Supporting Information

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SI Results

Infection by GAS and Lm Induces Distinct Species-Specific T-Cell Responses. Induction of TGF-β1 by GAS but not by Lm infection accounts for the different cellular responses. To confirm that Lm induces a Th1 cellular response distinct from the Th17 phenotype induced by GAS, mice were infected i.n. twice with GAS (2×10^7 CFUs) or with an equal dose of Lm at a 1-week interval, respectively (*Materials and Methods*). NALT cells were stained for CD4 and intracellular IL-17 and IFN-γ. As predicted, significantly higher frequencies of $CD4^+IL\text{-}17^+$ cells were detected in NALT from mice infected with GAS than from those infected with Lm after two i.n. infections (Fig. S3*A*). Conversely $CD4^+IFN\text{-}\gamma^+$ cells dominated NALT from mice infected with Lm (Fig. S3*B*). The smaller fraction of $CD4^+IFN\text{-}\gamma^+$ cells (~2.5%) observed in this experiment relative to that shown in Fig. 5*B* (~28%) is likely attributable to the small dose of streptococci used here. It was necessary to inoculate fewer bacteria because mice became severely ill when inoculated with Lm at the dose used in Fig. 5. When mice were first infected with GAS and then reinfected with Lm (Fig. S3*C*, column 4) or first infected with Lm and then with GAS (Fig. S3*C*, column 7), frequencies of $CD4^+IL\text{-}17^+$ cells were greater than in PBS controls (Fig. S3*C*, column 1) but still low relative to mice singly infected with either GAS or Lm (Fig. S3*C*, columns 2 and 5). This indicates that specific GAS antigen priming and rechallenge with GAS are required for maximal Th17 cell expansion.

The discovery that GAS predominantly induces a Th17 response, whereas Lm elicits primarily a Th1 response, suggested that the Th17 response is bacterial specific rather than dependent on NALT or the route of inoculation. This was confirmed by i.p. infection of mice with GAS or Lm followed by analyses of IL-17 secretion by splenocytes on ex vivo exposure to either heat-killed GAS or Lm. As predicted by the T-cell phenotypes above, splenocytes from mice infected with GAS secreted significantly more IL-17 than those from mice infected with Lm (Fig. S4*A*, columns 3 and 6; $P \leq 0.0001$). Moreover, ex vivo restimulation of splenocytes with Lm did not significantly induce IL-17 secretion, regardless of whether they had been primed by GAS or Lm infection (Fig. S4*A*, columns 4 and 6). Failure of Lm to induce TGF-β1 to levels comparable to GAS may account for the observed divergent T-cell responses to these different human pathogens. As predicted, TGF-β1 was elevated in response to GAS but not in response to Lm (Fig. S4*B*).

SI Materials and Methods

Human Tonsil Tissues. Palatine tonsil tissue specimens were obtained and manipulated as described by Kumar et al. (1). Briefly, tonsil tissues were obtained within 1–3 hr of completion of surgery. Single-cell suspensions from tonsils were prepared by forcing the tissue through a metal tissue sieve. Viable cells were further purified by Ficoll gradient. The resulting cells were plated in 48-well plates (10^6 cells per well) in complete RPMI [10% FBS (vol/vol), penicillin (100 $U \cdot mL^{-1}$) and streptomycin (0.1 $mg \cdot mL^{-1}$)] and incubated with heat-killed bacteria at a multiplicity of infection (MOI) from 5 to 20 in 5% CO_2 at 37 °C.

Single-Cell Suspension and ex Vivo Treatment with Bacteria for Cytokine Analyses. Single-cell suspensions of NALT, CLN, and spleen were prepared by scraping tissues over a nylon screen in 1 mL of complete RPMI. Live cells were plated in 24-well plates at 1.0–2.5 $\times 10^6$ cells per well and incubated with heat-killed bacteria at MOI (5–20) in 5% CO_2 at 37 °C. In TGF-β1 signaling inhibition assays, cells were pretreated with SB 431524 (10 μM) or an equal volume of DMSO (carrier) for 1 hr and then treated with heat-killed bacteria.

