total CRTH2\(^+\) CD4\(^+\) T cells (blue) and ex vivo magnetically enriched CRTH2\(^+\) allergen-specific CD4\(^+\) T cells tracked by pMHCII tetramer (red) stained with candidate cell surface markers. Data are representative of at least three allergic donors. (C) Real-time PCR analysis confirms that allergen-specific T\(_{H}12\) cells express CD161 but are not related to a type 17 phenotype. Data are means ± SEM from at least three subjects per group.
allergen-specific CD4+ T cells was concomitant with a lack of CD45RB and CD27 expression as well as coexpression of CD161 and CD49d (Fig. 2C and fig. S4). Collectively, these data identify the pathogenic allergen-specific TH2 cell subset in atopic individuals as highly mature (CD27− CD45RBlow) TH2 cells coexpressing CD161 and CD49d.

A distinct TH2 cell subset is associated with type 1 allergic diseases

We next sought to determine whether the pathogenic T cell signature identified on allergen-specific TH2 cells could be used to define a subset of the TH2 cells that would reflect an underlying allergic disease process. Although it has been argued that CRTH2+ CD4+ T cells are present at higher frequency in allergic subjects, we observed that this difference is marginal (fig. S5A). Despite a substantially lower proportion of CD161- expressing CRTH2+ T cells in nonatopic individuals, this subset was not restricted to allergic subjects. However, we observed that at least two markers (that is, CD161 and CR45RB or CD27) were needed to subset the CRTH2+ CD4+ T cells to identify an allergy-prone TH2 subset virtually absent in the nonatopic group, which includes the vast majority of allergen-specific T cells from allergic individuals (fig. S5, B and C). Using the gating strategy depicted in Fig. 3A, we...
observed that all allergic individuals tested exhibited a significantly higher number (n = 80; mean ± SEM, 3766 ± 413 cells per 10⁶ memory CD4⁺ T cells) of CD45⁺CD49d⁺CD27⁻ CRTh₂⁺CD161⁺ cells relative to nonatopic individuals (n = 34; mean ± SEM, 259 ± 37 cells per 10⁶ memory CD4⁺ T cells; P < 0.0001) (Fig. 3, A and B). Hence, we have named these proallergic T11₂ cells (which are unique to allergic individuals) the T11₂A cell subset.

Remarkably, both conventional T₁₁₂ and T₁₁₂A cell subsets retain their respective phenotype after long-term clonal expansion, suggesting that they did not differ in activation or maturation status and can thus be used as a stable and relevant surrogate marker (Fig. 3C). To confirm that the T₁₁₂A cell subset is specifically involved in type I allergic diseases, we next followed 10 grass pollen–allergic individuals before and during the grass pollen season (May to August), a window of time that correlates with increased allergy symptoms and with up-regulation of the activation marker CD38 within grass pollen–reactive CD4⁺ T cells (7, 16). Consistent with direct access to allergy-prone T₁₂ cells according to CRT₁₂, CD27, CD45RB, CD49d, and CD161 differential expression, we observed that CD38 expression was specifically up-regulated within the T₁₁₂A subset during grass pollen season but not within the conventional T₁₁₂ cell subset or outside pollen season (Fig. 3, D and E). Collectively, our data demonstrate that the T₁₁₂A cell subset represents a phenotypically distinct T₁₁₂ subpopulation, which may encompass the vast majority of pathogenic T₁₁₂ cells involved in type I allergic diseases.

The T₁₁₂A cell subset represents a suitable therapeutic target

To determine whether the T₁₁₂A cell subset constitutes a clinically relevant therapeutic target in the allergy context, we next performed a longitudinal study in a subset of peanut-allergic patients completing characterized oral desensitization immunotherapy (CODIT) with AR101, an experimental orally administered biological drug containing the antigenic profile found in peanuts. During this randomized, double-blinded, placebo-controlled trial (ARC001), coded samples from subjects were provided to the operator at baseline both before and after double-blind, placebo-controlled food challenges (DBPCFC) with peanut flour, as well as at the end of the maintenance visit before DBPCFC. The magnitude and quality of peanut–specific T cell responses were determined ex vivo using the CD154 up-regulation assay (19) after short restimulation of PBMCs with a pool of peanut peptides library derived from Ara h 1, Ara h 2, Ara h 3, Ara h 6, and Ara h 8 peanut-allergic components. As expected, the vast majority of peanut-reactive CD4⁺ T cells were bona fide T₁₁₂A cells at baseline, and the DBPCFC protocol led to significant increased expression of the cell surface activation marker CD38 (Fig. 4A and fig. S6A), concomitant with an increased average frequency of these cells (Fig. 4B). Accordingly, only T₁₁₂A cells, and not conventional T₁₁₂ cells, were specifically activated after peanut oral food challenge (OCF) (fig. S6B).

As reported elsewhere (20), 100 and 78% of patients who completed the active treatment regimen (n = 23) tolerated a cumulative amount of peanut protein of 443 and 1043 mg, respectively, compared to 19 and 0% in the placebo group (n = 26). In such a setting, we observed a direct correlation between decrease in peanut–specific T₁₁₂A cell frequency and achievement of peanut desensitization in the active group compared to placebo (Fig. 4, C and D, and fig. S6C). Together, our data demonstrate that T₁₁₂A cells play a critical role in allergic disease pathogenesis and reinforce previous data by our group that the allergen–specific T₁₁₂ cell subset may represent a suitable therapeutic target and surrogate marker of clinical efficacy during AIT (7, 16, 21).

T₁₁₂A cells differentially contribute to T₁₁₂-driven pathology

To determine whether allergic disease–related functional differences could be identified in the T₁₁₂A cell subset, freshly isolated T₁₁₂A, T₁₁₂ (CD161⁺CRT₁₁₂⁺CD27⁻), and T₁₁₁/T₁₁₇ (CD161⁺CRT₁₁₂⁻CD27⁻) cell subsets from allergic individuals were subjected to polychromatic intracellular cytokine profile analysis. After polyclonal activation with phorbol 12-myristate 13-acetate (PMA)/ionomycin, a significantly higher proportion of T₁₁₂A cells expressed IL-5 and IL-9 compared to conventional T₁₁₂ cells (Fig. 5A). Conversely, interferon-γ (IFN-γ) and IL-17, the respective cytokines for T₁₁₁ and T₁₁₇ cell subsets, were restricted to the CD161⁺CRT₁₁₂⁻CD27⁺ T₁₁₁ cell population. The T₁₁₂A cell subset was also more polyfunctional, with a significantly greater proportion of cells producing simultaneously multiple T₁₁₂ effector cytokines compared to conventional T₁₁₂ cells (Fig. 5, B and C). As a comparison, expression of cardinal T₁₁₂ cytokine was also investigated within ex vivo enriched allergen–specific CD4⁺ T cells in allergic
individuals and found to be restricted to the CD27^−CRTH2^−CD161^+ allergen-specific CD4^+ T cell subset (fig S7). Remarkably, the unique secretion pattern of TH2A cell lines was quite stable over time, even after multiple rounds of stimulations over sequential 6-week cultures (Fig. 5D).

Thus, human circulating TH2A cells may contribute differently to TH2-driven pathology than conventional TH2 cells by simultaneously producing multiple cardinal TH2 cytokines.

**Transcriptome analysis reveals unique pathway in TH2A cells**

To further investigate the pathophysiologic meaning of the allergic T cell signature, we performed microarray analysis (Gene Expression Omnibus accession GSE93219) on freshly isolated TH2A cells compared to known T cell subsets (that is, TH1, TH17, and TH2) from different donor pools, which contained blood from two to three donors. This was necessary to obtain sufficient numbers of cells for microarray experiments. From the data sets comparing TH2A with TH2 cells, epithelium-derived cytokines receptors, such as the IL-25 receptor (IL-25R), the IL-33 receptor (IL1RL1), and the thymic stromal lymphopoietin–receptor (CLRF2), which are well-known molecules involved in the allergic/asthmatic immune response (22–24), were more highly expressed in TH2A cells relative to conventional TH2 cells (Fig. 6, A and B). In addition, we confirmed that TH2A cells produced more IL-5 and IL-9 relative to conventional TH2 cells, whereas TH1- and TH17-related genes (IFN-γ, IL-17, RORC, IL23-R, and CCL20) were absent in TH1 and TH2A cell subset (Fig. 6B). TH2A cells also highly expressed genes involved in arachidonic acid signaling that have previously been linked to allergic disease such as hPGDS (10), the prostaglandin synthase PTGS2 (25, 26), the short-chain free fatty acid receptor GPR42 (27), and the peroxisome proliferator–activated receptor PPARγ (table S2) (21). Because of limitations of currently available anti-human ST2 and IL17RB reagents, we were unable to observe the differential expression of these two markers on the surface of peripheral CD4^+ T cells by using flow cytometry. Thus, we wished to determine whether up-regulation of IL-17RB and IL1RL1 transcript identified in the TH2A cell subset was specifically observed on allergen-specific T cells from allergic individuals. To this aim, we performed a real-time PCR expression analysis on sorted pMHCII tetramer-positive T cells tracking peanut-specific CD4^+ T cells in peanut-allergic subjects and in nonatopic individuals. Sorted conventional TH2 cells from the same allergic subjects were also used as control. As expected, we confirmed that gene transcripts, such as CD161, IL1RL1, and IL17RB, were expressed in allergen-specific CD4^+ T cells from allergic individuals but were absent both in conventional TH1 cells and in allergen-specific T cells from nonallergic individuals (Fig. 6C). Although not causal, these data imply that pathological differences between TH1 and conventional TH2 cells in allergic individuals are fundamental to disease development (fig S8).

