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Mechanism of CD1d-restricted natural killer T cell activation during microbial infection

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CD1d-restricted natural killer T (NKT) cells are important for host defense against a variety of microbial pathogens. How and when these T cells become activated physiologically during infection remains unknown. Our data support a model in which NKT cells use a unique activation mechanism not requiring their recognition of microbial antigens. Instead, weak responses to CD1d-presented self antigens were amplified by interleukin 12 made by dendritic cells in response to microbial products, resulting in potent interferon-γ secretion. NKT cells were among the first lymphocytes to respond during *Salmonella typhimurium* infection, and their activation *in vivo* also depended on interleukin 12 and CD1d recognition. We propose this mechanism of activation as a major pathway responsible for the rapid activation of NKT cells in different microbial infections.

The immune system has evolved the capability to present lipid antigens to T cells by CD1 molecules[1,2]. A growing body of evidence has established a function for CD1d-restricted T cells during the natural course of bacterial, protozoan, fungal and viral infections[2,3]. For example, pulmonary challenge with *Pseudomonas aeruginosa* in CD1d-deficient mice results in bacterial overgrowth at early stages of infection, which seems to be due to a lack of interferon-γ (IFN-γ)-dependent macrophage activation[4]. The absence of $V_\alpha14^+$ CD1d-restricted T cells during *Cryptococcus neoformans* infection leads to delayed pathogen elimination and impaired development of an adaptive T helper type 1 response[5]. During infection with *Trypanosoma cruzi*, absence of CD1d-restricted T cells leads to increased parasite burden and a decreased pathogen-specific antibody response[6]. CD1d-deficient mice also have impaired immune responses to several viral pathogens[7–9]. $V_\alpha14^+$ NKT cells contribute to liver injury induced by *Salmonella choleraesuis* infection; however, it is not known what influence CD1d-restricted T cells have on the outcome of salmonella infection in mice[10]. These studies show that CD1d-restricted T cells can affect a variety of infections, and indicate that the protective functions of these cells during infection are the result of their immediate cytokine production and their ability to contribute to the activation of other cells of the immune system. In addition, pharmacological activation of CD1d-restricted T cells by administration of the α-galactosylceramide (αGC) lipid antigen provides considerable protection during different microbial infections[2,3]. Yet how CD1d-restricted T cells become activated physiologically by microbes during the course of an infection is not known.

Most CD1d-restricted T cells demonstrate a notable restriction of their T cell receptor (TCR) repertoire by using an invariant TCRα chain ($V_\alpha24J_\alpha18$ in humans and $V_\alpha14J_\alpha18$ in mice) combined with a limited set of β chains[11,12]. CD1d-restricted T cells expressing the TCRα-invariant antigen receptor recognize αGC, an unusual α-glycosylated sphingolipid initially derived from a marine sponge[13]. Many CD1d-restricted T cells with these TCRα-invariant antigen receptors express the CD161 antigen (the NK cell marker NK1.1 in the mouse and NKR-P1A in humans) and are commonly referred to as NKT cells[14,15].

T cells restricted to CD1d were initially characterized on the basis of their autoreactivity to CD1 molecules[16,17]. Their activated memory surface phenotype even in the absence of exogenous stimulation indicates that self lipids presented by CD1d are sufficient for a certain degree of activation[18,19]. Yet it is not clear how the overt activation of CD1d-restricted T cells is induced during infection. Their *in vivo* self-reactivity does not elicit potent effector functions in homeostatic conditions, and recognition of foreign antigens, as might occur during infection, has not been demonstrated for CD1d-restricted T cells. Here, we examine the mechanism of activation of CD1d-restricted T cells in response to microbial products *in vitro* and during the course of microbial infection *in vivo*. We found that proinflammatory cytokines secreted by antigen-presenting cells (APCs) after exposure to microbial products amplified the basal weak responses of CD1d-restricted T cells to self antigens to yield potent effector functions. This is a mechanism that can account for the rapid activation of CD1d-restricted T cells in many different microbial infections.

RESULTS

Autoreactivity of CD1d-restricted T cells

To investigate the mechanism of CD1d-restricted T cell activation by microbial products, we developed an *in vitro* system with human

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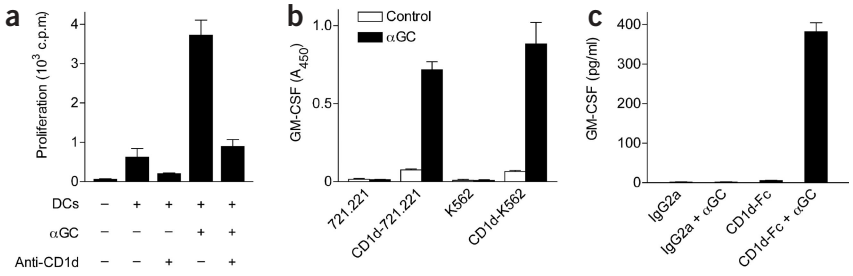