Total RNA Isolation from NALT and Real-Time RT-PCR Analysis. Mice were treated i.n with PBS or GAS strain HK-90-226. At 24 hr after infection, NALT was removed from mice and total RNA was purified from NALT tissue using the Qiagen RNeasy kit (Qiagen, Inc.) and on-column DNase digestion. RNA quality was verified using the Agilent Bioanalyzer and an RNA Nano chip (Agilent Techonologies) and quantified using a Nanodrop spectrophotometer (Nanodrop Technologies). Quality of RNA was checked by A260/A280, and all RNA samples had ratios >1.8. cDNAs were generated using the First Strand Synthesis kit from ABgene. PCR amplification was carried out with a SuperArray kit (proprietary sequence; RT2 Profiler PCR Array) according to the manufacturer's protocol. Expression of the target genes in NALT was normalized to expression under control conditions by means of the comparative threshold cycle (C_t) method and calculated and normalized to GADPH expression levels using the $2^{-\Delta\Delta Ct}$ method (2).

Enrichment and Adoptive Transfer of CD4 T Cells. Three groups of five mice [i.e., B6-immunized, B6 $IL\text{-}17^{-/-}$ (3) immunized by infection, and control B6-inoculated with PBS] were killed, and all lymph nodes were harvested and pooled separately for each group. Single-cell suspensions were made in 2 mL of ice-cold complete Eagle's high amino acid (cEHAA) medium, filtered through a nylon mesh, and pooled by group. For purifying CD4 T cells, a negative magnetic bead enrichment strategy was used (4). Single-cell suspensions were washed and incubated with a mixture of biotinylated anti-mouse CD11c (eBio), anti-mouse B220 (eBio), anti-mouse-CD8 (eBio), and anti-mouse-CD49b (clone DX5; eBio) for 15 min on ice (in a total volume of 300 μL of cEHAA medium and 3 μL of each antibody). Cells were washed twice with PBS and resuspended in 315 μL of labeling buffer (PBS + 5% FBS, vol/vol) before 35 μL of avidin-conjugated microbeads (Miltenyi Biotech) was added. After 15 min of incubation on ice, cells were washed twice with PBS and filtered through a nylon mesh to remove cell aggregates. Filtered cells were resuspended in 5 mL of labeling buffer and applied to a preprepared LS magnetized column (Miltenyi Biotech). Flow-through cells were collected, washed twice with PBS, and resuspended in 1 mL of PBS. Cell counts were performed on each enriched sample, and suspensions were diluted to a final cell concentration of 5×10^6 cells per 200 μL in PBS. A small volume from each sample was stained and analyzed by flow cytometry for the purity and phenotype of the cells. Female WT B6 mice aged 6 to 8 weeks were used as recipients (5 mice per group), and 5×10^6 purified cells were transferred to each mouse in a volume of 200 μL by tail vein injection. Twenty-four hours later, recipient mice (three groups) were challenged i.n. with 3×10^8 CFUs of GAS per mouse. All mice were killed 24 hr after the challenge; NALTs were harvested, and cell lysates were diluted and plated on sheep blood agar plates for CFU counts.

1. Kumar RB, Maher DM, Herzberg MC, Southern PJ (2006) Expression of HIV receptors, alternate receptors and co-receptors on tonsillar epithelium: Implications for HIV binding and primary oral infection. *Virol J* 3:25–37.
2. Hyland KA, et al. (2009) The early interferon response of nasal-associated lymphoid tissue to Streptococcus pyogenes infection. *FEMS Immunol Med Microbiol* 55: 422–431.
3. Nakae S, et al. (2002) Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses. *Immunity* 17: 375–387.
4. Winstead CJ, Fraser JM, Khoruts A (2008) Regulatory CD4+CD25+Foxp3+ T cells selectively inhibit the spontaneous form of lymphopenia-induced proliferation of naive T cells. *J Immunol* 180:7305–7317.