**DISCUSSION**

Although antigen-specific TH2 cells are at the core of the allergic process in atopic individuals, tracking and targeting these allergic...
disease–causing T cells without affecting other nonpathogenic T\textsubscript{H}2 processes have been a challenge. Using an ex vivo pMHCII tetramer-based T cell profiling, we have shown that in all type 1 allergic individuals, the differential expression of at least three markers (that is, CRTH2, CD161, and a differentiation stage marker such as CR45RB or CD27) is needed to define a pathogenic T\textsubscript{H}2 cell subset that is allergen-specific and virtually absent in nonatopic individuals (denoted here as T\textsubscript{H}2A subset).

Multiples lines of evidence suggested the pathogenic potential of T\textsubscript{H}2A cell subset in settings of allergic inflammatory disease. First, we observed that allergen-specific T\textsubscript{H}2 cells from allergic patients with either seasonal, perennial, fungus, or food allergy were virtually all contained in the terminally differentiated (CD27\textsuperscript{−}) memory T\textsubscript{H}1 cell subset that coexpresses CRT\textsubscript{H}2 and CD161. Second, the overall number of cells from this subset was markedly higher in all allergic individuals as compared to nonatopic individuals (denoted here as T\textsubscript{H}12A subset).

Fig. 6. T\textsubscript{H}2A cell subset shows distinct gene expression patterns. (A) Scatterplot of the average signal of T\textsubscript{H}2A versus conventional T\textsubscript{H}2 cell gene expression microarray data. Shown are genes whose transcription has been up-regulated (red) or down-regulated (blue) by a factor of 2. Genes that have previously been linked to allergic diseases are listed. (B) Hierarchical clustering heat map of all genes with expression fold changes of eight in one cell subset relative to the other three subsets. Data are mean normalized raw gene expression values from two independent microarray experiments on cells sorted from different donor pools (each pool containing blood from two to three donors). (C) Real-time PCR analysis showing mRNA expression profile of the most relevant genes up-regulated in T\textsubscript{H}2A cell subset in total CRT\textsubscript{H}2\textsuperscript{+} T cells (gray) and in allergen-specific T cells from nonallergic individuals (white) or allergic subjects (black). Data are means ± SEM from at least three subjects per group.

CD161 expression has been described as a hallmark of human T\textsubscript{H}17 cells (17, 18). Therefore, its expression on a T\textsubscript{H}12 cell subset that does not express CCR6, RORC, or IL-17 cytokine is of great interest. Given that lectin-like transcript 1, the CD161 ligand, is expressed on respiratory epithelial cells during respiratory virus infection (30), it likely indicates the specialized role of allergen-specific T\textsubscript{H}2 cells and thus may be implicated in allergic pulmonary inflammation and asthma exacerbation. CD161 expression also provides gut-specific homing properties to T cells (31), and a higher proportion of CD161\textsuperscript{+} circulating CD4\textsuperscript{+} T cells have been previously described in allergic patients compared to nonatopic individuals (10, 32). Expression of CD161 on T\textsubscript{H}12 cells was also associated with IL-5–producing T effector cells associated with eosinophilic gastrointestinal disease (3). In support of these findings, our results show that IL-5 and IL-9 cytokines have some of the greatest fold changes of all up-regulated transcripts in the T\textsubscript{H}12A subpopulation compared with conventional T\textsubscript{H}2 cells. Our functional analysis also confirmed that T\textsubscript{H}2A cells exhibited profoundly superior functional activity compared to conventional T\textsubscript{H}2 cells, with individual cells capable of producing a larger amount of a broad spectrum of T\textsubscript{H}2 cytokines upon TCR activation. Because each T\textsubscript{H}2 cytokine has a well-defined and relatively
specific function, it is likely that T_{H}2A cells have greater adverse activity relative to conventional T_{H}2 cells, which might reflect the wide array of clinical symptoms associated with allergic disorders (10, 33–35).

Understanding why some individuals elicit a pathogenic T_{H}2 response to allergen might facilitate the development of improved vaccination strategies. It therefore raises the question of the origin of T_{H}2A cells in atopic individuals. There is now growing evidence for a role of epithelium-derived cytokines in the differentiation of T_{H}2 cells and in the establishment of airway inflammation (36). IL-33 and IL-25 pathways have been also associated with the induction of both IL-9 and IL-5 production in human T_{H}2 cells that drive a cascade of downstream events (37–40). One possible mechanism to explain and integrate all these results into a cohesive schema is that upon allergen recognition, epithelial cells release cytokines that not only stimulate innate cell networks but may also act directly on CD4^{+} T cells to confer memory T_{H}2 cell pathogenicity in atopic individuals, as recently suggested by Endo et al. (39). Whether local epithelial cytokines influence allergen-specific T_{H}2 cell response requires further study, but our finding that T_{H}2A cells specifically express IL-17RB and IL1RL1 supports the notion of a local checkpoint that restricts the optimal pathogenic T_{H}2 responses to sites of tissue distress (10, 41). By establishing a clear link between the elimination of the allergen-specific T_{H}2A cell subset in peanut-allergic patients and the clinical benefit induced by oral immunotherapy, our data reinforce previous reports by our group that the current immunotherapy approach, using crude preparation of intact allergens, restores a desensitization state in the allergic patients by means of preferential exhaustion/deletion of allergen-specific T_{H}2 cells (7, 16, 42). T_{H}2A cell subset shares multiple functional features with CCR8^{+} (9), hPGDS^{+} (10), and IL-17RB^{+} (11) pathogenic T_{H}2 cell subsets that have been recently described in chronic atopic dermatitis, eosinophilic gastrointestinal diseases, and eosinophilic chronic rhinosinusitis, respectively. Therefore, it seems likely that T_{H}2A cell subset described in this study may encompass various types of pathogenic T_{H}2 cell populations involved in atopic diseases. Together, it supports the "disease induction model" proposed by Nakayama and colleagues (43–45), wherein the presence of a pathogenic CD4^{+} T cell subset with distinct phenotypic and functional properties might be sufficient for the pathogenesis of an immune-mediated disease, regardless of the balance of other T_{H}1 Subsets.

In summary, we have identified a proinflammatory human T_{H}2 cell subpopulation unique to atopic individuals that is defined by stable coexpression of CRT_{H}2, CD161, and CD49d and low expression of CD45RB and CD27. We suggest that T_{H}2A cells are important in the pathogenesis of allergic diseases and should facilitate the detailed analysis of allergen-specific T_{H}2 cell subset in allergic individuals. Therefore, further detailed studies focusing on the T_{H}2A cell subset may prove useful in the diagnosis, molecular characterization, or the discovery of novel therapeutic targets to enhance the power of allergen vaccines.

MATERIALS AND METHODS

Study design

The main research objective of this study was to determine whether allergic individuals have specific subpopulations of T_{H}2 cells associated with global atopic inflammatory disorders. To investigate allergic-related differences in peripheral T cells from allergic individuals, the profile of allergen-specific T_{H}2 cell subset ex vivo using direct pMHCII tetramer staining was determined and compared to the profile of total T_{H}2 cell subset. Candidate signature-associated markers were then tested in allergic patients and in nonatopic individuals. To evaluate this signature in the context of clinical intervention, a longitudinal study was conducted in patients receiving oral immunotherapy. Sample size was determined on the basis of the availability of fresh blood samples and with the intention to include samples before and after OFC and before and after therapy, where possible. All data generated were included in the analysis. Researchers performing the measurements were blinded to the treatment group and sample identity. To further explore the pathophysiologic meaning of this allergic T cell signature, we used real-time PCR, intracellular cytokine analysis and microarray analysis. Replication numbers for experiments are listed in the figure legends. Primary data for experiments where n < 20 are shown in table S3.

Subjects

Subjects were recruited at the Allergy Clinic at Virginia Mason Medical Center. All subjects were recruited with informed consent, and the study was approved by the Institutional Review Board of Benaroya Research Institute. Allergic subjects (n = 80) were selected on the basis of their clinical history, a positive prick test, and positive IgE reactivity to extract (test score, ≥ 0.35 kU/liter) using the ImmunoCAP test (Phadia AB). For subjects with no history of allergy (n = 34), the nonatopic status was confirmed by a lack of IgE reactivity and a negative in vitro basophil activation assay after stimulation with a pool of allergen extracts. All subjects were human leukocyte antigen (HLA)–typed by using sequence-specific oligonucleotide primers with UniTray SSP kits (Invitrogen).