Figure 1 Self-reactivity of CD1d-restricted T cell clones. (**a**) CD1d-restricted T cell clones were incubated alone or with monocyte-derived DCs in the presence (+) or absence (–) of αGC and/or blocking CD1d mAb. Proliferation was determined by standard [^{3}H]thymidine incorporation assay after 3 d. (**b**) CD1d-restricted T cell clones were incubated with CD1d-transfected or untransfected cell lines in the presence or absence of αGC for 16–24 h. Cytokine concentrations in culture supernatants were measured by ELISA. 721.221, lymphoblastoid cell line; K562, myelomonocytic cell line (**c**) CD1d-restricted T cell clones were incubated in the presence or absence of αGC- or DMSO-loaded plate-bound CD1d-Fc fusion protein or IgG2a for 16–24 h. Cytokine concentrations in culture supernatants were measured by ELISA. Data represent means ± s.d. of triplicate samples and are representative of at least three independent experiments.

CD1d-restricted T cell clones and CD1d-expressing APCs. When incubated with dendritic cells (DCs) or CD1d-transfected cell lines, CD1d-restricted T cell clones showed modest reactivity to the APCs, as determined by proliferation and cytokine secretion, even in the absence of added antigen or microbial products (**Fig. 1a,b**). NKT cell clones cultured with DCs showed 10-fold higher [^{3}H]thymidine incorporation (620 ± 225 c.p.m.) than did T cells alone (60 ± 11 c.p.m.). This self-reactivity was dependent on the expression of CD1d by the APCs, as monoclonal antibody (mAb) to CD1d inhibited the response by >60% (**Fig. 1a**). Similarly, NKT cell clones cultured in the presence of CD1d-expressing transfected cell lines secreted five- to eightfold more granulocyte-monocyte colony-stimulating factor than did those cultured in the presence of the untransfected parental cell line (**Fig. 1b**). Because no foreign antigens are added in these *in vitro* systems, the CD1d-restricted weak activation of NKT cell clones is likely to be mediated by endogenous cellular antigens. We noted strong CD1d-restricted activation of NKT cell clones after adding αGC to the cultures of T cells plus APCs, as shown by the 6- to 14-fold-increase in [^{3}H]thymidine incorporation and cytokine secretion compared with the amounts obtained in the absence of αGC (**Fig. 1a,b**). In an APC-free system of antigen presentation, with plate-bound CD1d-Fc fusion proteins to stimulate CD1d-restricted T cell clones, no notable activation occurred after exposure to CD1d molecules except when the CD1d molecules were loaded with αGC (**Fig. 1c**). The lack of stimulation of CD1d-restricted T cell clones by the recombinant CD1d-Fc fusion proteins indicates that presentation of specific cellular antigens is required for the self-reactivity of CD1d-restricted T cells, as noted before[20].

Microbial products activate NKT cell clones

We next examined CD1d-restricted T cell activation by microbes. CD1d-restricted T cell clones incubated with monocyte-derived immature dendritic cells (DCs) plus heat-killed *S. typhimurium* or *Staphylococcus aureus* produced substantial amounts of IFN-γ (**Fig. 2a**). The amounts of IFN-γ secreted by the CD1d-restricted T cell clones after stimulation with bacteria were 20–90% of those obtained by stimulation with αGC. We found that 12 independent CD1d-restricted T cell clones secreted IFN-γ when incubated with