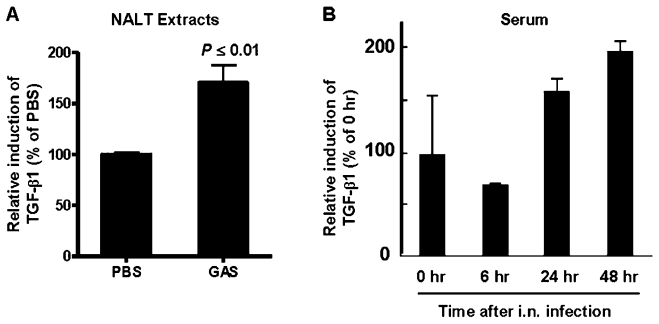

Fig. S1. Infection (i.n.) of mice induces TGF-β1 in NALT. Mice were infected i.n. once with viable GAS strain HK-90-226 or PBS. (*A*) Extracts of NALT cells were normalized for total protein concentration and then assayed for active TGF-β1 by ELISA. Data are mean ± SEM of six mice per group. (*B*) Two mice for each time point were inoculated with streptococci. Mice were euthanized at the indicated times, and blood was taken. Sera were assayed for active TGF-β1 using the luciferase reporter. Representative results from two independent experiments are shown.

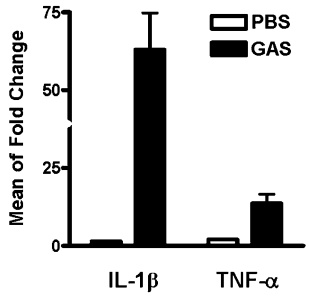

Fig. S2. Infection (i.n.) of mice induces IL-1β and TNF-α mRNA in NALT. NALTs from mice infected i.n. were removed after 24 hr, and total RNA was purified. PCR amplification was carried out with a SuperArray kit [Hyland KA, et al. (2009) The early interferon response of nasal-associated lymphoid tissue to Streptococcus pyogenes infection. FEMS Immunol Med Microbiol 55:422–431]. The values on the y axis are given as relative fold changes of mRNA expression in GAS-infected compared with PBS-inoculated NALT. Data are mean ± SEM of two mice per group.

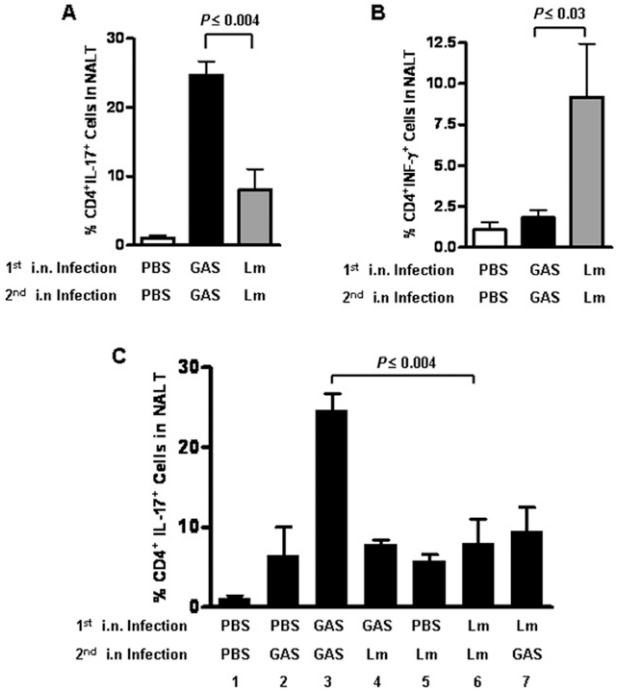

Fig. S3. GAS and Lm skew T-cell differentiation to Th17 and Th1 phenotypes, respectively. Mice were inoculated i.n. with PBS, viable 90-226 (GAS), or the same dose of Lm twice as indicated at a 1-week interval. Three days after the second inoculation, NALT cells were stained with specific antibodies and analyzed by flow cytometry. Percentages of $CD4^+IL\text{-}17^+$ cells (*A* and *C*) and percentages of $CD4^+IFN\text{-}\gamma^+$ cells (*B*) among total $CD4^+$ cells are shown. Data are mean ± SEM of four mice per group from two independent experiments.