CODIT study design and participants

In ARC001 (46), a multicenter, randomized, double-blind, placebo-controlled study of efficacy and safety of CODIT (Aimmune Therapeutics Inc.), peanut-allergic subjects aged 4 to 26 years were enrolled on the basis of clinical history of allergy to peanut, a serum IgE to peanut of ≥ 0.35 kU/liter (UniCAP) or positive skin prick test to peanut of > 3 mm compared to control, and an allergic reaction at or before 100 mg of peanut protein during a screening DBPCFC, conducted in accordance with PRACTALL (Practical Issues in Allergology, Joint United States/European Union Initiative) guidelines. Participants were randomly assigned (1:1) to active treatment with AR101 or matched placebo. Subjects initiated the study with a single dose of 0.5 mg of study product and escalated biweekly over the course of about 20 weeks to the target maintenance dose of 300 mg/day. The primary clinical efficacy end point was the proportion of subjects in each group who tolerated at least 300 mg (443 mg cumulative) of peanut protein with no more than mild symptoms at the exit DBPCFC. Of 55 subjects enrolled in the ARC001 study, 10 participants were consented for additional volume of blood (10 to 15 ml) to be collected before and after the screening DBPCFC, and 7 participants (3 placebo and 4 active) were consented for additional volume of blood to be collected before and after CODIT.

Tetramer reagents

Biotinylated HLA-DR molecules were generated and purified as described (47). T cell epitopes were identified by tetramer-guided epitope mapping (table S4) (48). Epitope-specific pMHCII tetramer reagents were generated by loading specific peptides onto biotinylated soluble DR monomers and subsequently conjugated with phycoerythrin (PE)–streptavidin (47).
Ex vivo analysis of allergen-specific CD4\(^+\) T cells

Twenty million PBMCs in culture medium at a concentration of 150 million cells/ml were treated with dasatinib (49) for 10 min at 37°C, followed by staining with of PE-labeled pMHCIi tetramers (20 μg/ml) at room temperature for 100 min. After tetramer staining, cells were then washed twice and incubated with anti-PE magnetic beads (Miltenyi Biotec) at 4°C for another 20 min. The cells were washed again and enriched using a magnetic column according to the manufacturer’s instructions (Miltenyi Biotec). Frequency was calculated as previously described (50). For unbiased FACS screen analysis, CRT1\(_{12}\)-labeled PBMCs and cells in the tetramer-bound fractions were both stained with antibodies against markers of interest (table S1) or corresponding isotype-matched monoclonal antibodies. A combination of the vital dye Via-Probe (BD Pharmingen) as a viability marker, CD19 (eBioscience), and CD14 (eBioscience) was used to exclude dead cells, B cells, and monocytes from the analysis, respectively. A FACSARia II was used for multiparameter analysis, and data were analyzed with FlowJo software (Tree Star, Inc.).

T\(_{H2A}\) cell subset analysis

T\(_{H2A}\) cells were defined as CD4\(^+\)CD45RO\(^-\)CD27\(^-\)CD45RB\(^{low}\)CRTH2\(^+\)CD161\(^-\)CD49d\(^-\) T cell subset. The following antibodies were used in flow cytometric analysis: fluorescein isothiocyanate (FITC)–conjugated anti-CD45RB (clone MEM-55, AbD Serotec), phycoerythrin-Texas Red (ECD)–conjugated anti-CD45RO (clone UCHL1, Beckman Coulter), Alexa Fluor 647–conjugated anti-CRT1\(_{12}\) (clone BM16, BD Biosciences), antigen-presenting cell (APC)–H7–conjugated anti-CD27 (clone M-T271, BD Biosciences), V450–conjugated anti-CD38 (clone HIT2, eBioscience), eFluor 650–conjugated anti-CD3 (clone OKT3, eBioscience), PE-conjugated anti-CD161 (clone HP-3G10, eBioscience), PE-Cy7–conjugated anti-CD49d (clone 9F10, BioLegend), and BV605–conjugated anti-CD4 (clone OKT4, BioLegend). CD45RB\(^{low}\) cells were identified using a cutoff of 35% among live memory CD4\(^+\) T cells.

T\(_H\) cell subset isolation

Freshly isolated PBMCs were labeled with V500–conjugated anti-CD4 (clone RPA-T4, BD Biosciences), Alexa Fluor 647–conjugated anti-CRT1\(_{12}\) (clone BM16, BD Biosciences), PE-Cy7–conjugated anti-CCR6 (clone R6H1, BD Biosciences), AF488–conjugated anti-CXCR3 (clone 1G6/CXCR3, BD Biosciences), APC–H7–conjugated CD27 (clone M-T271, BD Biosciences), ECD–conjugated anti-CD45RO (clone UCHL1, Beckman Coulter), PE-conjugated anti-CD161 (clone HP-3G10, eBioscience), and eFluor 650–conjugated anti-CD3 (clone OKT3, eBioscience). A combination of the vital dye Via-Probe (BD Pharmingen) as a viability marker, CD19 (eBioscience), and CD14 (eBioscience) was used to exclude dead cells, B cells, and monocytes from the analysis, respectively. T\(_{H2A}\) cells (CD4\(^+\)CD45RO\(^-\)CD27\(^-\)CRT1\(_{12}\)^{CD161\(^-\)}) (convventional T\(_{H2A}\) cells (CD4\(^+\)CD45RO\(^-\)CD27\(^-\)CRT1\(_{12}\)^{CD161\(^-\)}) (convventional T\(_{H2A}\) cells (CD4\(^+\)CD45RO\(^-\)CD27\(^-\)CRT1\(_{12}\)^{CD161\(^-\)}), T\(_{H17}\) cell subset (CD4\(^+\)CD45RO\(^-\)CRT1\(_{12}\)^{CCR6\(^+\)} CXCR3\(^-\)), T\(_{H1}\) cell subset (CD4\(^+\)CD45RO\(^-\)CRT1\(_{12}\)^{CCR6\(^+\)} CXCR3\(^+\))), and T\(_{H1}\) cell subset (CD4\(^+\)CD45RO\(^-\)CRT1\(_{12}\)^{CCR6\(^+\)} CXCR3\(^+\)CXCR3\(^-\))) were isolated to a purity over 96% using FACSARia II (BD Biosciences) (fig. S9).

Intracellular cytokine staining

Intracellular staining was performed by using the Cytofix/Cytoperm buffer set (BD Biosciences) according to the manufacturer’s instructions. Briefly, cells were incubated for 5 hours at 37°C with 5% CO\(_2\) with PMA (50 ng/ml), ionomycin (500 ng/ml), and GolgiPlug (BD Biosciences), permeabilized with Cytofix/Cytoperm buffer, and stained with APC-conjugated anti-IL-4 (clone JES1-39D10, Miltenyi Biotec), FITC-conjugated anti–IL-5 (clone 8D4-8, eBioscience), PE-conjugated anti–IL-9 (clone MH9A4, BioLegend), PerCP/Cy5.5–conjugated anti–IFN-γ (clone JES5-2A5, BioLegend), BV510–conjugated anti–IL-17 (clone BL168, BioLegend), and APC/Cy7–conjugated anti–IL-17 (clone BL168, BioLegend). After 30 min at 4°C, cells were washed and immediately analyzed by flow cytometry.

Real-time PCR expression analysis

The Fluidigm BioMark 96.96 Dynamic Array (51) was used to measure the gene expression in small cell populations. Ten cells per well were sorted by FACS in quadruplicate into 96-well plates containing a reaction mix for reverse transcription (CellsDirect One-Step qRT-PCR kit, Invitrogen) and preamplification with 96 selected gene primer pairs (Delta Gene assays, Fluidigm Corp.). After sorting, samples were reverse-transcribed and preamplified for 18 cycles. Primers and deoxynucleotide triphosphates were removed by incubation with Exonuclease I (New England Biolabs), and samples were diluted (five times) with TE buffer and stored at −20°C. Samples and assays (primer pairs) were prepared for loading onto 96.96 Fluidigm Dynamic Arrays according to the manufacturer’s recommendations. Briefly, the sample was mixed with 20× DNA binding dye sample loading reagent (Fluidigm Corp.) and 2× SooFast EvaGreen Supermix with Low ROX (Bio-Rad). Assays were mixed with 2× assay loading reagent (Fluidigm Corp.) and TE buffer to a final concentration of 5 μM. The 96.96 Fluidigm Dynamic Arrays (Fluidigm Corp.) were primed and loaded on an IPC Controller HX (Fluidigm Corp.), and real-time PCR was run on a BioMark HD (Fluidigm Corp.). Data were collected and analyzed using Fluidigm Real-Time PCR analysis software (v4.1.2).