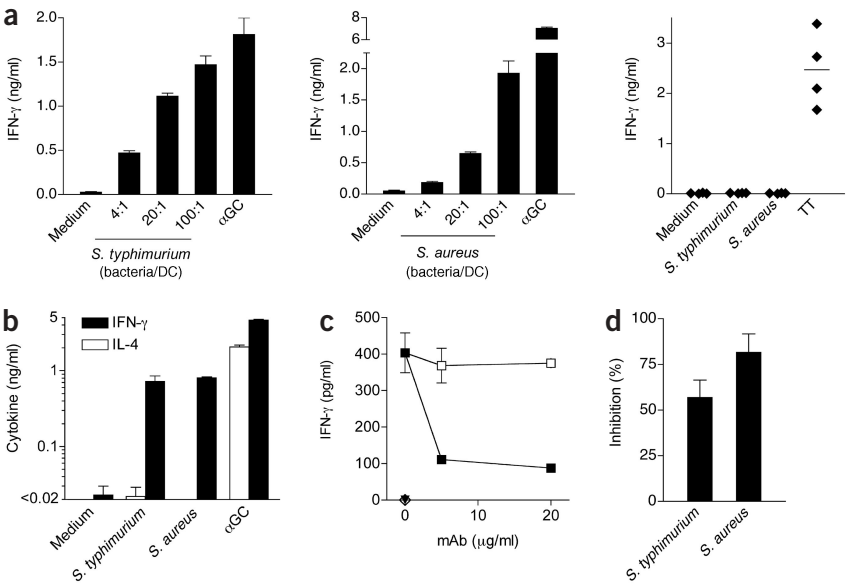

Figure 2 Activation of CD1d-restricted T cell clones by bacterial products. (**a**) CD1d-restricted T cell clones (left and middle) or MHC class II–restricted tetanus toxoid–specific T cell clones (right) were cultured with DCs in the presence or absence (Medium) of heat-inactivated *S. typhimurium* or *S. aureus*, αGC or tetanus toxoid (TT). Left and middle, bacteria/DC ratios are on horizontal axes; for tetanus toxoid–specific clones (right), 100 bacteria were added per DC and data are from four distinct clones. Cytokine concentrations were measured in culture supernatants after 16–24 h. (**b**) A CD1d-restricted T cell clone was cultured with DCs in the absence or presence of heat-inactivated bacteria or αGC for 16–24 h. Concentrations of IFN-γ and IL-4 were determined in culture supernatants. (**c**) DCs and T cells cocultured in the presence of heat-inactivated *S. typhimurium* and blocking CD1d (filled squares) or control (open squares) mAb (concentrations, horizontal axis). Reverse filled triangle, T cells alone; open diamond, DCs plus T cells without bacteria (symbols are overlapping). (**d**) DCs and T cells cocultured in the presence of *S. typhimurium* or *S. aureus* (100 bacteria per DC) and blocking CD1d mAb for 16–24 h. Data are shown as percent inhibition of IFN-γ secretion in culture supernatants, presented as summarized data from 12 independent CD1d-restricted T cell clones (means ± s.d.), and are representative of at least three independent experiments.

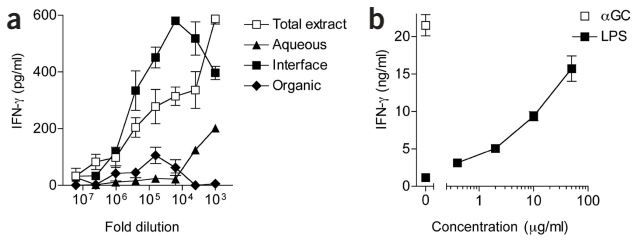

Figure 3 Purified bacterial products activate CD1d-restricted T cell clones in the presence of DCs. (**a**) Total aqueous extracts from *S. typhimurium* were extracted with chloroform-methanol and the resultant aqueous phase, interface and organic phase were examined in cultures of CD1d-restricted T cells plus DCs. Cytokine concentrations were determined by ELISA after 16–24 h. (**b**) Cultures of CD1d-restricted T cells plus DCs were incubated with purified LPS or αGC (concentrations, horizontal axis). IFN-γ concentrations in supernatants were measured by ELISA after 16–24 h of culture. Data represent means of triplicate supernatant samples and are representative of at least two independent experiments for two to five distinct clones.

DCs that were exposed to heat-killed *S. aureus*, whereas 11 of these 12 clones responded in the presence of DCs exposed to heat-killed *S. typhimurium* (data not shown). In contrast, tetanus toxoid–specific major histocompatibility complex (MHC) class II–restricted T cell clones did not produce detectable IFN-γ in response to the heat-killed bacteria, whereas they produced large amounts of IFN-γ in response to their cognate antigen (**Fig. 2a**, right). Although CD1d-restricted T cell clones secreted both IFN-γ and interleukin 4 (IL-4) when stimulated with αGC, we found little or no IL-4 when we stimulated clones with heat-killed *S. typhimurium* or *S. aureus* in the presence of DCs (**Fig. 2b**). Thus, several individual CD1d-restricted T cell clones produced IFN-γ potently when exposed to either Gram-positive or Gram-negative bacteria in the presence of DCs, whereas MHC class II–restricted T cell clones were not activated in these conditions.

To determine the requirement for a CD1d-TCR interaction for the activation of CD1d-restricted T cell clones by bacterial products, we did experiments with blocking CD1d mAb. The addition of blocking CD1d mAb inhibited secretion of IFN-γ of 12 independent CD1d-restricted T cell clones by means of 57 ± 9.5% and 82 ± 10% for *S. typhimurium* and *S. aureus*, respectively, in cultures of T cell clones and DCs plus bacteria, whereas isotype-matched control antibody had no such effect (**Fig. 2c**,**d**). Thus, the activation of CD1d-restricted T cell clones by microbial products in the presence of DCs was CD1d dependent.