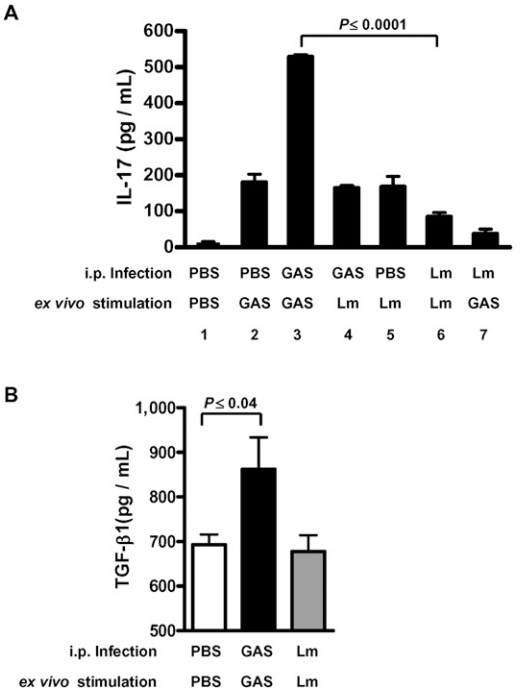

Fig. S4. Unlike GAS, Lm failed to induce TGF-β1 and induced lower levels of IL-17. Mice (n = 2) were inoculated i.p. once with live GAS or the same dose of Lm. Control mice were inoculated i.n. with PBS. At 48 hr after inoculation, spleens were taken and pooled splenocytes were restimulated with heat-killed GAS or Lm as indicated. Supernatants were collected after 72 hr of incubation and assayed for IL-17 (*A*) and TGF-β1 (*B*) by ELISA. Data are mean ± SEM of three independently treated splenocyte preparations and represent results from two separate experiments.

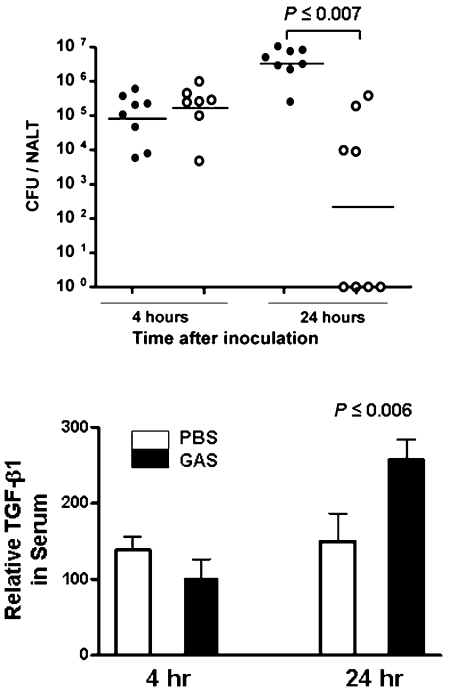

Fig. S5. Infection (i.n.) induced protective immunity. BALB/c mice were immunized by four i.n. inoculations, each separated by 1 week (○). Control mice were inoculated i.n four times with PBS (•). Groups of mice were challenged with 2×10^8 CFUs 2 days after the last immunization and then euthanized 4 or 24 hr later. (*Upper*) Viable streptococcal counts in single-cell suspensions of NALT were determined. The horizontal bars are Geomean of CFUs. (*Lower*) TGF-β1 in the blood at 4 and 24 hr was measured by a TGF-β1-luciferase reporter assay. The *P* value was determined by a Mann–Whitney *U* nonparametric *t* test.