Microarray analysis and data analysis

Conventional T\(_{H1}\) cells, conventional T\(_{H17}\) cells, T\(_{H2A}\) cells, and conventional T\(_{H12}\) cells were sorted from PBMCs of allergic subjects, as described above. Use of donor pools (each pool containing blood from two to three donors) was necessary to obtain sufficient numbers of cells for microarray experiments. Sorted T\(_{H1}\) subsets were stimulated for 6 hours with anti-CD3/CD28 beads (Life Technologies) or left unstimulated before extraction of RNA (RNeasy Mini kit, Qiagen). Replicates of RNA were obtained from each sample that passed quality control. Complementary RNA was prepared by amplification and labeling using the Illumina TotalPrep RNA Amplification kit (Life Technologies) and hybridized to human HT-12 Beadarray chips (Illumina). Beadchips were scanned on a HiScanSQ Beadarray chips (Illumina). Background-subtracted data were generated using GenomeStudio software (Illumina). Data were processed by customized R/Bioconductor pipeline, including quantile normalization (52), flooring, log, transformation, and PALO filtering (Present At Least Once; at least one sample must have had detection P < 0.01). Analyses were performed using R.

Statistical analysis

Prism software (GraphPad) was used for statistical analysis of flow cytometry data. No randomization or exclusion of data points was used. The nonparametric Mann-Whitney U test was used for unpaired comparisons between groups, whereas the nonparametric Wilcoxon matched pairs test was used for paired comparison.


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Eradication of spontaneous malignancy by local immunotherapy

Idit Sagiv-Barfi, Debra K. Czerwinski, Shoshana Levy, Israt S. Alam, Aaron T. Mayer, Sanjiv S. Gambhir, Ronald Levy*

It has recently become apparent that the immune system can cure cancer. In some of these strategies, the antigen targets are preidentified and therapies are custom-made against these targets. In others, antibodies are used to remove the brakes of the immune system, allowing preexisting T cells to attack cancer cells. We have used another noncustomized approach called in situ vaccination. Immunoenhancing agents are injected locally into one site of tumor, thereby triggering a T cell immune response locally that then attacks cancer throughout the body. We have used a screening strategy in which the same syngeneic tumor is implanted at two separate sites in the body. One tumor is then injected with the test agents, and the resulting immune response is detected by the regression of the distant, untreated tumor. Using this assay, the combination of unmethylated CG–enriched oligodeoxynucleotide (CpG)—a Toll-like receptor 9 (TLR9) ligand—and anti-OX40 antibody provided the most impressive results. TLRs are components of the innate immune system that recognize molecular patterns on pathogens. Low doses of CpG injected into a tumor induce the expression of OX40 on CD4+ T cells in the microenvironment in mouse or human tumors. An agonistic anti-OX40 antibody can then trigger a T cell immune response, which is specific to the antigens of the injected tumor. Remarkably, this combination of a TLR ligand and an anti-OX40 antibody can cure multiple types of cancer and prevent spontaneous genetically driven cancers.

INTRODUCTION

T cells that recognize tumor antigens are present in the tumor microenvironment, and their activity is modulated through stimulatory and inhibitory receptors. Once cancer is well established, the balance between these inputs is tipped toward immunosuppression (1, 2). The inhibitory signals on T cells are delivered through molecules such as cytotoxic T lymphocyte–associated protein 4 (CTLA4) and programmed cell death protein 1 (PD1) by interaction with their respective ligands expressed on cancer cells and/or antigen-presenting cells (APCs). However, these same tumor-reactive T cells express stimulatory receptors including members of the tumor necrosis factor receptor (TNFR) superfamily. Therefore, many attempts are being made to relieve the negative checkpoints on the antitumor immune response and/or to stimulate the activation pathways of the tumor-infiltrating effector T cells (Teff).

Here, we conducted a preclinical screen to identify candidate immunostimulatory agents that could trigger a systemic antitumor T cell immune response when injected locally into one site of tumor. We found that Toll-like receptor 9 (TLR9) ligands induce the expression of OX40 on CD4 T cells in the tumor microenvironment. OX40 is a costimulatory molecule belonging to the TNFR superfamily, and it is expressed on both activated T eff and regulatory T cells (Treg). OX40 signaling can promote Teff activation and inhibit Treg function.

The addition of an agonistic anti-OX40 antibody can then provide a synergistic stimulus to elicit an antitumor immune response that cures distant sites of established tumors. This combination of TLR9 ligand and anti-OX40 antibody can even treat spontaneous breast cancers, overcoming the effect of a powerful oncogene. This in situ vaccine maneuver is safe because it uses low doses of the immunoenhancing agents and practical because the therapy can be applied to many forms of cancer without prior knowledge of their unique tumor antigens.

RESULTS

In situ vaccination with a TLR9 ligand induces the expression of OX40 on intratumoral CD4 T cells

TLRs are known to signal the activation of a variety of cells of the innate and adaptive immune system. To exploit this for cancer therapy, we subcutaneously implanted a tumor into syngeneic mice, and after the tumor had become established, we injected a CpG oligodeoxynucleotide—ligand for TLR9—into the tumor nodule. We then analyzed the intratumoral T cells for their expression of inhibitory and activation markers. Before treatment, we observed that OX40 was expressed on CD4 cells in the tumor microenvironment (Fig. 1A, top) and that this was restricted mainly to the Treg, as has been previously reported (3–5) (Fig. 1B, top). After intratumoral injection of CpG, there was up-regulation of OX40 on CD4 T cells (Fig. 1A, middle), mostly among the effector CD4 cells that greatly outnumber the Treg (Fig. 1, A and B, bottom). This inductive effect was specific to the activating receptor OX40 and did not occur for inhibitory T cell checkpoint targets such as CTLA4 and PD1 (fig. S1A). Moreover, this OX40 up-regulation on CD4 cells also occurred in a patient with follicular lymphoma that had been treated with low-dose radiation and intratumoral injection of CpG (Fig. 1C) and in tumor-infiltrating cell populations from lymphoma patients’ samples that were exposed to CpG in vitro (Fig. 1, D and E, and fig. S2). In these human cases, the enhancement of OX40 expression was observed on both Teff and Treg (Fig. 1D). All of these changes occurred only in the tumor that was injected with CpG and not in the tumor at the untreated site (fig. S1B).

CpG induces OX40 as revealed by in vivo imaging

The enhancement of OX40 expression by intratumoral injection of CpG could be visualized in mice by whole-body small-animal positron emission tomography (PET) imaging after tail-vein administration.

1Division of Oncology, Department of Medicine, Stanford University, Stanford, CA 94305, USA. 2Department of Radiology, Molecular Imaging Program at Stanford (MIPS), Stanford University, Stanford, CA 94305, USA.
*Corresponding author. Email: levy@stanford.edu
Fig. 1. CpG induces the expression of OX40 on CD4 T cells. (A) A20 tumor–bearing mice were treated either with vehicle (top) or CpG (middle). Forty-eight hours later, tumors were excised and a single-cell suspension was stained and analyzed by flow cytometry. (B) OX40 expression within the CD3+CD4+ subset was separately analyzed for FoxP3-negative [effector T cell (Teff)] and FoxP3-positive [regulatory T cell (Treg)] subsets. Fold changes of OX40+ cells were calculated according to their frequencies in the vehicle versus CpG treatment (n = 2). (C) Fine needle aspirates from CpG-injected and noninjected tumors of a follicular lymphoma patient were obtained 22 hours after treatment. Fluorescence-activated cell sorting (FACS) plots of OX40 expression within the CD4+ subset after a 24-hour rest in media. Top: Nontreated lesion. Bottom: CpG-treated site (n = 2). (D) Single-cell suspensions from biopsy specimens of human lymphoma (five mantle cell lymphomas and five follicular lymphomas) were exposed in vitro to CpG for 48 hours and analyzed for OX40 expression as in (B). (E) CpG-stimulated human lymphoma–infiltrating CD4+ T cells, CD8+ T cells, and CD19+ B cells were gated and visualized in tSNE (t-Distributed Stochastic Neighbor Embedding) space using Cytobank software. The ViSNE map shows the location of each CD4+, CD19+, and CD8+ cell population (green, blue, and orange, respectively; bottom). Cells in the ViSNE maps were colored according to the intensity of OX40 expression. CpG up-regulation of OX40 expression on a subset of CD4+ T cells is highlighted by a red box. (F) BALB/c mice were implanted subcutaneously with A20 lymphoma cells (5 × 106) on both the right and left shoulders. When tumors reached between 0.7 and 1 cm in the largest diameter (typically on days 8 to 9 after inoculation), phosphate-buffered saline and CpG (50 µg) were injected into one tumor site (left tumor). Sixteen hours later, 64Cu-DOTA-OX40 was administered intravenously via the tail vein. Positron emission tomography imaging of mice was performed 40 hours after in situ treatment. Left: Vehicle-treated. Right: CpG-treated. These images are representative of six mice per group. (G) Fresh A20 tumors were excised from animals (typically 5 to 6 days after inoculation), and either whole tumors (left), T cells purified from the tumor (middle), or whole tumor depleted of CD11b+ and CD11c+ cells (right) were treated for 48 hours with media (top) or CpG (bottom) and were analyzed for their expression of OX40 by flow cytometry. (H) Left: A20 tumors were excised as in (F). Right: Single-cell suspensions from biopsy specimens of human follicular lymphoma. Tumors were treated for 48 hours with media and CpG with or without antibodies (1 µg/ml) to interleukin-2 (IL-2), IL-4, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-12, interferon-γ (IFN-γ), or tumor necrosis factor–α (TNF-α) and were analyzed for their expression of OX40 by flow cytometry. α–IL-2, *P = 0.0144; α–IFN-γ, **P = 0.0032; α–TNF-α, ***P = 0.008, unpaired t test, either depleting antibody versus CpG alone.
of an anti-OX40 antibody labeled with $^{64}$Cu (Fig. 1F). Remarkably, the systemically injected antibody revealed that OX40 was induced in the microenvironment of the injected tumor, as opposed to a second non-injected tumor site in the same animal. This result indicates that the effect of CpG at this low dose to up-regulate OX40 expression is predominately local.