Lipopolysaccharide can activate NKT cell clones

To identify the microbial product(s) causing the activation of CD1d-restricted T cells by *S. typhimurium*, we did lipid extraction and fractionation experiments that have been used before to identify CD1b- and CD1c-presented microbial lipid antigens from *Mycobacterium tuberculosis* as well as CD1d-presented mammalian cellular lipids[1,20–22]. Organic chloroform-methanol extracts of *S. typhimurium* containing many bacterial lipids were unable to stimulate CD1d-restricted T cell clones when cultured with DCs or CD1d-expressing cell lines, or when added to the APC-free antigen-presentation assay with plate-bound CD1d-Fc fusion proteins (data not shown). However, aqueous extracts of *S. typhimurium* (total extract) stimulated CD1d-restricted T cell clones when cultured in the presence of DCs. When aqueous *S. typhimurium* extracts were further extracted with chloroform-methanol according to a published phase-separation method[23], material in the interphase retained the ability to induce IFN-γ secretion from CD1d-restricted T cell clones in the presence of DCs (**Fig. 3a**). The active material in aqueous *S. typhimurium* extracts was retained by a membrane with a molecular weight cut-off of 100 kDa and was protease resistant (data not shown). When DCs were incubated with aqueous *S. typhimurium* extracts or whole heat-killed bacteria and subsequently fixed with glutaraldehyde, they were no longer able to stimulate CD1d-restricted T cell clones (data not shown). This finding raised the possibility that activation of CD1d-restricted T cell clones by *S. typhimurium* in the presence of DCs might not result from the presentation of foreign microbial antigens by CD1d. In a Folch aqueous-organic phase-separation, most bacterial lipids partition to the organic phase, whereas amphipathic molecules like lipopolysaccharide (LPS) partition into the interphase[23,24]. We found that purified LPS was able to stimulate IFN-γ secretion from CD1d-restricted T cell clones in the presence of DCs (**Fig. 3b**).

APC-free reconstitution of NKT cell activation

Because LPS is a known agonist for Toll-like receptor 4 (ref. 25), we considered the possibility that microbial products like LPS from *S. typhimurium*, instead of activating CD1d-restricted T cells as CD1d-presented microbial antigens, might activate the APCs through Toll-like receptors, and that the CD1d-restricted T cells are activated, in part, through stimulation by an APC derived factor. The additional requirement for TCR-CD1d interaction shown here could be fulfilled by recognition of CD1d-presented self lipids. To investigate whether the response to a weak TCR stimulus would be enhanced by DC-derived factors, we exposed T cell clones to a TCR stimulus that we could manipulate. We used a suboptimal amount of plate-bound antibody to CD3 (anti-CD3) as a weak TCR stimulus in the presence or absence of supernatants obtained from DCs exposed to *S. typhimurium* or *S. aureus* (**Fig. 4a**). Suboptimal anti-CD3 stimulation alone resulted in minimal secretion of IFN-γ, and the addition of conditioned medium derived from DCs exposed to *S. typhimurium* or *S.aureus* enhanced IFN-γ secretion by a CD1d-restricted T cell clone 12-fold or 7-fold, respectively. Transfer of conditioned medium alone onto CD1d-restricted T cells in the absence of anti-CD3 stimulation did not result in detectable IFN-γ secretion (data not shown).

To identify a potential DC-derived cytokine activating the CD1d-restricted T cell clones, we determined the concentration of cytokines in supernatants from DCs exposed to heat-killed *S. typhimurium* or *S. aureus*. The inflammatory cytokines IL-1β, IL-6, IL-12 and tumor necrosis factor-α (TNF-α) were produced in large quantities when bacteria were present with the DCs, whereas they were not detectable when DCs were cultured in medium alone (**Supplementary Table 1** online). DCs exposed to *S. aureus* produced IL-1β, IL-6 and large amounts of IL-12p70 and TNF-α. Exposure of DCs to *S. typhimurium* resulted in comparatively lower amounts of IL-12p70 and TNF-α and higher amounts of IL-1β and IL-6. IL-18, a cytokine known to be induced by microbial products and to contribute to IFN-γ production by lymphocytes[26,27] was present only in very low concentrations.

We next tested whether any of the inflammatory cytokines detected in the supernatants from DCs exposed to heat-inactivated bacteria was sufficient to reconstitute the effects of conditioned media in the APC-free system. We added purified recombinant IL-1β, IL-6, IL-12 and TNF-α alone, or IL-1β, IL-6 and TNF-α in combination, to the T cell clones with or without suboptimal anti-CD3 stimulation. Only IL-12 in combination with suboptimal anti-CD3 stimulation notably enhanced IFN-γ secretion by the T cell clones (**Fig. 4b**).