**CpG induces cytokine secretion by myeloid cells which in turn induces OX40 expression on T cells**

Purified tumor-infiltrating T cells do not up-regulate OX40 when exposed to CpG in vitro (Fig. 1G). The T cells within whole tumor cell populations similarly fail to up-regulate OX40 after depletion of macrophages and dendritic cells (Fig. 1G). From these results, we conclude that myeloid-derived cells communicate the CpG signal to T cells. Therefore, we tested for the role of several cytokines in this cellular cross-talk. In human and mice tumors, antibody neutralization of interleukin-12 (IL-12), interferon-$\gamma$ (IFN-$\gamma$), and TNF-α each prevented the CpG-induced up-regulation of OX40 on T cells in these tumor cell populations (Fig. 1H). In contrast, neutralization of IL-2, IL-4, IL-10, and granulocyte-macrophage colony-stimulating factor (GM-CSF) had no effect (Fig. S3).

**In situ vaccination with a TLR ligand and anti-OX40 antibody induces T cell immune responses that cure established cancers**

On the basis of the results above, we hypothesized that an agonistic anti-OX40 antibody could augment CpG treatment and help to induce antitumor immune responses. To test this hypothesis, we implanted mice with A20 B cell lymphoma tumors at two different sites in the body, allowed the tumors to become established, and then injected a TLR agonist together with a checkpoint antibody into only one tumor site (Fig. 2A). The animals were then monitored for tumor growth at both the injected and the distant sites (Fig. 2B). The tumors of vehicle-treated mice grew progressively at both sites. CpG caused complete regression of tumors at the local injected site but had only a slight delay in growth of the distant nontreated tumor. The anti-OX40 antibody alone induced a slight delay in growth of both the treated and nontreated tumors. However, the combination of CpG and anti-OX40 resulted in complete regression of both injected and nontreated tumors. Consistent with the time needed to induce an adaptive T cell response, the kinetics of regression at the two sites was different, with the distant site following the local site by several days (Fig. S1C). Tumor regressions in response to the combined treatment were long-lasting and led to cure of most of the mice (Fig. 2B, bottom).

The systemic antitumor response required the presence of both CD4+ and CD8+ T cells because mice treated with the corresponding depleting antibodies were unable to control tumor growth (Fig. 2C). CD8+ T cells derived from mice treated with both CpG and anti-OX40 antibody responded to tumor cells in vitro as measured by IFN-$\gamma$ production (Fig. 2D). CD4+ T cells from mice treated by the combination also responded to tumors in vitro but with a lesser magnitude (Fig. S4). Immediately after CpG and anti-OX40 injection, the proportion of the CD4 effector/memory T cell subset increased at the treated site. Twenty-four hours later, this subset increased in the spleen, and 5 days later, the same occurred at the distant, nontreated site (Fig. S5).

Distant tumors occasionally did recur in mice treated with the effective combination (3 of 90 mice), and interestingly, these late recurring tumors were sensitive to retreatment by anti-OX40 and CpG (Fig. S6). An alternative TLR agonist, resiquimod (R848), a ligand for TLR7/8, in combination with anti-OX40 induced a similar systemic antitumor immune response (Fig. S7A). Anti-OX40 antibody was especially effective compared to other immune checkpoint antibodies, such as anti-PDL1 and anti–PDL1 (programmed death-ligand 1) (Fig. S7B), which delayed tumor growth in the nontreated site but were not curative.

In situ vaccination with CpG and anti-OX40 was effective not only against lymphoma but also against tumors of a variety of histologic types, such as breast carcinoma (4T1), colon cancer (CT26), and melanoma (B16-F10) (Fig. S8, A to C). In all these tumor models, the systemic therapeutic effects were induced by extremely low doses of both the CpG (typically 50 μg) and the anti-OX40 antibody (typically 8 μg) or even lower (Fig. S9). However, the TLR agonist worked best when it was injected directly into the tumor, consistent with its action to up-regulate the OX40 target in the T cells of the tumor microenvironment. Similar systemic effects were obtained when the OX40 antibody was given systemically, rather than into the tumor, but at higher doses (Fig. S10).

**In situ vaccination protects animals genetically prone to spontaneous breast cancers**

Female FVB/N-Tg(MMTV-PyVT)634Mul/J mice (also known as PyVT/PyMT) develop highly invasive mammary ductal carcinomas that give rise to a high frequency of lung metastases (6). By 6 to 7 weeks of age, all female carriers develop the first palpable mammary tumor (7), and eventually, tumors develop in all of their 10 mammary fat pads. This provided an opportunity for therapeutic intervention in a spontaneous tumor model where the site of tumor development is known and accessible for in situ vaccination.

Young mice were observed, and as their first tumor reached 50 to 75 mm$^3$, we injected it with CpG and anti-OX40 antibody (Fig. 3A). In some cases, a second tumor was present at the beginning of therapy, and in these mice with coincident tumors, treatment at a single tumor site with CpG and anti-OX40 led to significant retardation of growth of the contralateral tumor (Fig. 3B), establishing the combination as a therapy for established and disseminating tumors. The injected and the noninjected tumors regressed, and remarkably, the treated mice were protected against the occurrence of independently arising tumors in their other mammary glands (Fig. 3C). The treated mice had significantly lower eventual total tumor burdens (Fig. 3, C and D) and developed far fewer lung metastases (Fig. 3E). This in situ vaccination with CpG and anti-OX40 not only caused tumor regression and reduced tumor incidence but also had a major effect on the survival of these cancer-prone mice (Fig. 3F). After CpG and anti-OX40 treatment, these mice developed antitumor CD8 T cells in their spleens as indicated by their ability to produce IFN-$\gamma$ when exposed in vitro to autologous tumor cells from the noninjected tumor site (Fig. 3G). These results establish that the antitumor immune response was elicited against tumor antigens shared by all the independently arising tumors in these mice, rather than antigens unique to the injected tumor, and accounted for the impressive therapeutic effects seen.

**Therapeutic effect of in situ vaccination is antigen-specific and triggered at the site of local injection**

The results of cross-protection against independently arising tumors in the spontaneous breast cancer model raise the question of antigen specificity. We approached this question using two different tumors that are antigenically distinct. Mice cured by in situ vaccination of the A20 lymphoma were immune to rechallenge with the same tumor (A20) but not to a different tumor (CT26) (Fig. S11). Conversely, mice cured of the CT26 tumor were immune to rechallenge with CT26 but not with A20. Therefore, these two tumors are antigenically distinct.
Fig. 2. In situ vaccination of CpG in combination with anti-OX40 antibody cures established local and distant tumors. (A) Treatment schema. BALB/c mice were implanted subcutaneously with A20 lymphoma cells ($5 \times 10^6$) on both the right and left sides of the abdomen. When tumors reached between 0.5 and 0.7 cm in the largest diameter (typically on days 4 to 5 after inoculation), αOX40 (4 µg) and CpG (50 µg) were injected into one tumor site every other day for a total of three doses. Tumors sizes were serially measured with a caliper. (B) Tumor growth curves. Left column: Treated tumors (Tr). Right column: Nontreated tumors (NT). Top to bottom: Vehicle, CpG, αOX40, and CpG and αOX40 and survival plots of the treated mice ($n = 10$ mice per group). **** $P < 0.0001$, unpaired t test. Shown is one representative experiment out of nine. (C) Effect of CD4/CD8 depletion. Mice were implanted with bilateral tumors, and one tumor was injected with CpG and αOX40 antibody according to the schema in (A). CD4 (0.5 mg)– and CD8 (0.1 mg)–depleting antibodies were injected intraperitoneally on days 6, 8, 12, and 15 ($n = 10$ mice per group). (D) CD8 T cell immune response. Splenocytes from the indicated groups obtained on day 7 after treatment were cocultured with media, $1 \times 10^6$ irradiated 4T1 cells (unrelated control tumor), or A20 cells (homologous tumor) for 24 hours. Intracellular IFN-γ was measured in CD8+ T cells by flow cytometry as a percentage of CD44hi (memory CD8) T cells shown in dot plots and bar graph, summarizing data from three experiments ($n = 9$ mice per group). **** $P < 0.0001$, unpaired t test.
To further demonstrate the specificity of the antitumor response, we implanted tumors into mice at three different body sites: two with the A20 and one with CT26 (Fig. 4A). One A20 tumor site was then injected with CpG and anti-OX40 antibody. Both A20 tumors, the injected one and the noninjected one, regressed but the unrelated CT26 tumor continued to grow (Fig. 4A). In a reciprocal experiment, we injected mice with two CT26 tumors and one A20 tumor and treated one CT26 tumor. Once again, only the homologous distant tumors (in this case, CT26) regressed but not the unrelated A20 tumor (Fig. 4B). This result confirmed that the immune response induced by the therapy was tumor-specific. Furthermore, it demonstrated that in situ vaccination with these low doses of agents works by triggering an immune response in the microenvironment of the injected site rather than by diffusion of the injected agents to systemic sites.