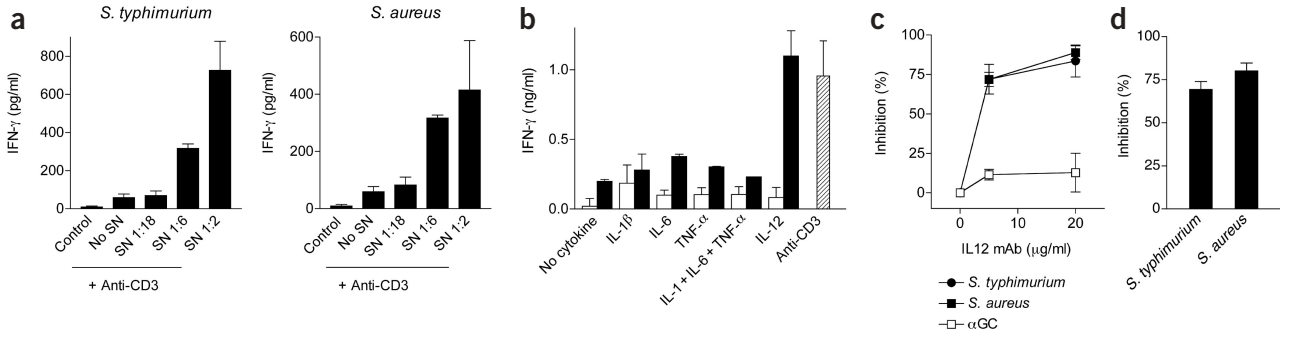

Figure 4 CD1d-restricted T cell activation by bacterial products is IL-12 dependent. (**a**) CD1d-restricted T cell clones were incubated in 96-well plates coated with suboptimal concentrations of CD3 mAb. Supernatants (SN) from 24-hour cultures of DCs plus bacteria were filtered (0.22-μm pore size) and added to the T cell cultures (dilutions, horizontal axes). IFN-γ concentrations in culture supernatants were measured by ELISA after 16–24 h. (**b**) CD1d-restricted T cell clones were incubated in 96-well plates coated with suboptimal amount of CD3 mAb (filled bars) or control antibody (open bars) in the presence of recombinant cytokines. Maximal anti-CD3 stimulation served as a control (hatched bar). IFN-γ concentrations in culture supernatants were measured by ELISA after 16–24 h. (**c**) CD1d-restricted T cell clones were incubated with heat-inactivated bacteria or αGC in the presence of blocking IL-12 mAb (concentrations, horizontal axis). IFN-γ concentrations in culture supernatants were measured by ELISA after 16–24 h. Data represent percent inhibition of the maximal response. (**d**) Percent inhibition of IFN-γ secretion in supernatants of cultures of DCs plus T cells in the presence of heat-inactivated *S. typhimurium* or *S. aureus* and blocking IL-12 mAb. Data represent summarized data of 12 independent CD1d-restricted T cell clones (means ± s.d.) and are representative of at least three independent experiments.

To confirm the function of IL-12 in activating CD1d-restricted clones in the presence of DCs exposed to bacteria, we tested the effect of blocking IL-12 mAb in our DC–NKT cell clone culture system. We found that mAb to IL-12 reduced the secretion of IFN-γ induced by *S. typhimurium* and *S. aureus* in 12 independent CD1d-restricted T cell clones by means of 69 ± 4.6% and 80 ± 4.6%, respectively (**Fig. 4c**,**d**). In contrast to the bacteria-stimulated IFN-γ secretion, αGC-stimulated secretion of IFN-γ by CD1d-restricted T cells was inhibited by IL-12 mAb only slightly, by a mean of 9.8 ± 9.5% (**Fig. 4c**). Thus, for the activation of CD1d-restricted T cell clones by bacterial products, the production of IL-12 by DCs was necessary to activate the T cells, whereas in the presence of the potent antigen αGC, IL-12 did not have such a function.

IL-12 plus self-reactivity activate NKT cells

Next, we investigated whether recognition of self lipids presented by CD1d combined with exposure to IL-12 is sufficient to induce IFN-γ secretion by CD1d-restricted T cells. We cultured CD1d-restricted T cell clones with DCs in the absence of microbial products, a condition in which NKT cell stimulation is minimal (**Fig. 1**). The addition of recombinant IL-12 to CD1d-restricted cultures of T cells plus DCs led to notably increased IFN-γ secretion by the T cell clones, compared with the addition of IL-12 to T cells alone (**Fig. 5a**). At IL-12 concentrations as low as 0.22 ng/ml, the secretion of IFN-γ by a CD1d-restricted T cell clone increased 28-fold when DCs were present compared with exposure of the T cells to DCs alone. CD1d mAb inhibited increases in IL-12-induced IFN-γ secretion in the presence of DCs by 90%, whereas isotype-matched control antibodies had no such effect (**Fig. 5b**). In similar conditions, the addition of IL-12 to MHC class II–restricted tetanus toxoid–specific clones and DCs in the absence of added microbial products did not induce secretion of detectable amounts of IFN-γ (**Fig. 5a**).

Next we determined whether freshly isolated NKT cells respond to IL-12 in the same way as human CD1d-restricted T cell clones. We added recombinant IL-12 to mouse splenocyte cultures in the presence of blocking CD1d mAb or isotype-matched control mAb. The resulting IFN-γ production was much less in the presence of blocking CD1d mAb than with the isotype-matched control mAb (**Fig. 5c**). These results indicate that a substantial amount of the IFN-γ produced by mouse splenocytes in the presence of IL-12 is dependent on the