Naturally arising tumors can show intratumoral antigenic heterogeneity. To test whether CpG and anti-OX40 treatment can trigger an
Fig. 4. Immunizing effects of intratumoral CpG and anti-OX40 are local and tumor-specific. (A) Three-tumor model. Each mouse was challenged with three tumors, two of them A20 lymphoma (blue) and one CT26 colon cancer (red). Mice were treated at the indicated times (black arrows). Tumor growth curves of the treated tumor (bottom left), the homologous nontreated A20 tumor (top right), and the heterologous CT26 tumor (bottom right). Photos of a representative mouse at day 11 after tumor challenge from the vehicle-treated group and from the group with A20 tumors treated with intratumoral CpG and oOX40 (\(n = 10\) mice per group) are shown. (B) Reciprocal three-tumor model with two CT26 tumors and one A20 tumor. Treatment was given to one CT26 tumor, and growth curves are shown for the treated CT26 tumor site (bottom right), the nontreated homologous CT26 tumor site (top right), and the heterologous A20 tumor (bottom right). Photos of a representative mouse from this experiment (\(n = 10\) mice per group) are shown. (C) Mixed three-tumor model. Each mouse was challenged with three tumors: one A20 (blue, top right abdomen), one CT26 (red, bottom right abdomen), and one mixture of A20 and CT26 tumor cells (blue and red gradient, left abdomen). Mice were treated only in the mixed tumor at the indicated times (black arrows). Tumor growth curves of the treated tumor (bottom left), the nontreated A20 tumor (top right), and the nontreated CT26 tumor (bottom right). Photos of a representative mouse at day 11 after tumor challenge from the vehicle-treated group (top) and at day 17 from the intratumoral CpG and oOX40 (\(n = 8\) mice per group) are shown.

Immune response against multiple different tumor antigens at the same time, we injected mice with a mixture of A20 and CT26 tumor cells at one site, treated that site with local CpG and anti-OX40 antibody, and monitored two additional sites of tumor containing each of the single tumor cells (A20 and CT26, respectively). In situ vaccination of the mixed tumor site simultaneously induced immune responses protective of each of the respective other two pure tumor sites (Fig. 4C). These results demonstrate the power of in situ vaccination to simultaneously immunize against a panoply of different tumor antigens.

**Fc competency is required for efficacy of the anti-OX40 antibody**

OX40 is expressed on both intratumoral FoxP3\(^+\) T\(_{reg}\) and activated T\(_{eff}\) (Fig. 1B). The immunoenhancing activity of the anti-OX40 antibody could therefore be mediated by inhibition/depletion of T\(_{reg}\) by stimulation of T\(_{eff}\) or by a combination of both. We tested the anti-OX40 T\(_{reg}\) depletion hypothesis by replacing it with an antibody against folate receptor 4 (FR4), a T\(_{reg}\)-depleting agent (fig. S12A) (8). T\(_{reg}\) were partially depleted (43% reduction) by the anti-FR4 in combination with CpG, but no distant therapeutic effect occurred (Fig. 5A). We further investigated this question using mice genetically engineered to express diphtheria toxin under the FoxP3 promoter (9, 10). Injection of diphtheria toxin led to complete T\(_{reg}\) depletion in these mice (fig. S12B). However, when combined with intratumoral CpG, no distant therapeutic effect was observed (Fig. 5B). As others have shown, stimulation of T\(_{reg}\) through OX40 can impair their function (4, 11, 12), which we confirm here (fig. S13, A and B). Therefore, we conclude that T\(_{reg}\) stimulatory impairment but not depletion is involved in the mechanism of therapeutic synergy with CpG. To dissect the mechanism of these potent therapeutic effects, we compared two different forms of the anti-OX40 antibody that differ in their ability to bind to CD16: the activating Fc receptor on natural killer (NK) cells and macrophages. When used in combination with CpG, the native, Fc-competent version of anti-OX40 antibody induced systemic antitumor immunity, whereas the Fc-mutant version did not (Fig. 5A). We repeated the in situ vaccination experiment in mice deficient in the Fc common \(\gamma\) chain, a component of the activating Fc \(\gamma\) receptors I, III, and IV (13). Once again, in the absence of Fc receptor interaction, this time at the level of the host, the effect of in situ vaccination with CpG and anti-OX40 antibody was lost (fig. S14). These results could implicate ADCC (antibody-dependent cellular cytotoxicity) function of the antibody or alternatively an Fc-dependent agonistic action of the anti-OX40 antibody (14, 15).
Fig. 5. A competent Fc is required for the antitumor immune response. (A and B) Effect of Treg depletion. (A) Tumors were implanted according to the schema in Fig. 2A. Mice were treated with either CpG and anti-folate receptor 4 (FR4) antibody (15 μg) or CpG and αOX40 as described in Fig. 2A, and the NT was measured over time. **** P < 0.0001, unpaired t test (n = 10 mice per group). (B) DEREGR mice were implanted with B16-F10 melanoma cells (0.05 × 10⁶) on both the right and left sides of the abdomen. Diphtheria toxin (DT; 1 μg) was injected intraperitoneally on days 1, 2, 7, and 14. CpG or combination of CpG and anti-OX40 was given on days 7, 9, and 11. The NT was measured over time. * P = 0.0495, unpaired t test (n = 4 mice per group). (C) A20 cells were inoculated and treated as described in Fig. 2A, tumor volumes were measured after treatment of CpG with either αOX40 rat immunoglobulin G1 (IgG1) (red) or αOX40 rat IgG1 Fc mutant (black). **** P < 0.0001, unpaired t test (n = 10 mice per group). WT, wild type. (D) Tumors from control and treated mice were excised at the indicated times after a single treatment, and the cell populations from the different groups were differentially labeled (barcoded) with two different levels of violet tracking dye (VTD) and mixed together, stained, and analyzed as a single sample (n = 3 mice per group (C to F)). (E to H) Dot plots for single time point and bar graphs for replicates of multiple time points. (E) Number of F4/80+CD11b+ myeloid cells. ** P = 0.009 (8 h), Fc WT versus vehicle. (F) CD137 expression on natural killer (NK) cells. * P = 0.0035 (2 h), * P = 0.0343 (8 h), unpaired t test, Fc WT versus Fc mutant. (G) CD8 expression on CD8+ T cells. * P = 0.025 (8 h), ** P = 0.0064 (24 h), unpaired t test, Fc WT versus Fc mutant. (H) Treg cell proliferation. *** P = 0.0003 (24 h), unpaired t test, Fc WT versus Fc mutant.
Therefore, we examined immune cells in the tumor microenvironment during the early phases of treatment with intratumoral CpG and compared the changes induced with the Fc-competent to those induced by the Fc-mutant version of the anti-OX40 antibody. Early after in situ vaccination, within 24 hours, the tumor-infiltrating cell populations from animals treated with Fc-competent or Fc-mutant antibodies were barcoded (16), pooled, and then costained by a panel of antibodies to identify subsets of immune cells and their activation states (Fig. 5B). The cell populations derived from the different treatment groups were then separately identified by their barcodes. In response to the anti-OX40 antibody with the native Fc, there was an increase in myeloid cell infiltration (Fig. 5C), a cell population important in the cross-talk between CpG and the T cells (see above; Fig. 1, F and G). NK cells showed an Fc-dependent up-regulation of their CD137 activation marker (Fig. 5D). In addition, the Fc-competent but not the Fc-mutant antibody induced activation of a population of CD8 T cells, as indicated by increased CD69 expression (Fig. 5E). Treg were inhibited in their proliferation by comparison to those exposed to the antibody with the mutated Fc region (Fig. 5F). Neither Treg nor Teff were killed by the Fc-competent antibody (fig. S15). These early cellular changes occurred only in the tumor microenvironment of the treated site and were not evident at other sites throughout the mouse (fig. S1B). These results imply that anti-OX40 antibodies, in conjunction with TLR ligands, can induce therapeutic systemic antitumor immune responses by a combination of NK cell activation, Treg inhibition, and Teff activation, all at the treated tumor site.