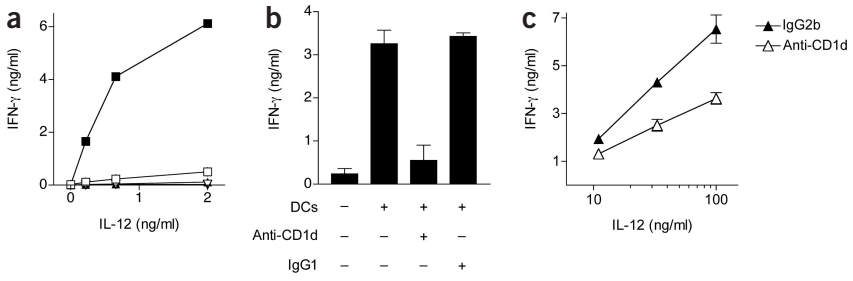

Figure 5 Self-reactivity of CD1d-restricted T cell clones is amplified by IL-12. (**a**) CD1d-restricted (squares) or MHC class II–restricted (triangles) T cell clones were incubated in the presence (filled symbols) or absence (open symbols) of DCs and IL-12 (concentrations, horizontal axis). Data for the MHC class II–restricted T cell clone in the presence or absence of DCs are almost identical and therefore symbols (triangles) are overlapping. Results are representative of at least three independent experiments. (**b**) A CD1d-restricted T cell clone was incubated in the presence (+) or absence (–) of DCs in complete RPMI media containing IL-2 and IL-12 in the presence (+) or absence (–) of CD1d or control mAbs. Cytokine concentrations in tissue culture supernatants were determined by ELISA after 16–24 h. Data are representative of at least three independent experiments. (**c**) Freshly isolated mouse splenocytes were incubated with recombinant mouse IL-12 (concentrations, horizontal axis) in the presence of blocking CD1d or isotype-matched control mAbs (10 μg/ml). Cytokine concentrations in tissue culture supernatants were determined by ELISA after 18–24 h. Data represent means of triplicate analysis ± s.d. and are representative of two experiments.

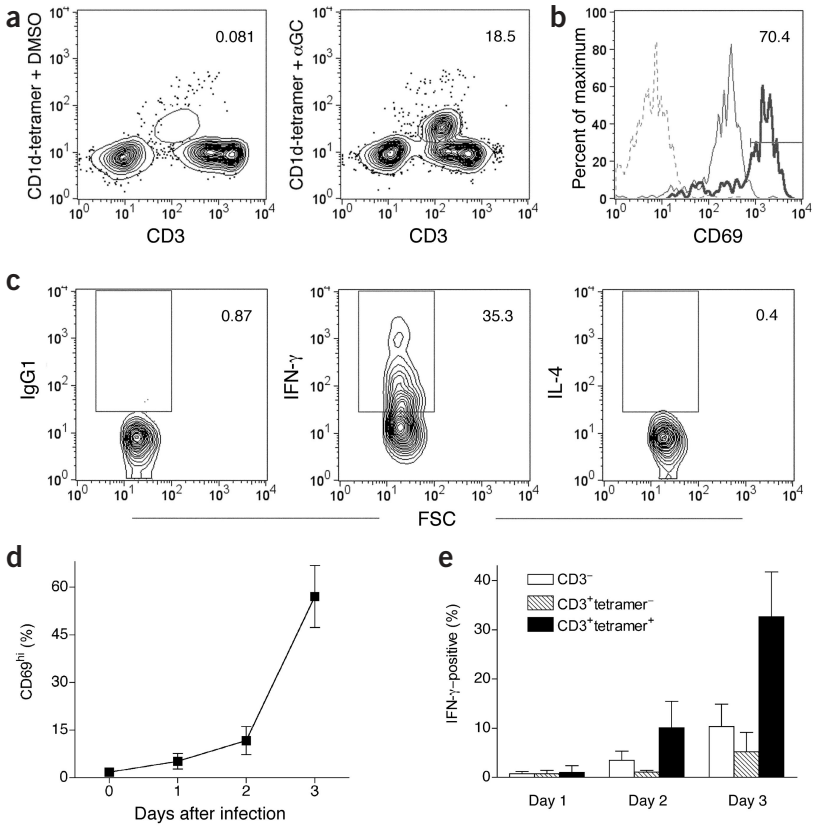

Figure 6 *In vivo* activation of CD1d-restricted T cells during *S. typhimurium* infection. Lymphocytes were isolated from livers of mice infected with *S. typhimurium* or uninfected mice and analyzed by flow cytometry. Numbers in flow cytometry panels indicate the percentage of gated lymphocytes. (**a**) Detection of CD1d-restricted T cells by staining with αGC- or DMSO-loaded CD1d tetramers and anti-CD3. (**b**) CD69 surface staining of CD1d tetramer–positive lymphocytes before infection (thin line) and at day 3 after infection (bold line), or staining with isotype control antibodies (broken line). (**c**) CD1d tetramer–positive cells in mice 3 d after infection and intracellular cytokine (IFN-γ or IL-4) or control antibody staining. (**d**) Percentage of $CD69^{hi}$-expressing CD1d tetramer–positive lymphocytes at different times after infection. Data were pooled from three independent experiments and represent mean ± s.d. for four to six mice. (**e**) Percentages of IFN-γ-positive cells on days 1, 2 and 3 after infection of $CD3^-$ (containing NK cells), $CD3^+$ tetramer-negative and $CD3^+$ tetramer-positive lymphocytes. Data were pooled from three independent experiments and represent mean ± s.d. for four to six mice.

interaction of NKT cells with CD1d on APCs. As no exogenous antigens are added in this system, the stimulation provided by CD1d recognition is likely to be due to recognition of self-lipids. Thus, weak responses of CD1d-restricted T cell clones and freshly isolated CD1d-restricted T cells to self antigens presented by CD1d can be amplified considerably by the proinflammatory cytokine IL-12 to elicit potent secretion of IFN-γ.

NKT cell activation during *S. typhimurium* infection

Next, we analyzed CD1d-restricted T cell activation in a mouse model of *S. typhimurium* infection *in vivo* (**Fig. 6**). We infected mice intravenously with 1×10^3 to 1×10^4 live bacteria. We isolated lymphocytes from livers and spleens of infected and uninfected mice and detected CD1d-restricted T cells by staining with soluble fluorescent CD1d multimers (CD1d tetramers) that were loaded with αGC or 'mock treated' (**Fig. 6a**). Total numbers of CD1d tetramer positive cells isolated from livers or spleens were not substantially different between uninfected and infected mice on days 1, 2 and 3 after infection (**Supplementary Table 2** online). CD1d tetramer–positive T cells from livers of uninfected mice stained at intermediate intensity for the early activation marker CD69 (mean fluorescence intensity (MFI), 106 ± 48), similar to published results[14] (**Fig. 6b**). At 1 d after infection, a mean of 5.2 ± 2.4% of CD1d tetramer–positive cells became $CD69^{hi}$ (MFI, 135 ± 81), increasing to means of 12 ± 4.4% (MFI, 193 ± 146) and 57 ± 9.8% (MFI, 790 ± 493) on days 2 and 3 after infection, respectively, compared with a mean of 1.8 ± 0.2% of uninfected controls (**Fig. 6d**). Thus, most CD1d-restricted T cells became rapidly activated during *S. typhimurium* infection. Intracellular cytokine staining on day 1 after infection showed a mean of 1.0 ± 1.2% of the tetramer-positive T cells staining positive for IFN-γ, increasing to 10 ± 4.6% and 33 ± 8.3% on days 2 and 3, respectively (**Fig. 6c**,**e**). In uninfected controls, a mean of 1.0 ± 1.0% of CD1d tetramer–positive cells stained positive for IFN-γ. Thus, the expression of intracellular IFN-γ by CD1d-restricted T cells during *S. typhimurium* infection followed a time course similar to that of CD69 upregulation. No intracellular IL-4 could be detected in CD1d tetramer–positive cells on days 1 and 2 after infection, whereas on day 3 after infection, a mean of 2.0 ± 2.2% of the tetramer-positive cells stained positive for IL-4, compared with 0.4 ± 0.3% of uninfected controls (**Fig. 6c** and data not shown). We also noted an increase in IFN-γ production by CD1d-restricted T cells during the first 3 d of infection in spleen (**Supplementary Table 3** online).

During the first 3 d of *S. typhimurium* infection, other lymphocyte populations in the liver also expressed intracellular IFN-γ. Concomitant with the appearance of T cells positive for both CD1d tetramer and IFN-γ on day 2 after infection, a mean of 3.5 ± 1.6% of $CD3^-$ lymphocytes stained positive for IFN-γ, increasing to 10 ± 4.1% on day 3 (**Fig. 6e**). In addition, means of 1.1 ± 0.3% and 5.2 ± 3.6% of other T cells (CD1d tetramer–negative negative) stained positive for IFN-γ on days 2 and 3, respectively. Of the total IFN-γ-positive lymphocytes in liver on day 2 and 3 after infection, means of 4.2 ± 2.0% and 8.9 ± 2.8%, respectively, were CD1d tetramer positive; we obtained similar results with the spleen (**Supplementary Table 3** online).

This analysis shows that during *S. typhimurium* infection, CD1d-restricted T cells become activated within 24 h of infection, and are among the first lymphocytes to produce IFN-γ. Moreover, a large proportion of the CD1d-restricted T cells detected by αGC–CD1d tetramer staining became activated and secreted IFN-γ. However, the largest subset of lymphocytes that produced IFN-γ in the first 3 d

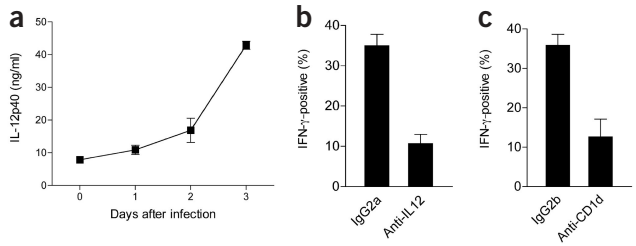

Figure 7 IFN-γ production by CD1d-restricted T cells during *S. typhimurium* infection is dependent on IL-12. (**a**) IL-12p40 concentrations were determined by ELISA in sera of mice infected with *S. typhimurium* at different times after infection. Data were pooled from two independent experiments and represent means ± s.d. for four mice per group. (**b**) Mice were infected with *S. typhimurium* and received blocking IL-12 or control mAbs intravenously 1 d before and 2 d after infection. Lymphocytes were isolated from mouse livers 3 d after infection and analyzed by flow cytometry. Data represent percent of CD1d tetramer–positive cells that are IFN-γ-positive, and are the means ± s.e.m. of cumulative data from three independent experiments and six mice per group. (**c**) Mice were infected with *S. typhimurium* and received blocking CD1d or control mAbs intravenously 1 d before and 2 d after infection. Lymphocytes were isolated from mouse livers 3 d after infection and analyzed by flow cytometry. Data represent percent of CD1d tetramer–positive cells that are IFN-γ positive and are the means ± s.e.m. of cumulative data from two independent experiments and four mice per group.

of infection was $CD3^-$, and was thus likely to be NK cells (**Supplementary Table 3** online). CD1d tetramer–stained cells represented means of 25 ± 4.8% and 13 ± 6.9% of the IFN-γ positive T cells in the liver on days 2 and 3 after infection, respectively (data not shown). We obtained similar results from our analysis of splenocytes (**Supplementary Table 3** online).