**DISCUSSION**

We have developed a practical strategy for immunotherapy of cancer. It takes advantage of the preexisting T cell immune repertoire within the tumor microenvironment. The combination of a TLR agonist and an activating antibody against OX40 amplifies these antitumor T cells and induces their action throughout the body against tumor at nontreated sites. This in situ vaccination does not require knowledge of the tumor antigens. Potential drawbacks include reliance on adequate immune infiltrates and the availability of a suitable injectable site of tumor.

After screening a series of immune activators and checkpoint antibodies, we identified the combination of CpG oligodeoxynucleotide (TLR9 ligand) and anti-OX40 antibody to be the most potent form of in situ vaccination in multiple mouse models. TLR7/8 agonists could substitute for CpG, but checkpoint antibodies against PD1, PDL1, or CTLA4 could not substitute for anti-OX40.

The synergistic therapeutic effect between locally injected CpG and anti-OX40 antibodies is explained by the fact that CpG induced the expression of the OX40 target on CD4+ T cells in the tumor microenvironment. CpG also induced OX40 in CD4+ T cells in the tumor microenvironment of human lymphoma tumors, and therefore, our results are likely to translate to human cancer.

It has been reported that local intratumoral administration of CpG together with systemic antibody against IL-10R leads to rejection of the injected tumor and distant metastases (17, 18). This combination was shown to deflect M2 to M1 macrophages in the tumor microenvironment (19). Therefore, we examined the requirement for induction of OX40 expression on CD4 T cells by CpG in our system. We found that it was dependent on cytokines secreted by myeloid cells, including IL-12, IFN-γ, or TNF-α but not IL-2, IL-4, IL-10, and GM-CSF.

The therapeutic effect at the distant sites was specific for antigens expressed by the tumor at the injected site that were shared with the tumor cells at the distant sites. This result not only established the tumor specificity of the immunization but also proved that it was the local effect of the injected agents in the tumor microenvironment rather than their systemic delivery that triggered the systemic antitumor immune response.

Autoimmune toxicities are a common complication of systemically administered immune checkpoint antibodies (20–24). In contrast, direct injection of the antibodies into the tumor at very low doses can avoid these side effects (25, 26). In our experiments, in situ injection of microgram quantities of immune stimulants and checkpoint antibodies proved to be sufficient to induce the required local immune modulation, resulting in a systemic antitumor immune response.

A major challenge in tumor immunotherapy lies in breaking tumor immune tolerance. In a previous report, we showed that depletion of tumor-specific Treg by the addition of anti-CTLA4 antibody was associated with enhanced antitumor efficacy (27). However, we find here that activating antibody against OX40 is sufficient. It is known that OX40 is expressed on both Treg and Teff in the tumor microenvironment, and as we now realize, OX40 can be further induced on CD4+ T cells in response to CpG. Modulating both Teff and Treg is essential to obtain therapeutic effect (28–30). Antibodies to OX40 costimulate Teff (31–37), and they also inhibit the function of Treg (12, 27, 38–40).

Having demonstrated the potent therapeutic efficacy of in situ immunotherapy in several different transplanted tumor types, we assessed this form of therapy in a spontaneous arising tumor. The MMTV-PyMT mouse model recapitulates several of the characteristics of virulent human breast cancer, among them showing histology similarity, having loss of estrogen and progesterone receptors, and overexpressing ErbB2/Neu and cyclin D1 (6, 41, 42). Although the tumors within a mouse arise independently in different mammary glands, they all share the expression of the PyMT antigen (43). Injection of CpG and anti-OX40 antibody into the first tumor to occur in each mouse resulted in reduced tumor load in the other mammary fat pads and prevented lung metastases. These results demonstrate the potency of the in situ vaccine maneuver in a situation of spontaneous cancer-driven by a strong oncogene, suggesting the possibility of a direct application to human cancer. By analogy to the genetically prone mice, we can imagine administering an in situ vaccine at the site of the primary tumor before surgery in patients at high risk for the occurrence of metastatic disease and/or in patients genetically prone to develop secondary primary cancers, such as those with inherited mutation in the BRCA genes.

The CpG used here, SD-101, is currently being tested in patients as a single agent and in combination with other therapeutic modalities (NCT02927964, NCT02266147, NCT01745354, NCT02254772, and NCT02521870). Anti-OX40 antibody is also currently being studied in phase 1 clinical trials (NCT02559024, NCT01644968, NCT02221960, NCT02318394, NCT02274155, NCT01862900, NCT01303705, and NCT02053333). The results from our current preclinical studies provide strong rationale for combining CpG with agonistic anti-OX40 antibodies in a therapeutic format of in situ vaccination in patients with lymphoma and solid tumors. As we have shown, CpG and anti-OX40 antibodies work locally at very low doses that should provide the advantage of avoiding toxicities that occur with their systemic administration.
### MATERIALS AND METHODS

#### Study design

Our objective was to develop a new immunotherapy for cancer by using the tumor itself as a source of antigen, of immune reactive cells and as a site for injecting immune activating agents—in situ vaccination. Our general strategy was to implant the same syngeneic tumor at two separate sites in the body of mice. One tumor was then injected with the test agents, and tumor size was measured in both the treated and nontreated sites. Using this assay for abscopal therapeutic effects, we identified the combination of unmethylated CG–enriched oligodeoxynucleotide (CpG)—a TLR9 ligand—and an agonistic antibody against OX40 as the most promising immunostimulatory regimen.

Because transplanted syngeneic tumor models lack certain aspects of naturally occurring tumors, we also studied the effects of our combination in a spontaneous model of breast cancer. This model is driven by the polyoma middle T oncogene under the control of the MMTV promoter, and the female mice develop independently arising breast cancers in all of their mammary glands between 5 and 14 weeks of age.

We observed the mice, and when their first breast cancer tumor arose, we injected it with our combination of CpG and anti-OX40 antibody. Each mouse was then monitored for the regression of simultaneously present second tumors, for the occurrence of newly arising tumors, for metastatic disease in their lungs, and for their survival. Data were analyzed by Kaplan-Meier curves with events scored as the time to reach 2 cm in the largest diameter at which time the mouse was sacrificed. Data were analyzed using the log-rank test.

We studied the mechanism of the therapy by examining the requirement for T cells and their subsets, including Treg, by the requirement for Fc function of the anti-OX40 antibody. These requirements were tested by depleting specific T cells and by substituting an Fc mutant for the native anti-OX40 antibody.

In all therapy experiments, to ensure similar tumor sizes in all treatment groups, mice were randomized only after tumors were established. To ensure statistical power, experimental groups were typically composed of 10 animals each. For each experiment, mice numbers, statistical tests, and numbers of experimental replicates are described in the figure legends. Data include all outliers. Investigators were not blinded during evaluation of the in vivo experiments. Raw data for all therapy experiments are provided in table S1.

#### Reagents

Cpg SD-101 was provided by Dynavax Technologies. Anti-mouse CD8a (clone 2.43) and anti-mouse CD4 (clone GK1.5) antibodies were purchased from BioXCell. Anti-OX40 (CD134) monoclonal antibody (mAb) [rat immunoglobulin G1 (IgG1), clone OX86; European Collection of Cell Cultures], isotype control rat hybridoma, SFR8-B6 [American Type Culture Collection (ATCC) HB–152] were produced as ascites in severe combined immunodeficient mice by Bionexus. FC-silent Anti-OX40 (CD134) mAb was purchased from Absolute Antibody.

The following mAbs were used for flow cytometry: CD4-PerCP (peridinin chlorophyll protein) Cy5.5, CD3-PerCP Cy5.5, or BV786; CD4-BV650, CD8a–FITC (fluorescin isothiocyanate), or APC (allophycocyanin) H7; CD44-APC, IFN-γ–PE (phycoerythrin), B220-PerCP Cy5.5, CD49b–PE Cy7, CD69-BV650, CD137–PE, and ICOS (CD278)–PE Cy7; FoxP3–PE; and Ki-67–BV711. These antibodies and their isotype controls were purchased from BD Biosciences, BioLegend, or eBioscience.

#### Cell lines and mice

A20 B cell lymphoma, B16-F10 melanoma, and CT26 colon carcinoma lines were obtained from ATCC, and 4T1-Luc breast carcinoma cell line was a gift from the S. Strober laboratory and the C. Contag laboratory (both at Stanford University). Tumor cells were cultured in complete medium (RPMI 1640; Dulbecco’s modified Eagle’s medium for B16-F10; Cellgro) containing 10% fetal bovine serum (FBS; HyClone), penicillin (100 U/ml), streptomycin (100 μg/ml), and 50 μM 2-mercaptoethanol (Gibco). Cell lines were routinely tested for mycoplasma contamination.

Six- to 8-week-old female BALB/c and C57BL/6 were purchased from Charles River (www.crivel.com). FVB/N-Tg(MMTV-PyVT)634Mul/J male FVB/NJ females [C57BL/6-Tg(Foxp3-DTR/EGFP)23.2Spar; also known as DEREg mice] were purchased from The Jackson Laboratory (http://jaxmice.jax.org/). Mice were housed in the Laboratory Animal Facility of the Stanford University Medical Center (Stanford, CA). All experiments were approved by the Stanford administrative panel on laboratory animal care and conducted in accordance with Stanford University animal facility guidelines.

#### Tumor inoculation and animal studies

A20, CT26, 4T1, and B16-F10 tumor cells (5 × 10^6, 0.5 × 10^6, 0.01 × 10^6, and 0.05 × 10^6, respectively) were injected subcutaneously at sites on both the right and left sides of the abdomen. When tumor size reached 0.5 to 0.7 cm in the largest diameter, mice were randomized to the experimental groups. CpG and anti-OX40 were injected into the tumor only on the right side of the animals in a volume of 50 μl. Tumor size was monitored on both sides of the animals with a digital caliper (Mitutoyo) every 2 to 3 days and expressed as volume (length × width × height). Mice were sacrificed when tumor size reached 1.5 cm in the largest diameter as per guidelines. All mice that developed tumors on both sides of the abdomen were included in the experiments. The investigator was not blinded to the group allocation during the experiment and/or when assessing the outcome.

4T1 tumor–challenged mice were analyzed for lung metastasis by injecting India ink through the trachea after euthanasia. Lungs were then excised, washed once in water, and fixed in Fekete’s solution (100 ml of 70% alcohol, 10 ml of formalin, and 5 ml of glacial acetic acid) at room temperature. Surface metastases subsequently appeared as white nodules at the surface of black lungs and were counted under a microscope.

DEREG mice were implanted with B16-F10 tumor cells as described above. Diphtheria toxin (1 μg; Sigma-Aldrich) was injected intraperitoneally on days 1, 2, 7, and 14 after tumor implantations. CpG or combination of CpG and anti-OX40 was given on days 7, 9, and 11 after tumor implantations.

#### Flow cytometry

Cells were surface-stained in phosphate-buffered saline (PBS), 1% bovine serum albumin, and 0.01% sodium azide, fixed in 2% paraformaldehyde, and analyzed by flow cytometry on a FACSCalibur or LSR II (BD Biosciences). Data were stored and analyzed using Cytobank (www.cytobank.org).

#### Multiplex flow cytometry—fluorescent cell barcoding

Excised tumors from mice treated with an Fc-competent OX40 antibody, an FC-silent OX40 antibody (Absolute Antibody), or saline were mechanically processed into single-cell suspensions and barcoded using three different concentrations of CellTrace Violet Proliferation
were cocultured in 96-well plate in the presence of anti-CD3 and 1 × 10^6 irradiated A20 or 4T1 cells for 24 hours at 37°C and potassium buffer (Quality Biological). Splenocytes were then

### IFN-γ production assay

Single-cell suspensions were made from spleens of treated mice (on day 7 after treatment), and red cells were lysed with ammonium chloride and potassium buffer (Quality Biological). Splenocytes were then cocultured with 1 × 10^6 irradiated A20 or 4T1 cells for 24 hours at 37°C and 5% CO₂ in the presence of 0.5 μg of anti-mouse CD28mAb (BD Pharmingen). Monensin (GolgiStop; BD Biosciences) was added for the last 5 to 6 hours. Intracellular IFN-γ expression was assessed using BD Cytofix/Cytoperm Plus Kit as per the manufacturer’s instructions.

### Depletion of CD4 and CD8 T cells

Anti-CD4 (clone GK1.5, rat IgG2b) and anti-CD8 (clone 2.43, rat IgG2b) mAbs (BioXCell) were injected 2 days and 1 day before therapy, on the day therapy was begun, and at 5, 8, and 19 days after the beginning of therapy at a dose of 0.5 or 0.1 mg per injection for CD4 and CD8, respectively. The depletion conditions were validated by flow cytometry of blood showing specific depletion of more than 95% of each respective cell subset.

### In vitro assessment of OX40 expression

Fresh tumor cells were excised from mice, processed into single-cell suspensions, and incubated for 48 hours with CpG (1 μg/ml). Cells were then stained for the surface antigens CD3, CD4, CD8, and OX40. They were then fixed and permeabilized using reagents from eBioscience, followed by FoxP3 staining. T cell isolation and depletion of T cells and CD11b- and CD11c-expressing cells from tumors used kits from Miltenyi Biotec.

### Tetramer staining

PE-conjugated H-2Ld tetramer to peptide SPSYYVYHQF (MuLV env gp70, 423 to 431) was purchased from ProImmune, and PE-conjugated H-2Ld tetramer to peptide IASNENMETMESSTLE (influenza nucleoprotein 365 to 380) was a gift from the M. Davis laboratory (Stanford University). Antibodies were used at 5 μg/ml, and tetramer staining was performed in fluorescence-activated cell sorting buffer for 10 min at room temperature and followed by surface staining on ice for 20 min.

### Activation and suppression assay

#### T cell activation assay

C57BL/6 splenocytes were isolated, violet tracking dye (VTD)–labeled, and incubated in the presence of anti-CD3 antibody (0.05 μg/ml) for 72 hours with or without anti-OX40 antibodies. T cell activation and proliferation were determined by VTD dilution and the expression of the activation marker CD69.

#### Treg suppression assay

To determine the impact of OX40 antibodies on Treg activity, VTD-labeled splenic cells were cocultured with OX40–KO (knockout) Tregs, [OX40 wild-type (WT) splenocytes/OX40 KO Tregs = 1:1]. Cells were cocultured in 96-well plate in the presence of anti-CD3 and anti-CD28 beads (Thermo Fisher Scientific) for 96 hours with or without anti-OX40 antibody (1 μg/ml). Proliferation of the WT-labeled Tregs was measured by flow cytometry and calculated by VTD dilution. Tregs were isolated using kits from Miltenyi Biotec.

### PET imaging

PET imaging of mice was performed using the microPET/CT hybrid scanner (Inveon, Siemens). PET images were reconstructed using 2 iterations of three-dimensional ordered subset expectation maximization (3DOSEM) algorithm (12 subsets) and 18 iterations of the accelerated version of 3D-MAP (that is, FASTMAP)—matrix size of 128 × 128 × 159. Computed tomography (CT) images were acquired just before each PET scan to enable attenuation correction of the PET data set and provide an anatomic reference for the PET image. Mice were anesthetized using isoflurane gas (2.0 to 3.0% for induction and 2.0 to 2.5% for maintenance). 64Cu-DOTA-OX40 (80 to 110 μCi; radiochemical purity of 99% as determined by thin-layer chromatography; and specific activity is 185 MBq/mg) was administered intravenously via the tail vein 16 hours after CpG and vehicle intratumoral injections. Static PET scans (10 min) were acquired 16 hours after intravenous administration of 64Cu-DOTA-OX40 (40 hours after intratumoral injections). Once reconstructed using a 3DOSEM algorithm, PET images were coregistered with CT images to generate figures using the IRW (Inveon Research Workplace) image analysis software (version 4.0; Siemens).

### Up-regulation of OX40 on CD4 T cells infiltrating human B cell tumors

Tumor samples from patients with Follicular and Mantle Cell B cell lymphoma who were part of ongoing clinical trials [Stanford International Review Board (IRB) protocols IRB-31224, IRB-36750, and IRB-5089] were available for in vitro analysis. Single-cell suspensions were incubated for either 24 or 48 hours in RPMI medium containing 5% FBS (HyClone), penicillin (100 U/ml), and streptomycin (100 μg/ml) and then stained for T cell surface antigens including CD3, CD4, CD8, and OX40. They were also fixed and permeabilized using reagents from eBioscience and then stained for FoxP3. For in vitro stimulation studies, CpG at a concentration of 1 μg/ml was added to the medium.

For response of tumor-infiltrating cells to CpG in vivo, a sample was obtained from a site of tumor that had been injected with CpG (3 mg) 24 hours before and compared to a site of tumor that had not been injected. Both of these sites shared as part of the clinical protocol an exposure to low-dose radiation (2 grays on each for two successive days). Single-cell suspensions were rested in the medium with no further exposure to CpG for 24 hours before analysis of OX40 expression by flow cytometry.

### Statistical analysis

Prism software (GraphPad) was used to analyze tumor growth and to determine statistical significance of differences between groups by applying an unpaired Student’s t test. P values <0.05 were considered significant. The Kaplan-Meier method was used for survival analysis. P values were calculated using the log-rank test (Mantel-Cox).

### Supplementary materials

- Fig. S1. In situ vaccination with a TLR9 ligand induces the local expression of OX40 but not that of PD1 or CTLA4.
- Fig. S2. CpG induces the expression of OX40 on CD4 T cells.
REFERENCES AND NOTES


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