IL-12 and CD1d blocking *in vivo*

Next we assessed whether IL-12 was required for the activation of CD1d-restricted T cells during *S. typhimurium* infection, as was the case in the *in vitro* experiments described above. We first determined whether IL-12 secretion correlated with the activation of CD1d-restricted T cells during *S. typhimurium* infection. IL-12p40 concentrations in sera of infected mice increased to a mean of 11 ± 1.4 ng/ml on day 1 and then to 17 ± 3.7 ng/ml and 43 ± 1.2 ng/ml on days 2 and 3 after infection, respectively, compared with a mean of 7.9 ± 0.9 ng/ml in uninfected mice (**Fig. 7a**). CD69 surface expression rose on CD1d-restricted T cells by day 1 after infection, whereas IFN-γ secretion was first detectable in these cells by day 2 after infection (**Fig. 6d**). Thus, during infection with *S. typhimurium*, cytokine production of CD1d-restricted T cells occurred subsequent to IL-12 secretion.

We used blocking mAb to IL-12 to determine the function of IL-12 for activation of CD1d-restricted T cells during *S. typhimurium* infection. Mice infected with *S. typhimurium* received blocking IL-12 mAb or isotype-matched control mAb 1 d before infection and on day 2 after infection. On day 3 after infection in mice that were treated with IL-12 mAb, a mean of 11 ± 2.3% of the tetramer-positive hepatic lymphocytes stained positive for IFN-γ, whereas in mice that received control antibody, a mean of 35 ± 2.8% of the tetramer-positive cells stained positive for IFN-γ, demonstrating a 70% reduction of the IFN-γ production by treatment with blocking IL-12 mAb (**Fig. 7b**). We obtained similar results with the spleen (data not shown).

To investigate whether activation of CD1d-restricted T cells during *S. typhimurium* infection requires recognition of CD1d, mice received blocking CD1d mAb or isotype-matched control antibody during infection. On day 3 after infection, a mean of 12.6 ± 4.5% of the tetramer-positive hepatic lymphocytes stained positive for IFN-γ in mice that were treated with blocking CD1d mAb, whereas in mice that received control antibody, a mean of 35.9 ± 2.8% of the tetramer-positive cells stained positive for IFN-γ, demonstrating a 65% inhibition (**Fig. 7c**). We obtained similar results with the spleen (data not shown). Thus, IFN-γ production by CD1d-restricted T cells during *S. typhimurium* infection *in vivo* is dependent on IL-12 and recognition of CD1d.

DISCUSSION

CD1d-restricted T cells have been shown to be essential in several models of microbial infection. However, it is unclear how these T cells become activated in natural conditions during the course of an infection. We found that microbe-induced activation of CD1d-restricted T cells did not require recognition of foreign antigens. Instead, our *in vitro* experiments demonstrated that in the presence of IL-12, TCR-mediated recognition of self antigens presented by CD1d was sufficient to induce productive activation of CD1d-restricted T cells. Our *in vivo* experiments showed early activation of CD1d-restricted T cells during *S. typhimurium* infection and confirmed the requirement for signals from IL-12 and CD1d for their cytokine production *in vivo*. Thus, our data indicate a model for the activation of CD1d-restricted T cells that does not require their recognition of microbial antigens. Instead, weak responses to CD1d-presented self-antigens are amplified by IL-12 made by DCs in response to microbial products. These features might allow CD1d-restricted T cell activation to occur early and in the setting of any microbial challenge that stimulates the production of IL-12. However, our studies do not exclude the possibility that additional TCR-mediated recognition of microbial antigens by CD1d-restricted T cells might occur in some circumstances.

Studies in murine models of *S. typhimurium* infection have shown that MHC class II–restricted immune responses do not start until 5–7 d after infection and peak at 2–3 weeks after infection[28]. Similar results were obtained for $CD8^+$ T cell responses[29]. These findings are contrasted by the very early activation of salmonella-specific MHC class II–restricted T cells noted in studies with adoptive transfer of TCR-transgenic T cells[30]. For oral infection with *S. typhimurium*, these studies found T cell activation within 3 h of infection in Peyer's patches, but negligible T cell activation in other organs, including liver and spleen. The precursor frequency of antigen-specific T cells in previously uninfected nontransgenic mice is very low, and proliferation of these T cells would be required before substantial numbers are present. Hence, in nontransgenic mice, rare T cells in proximal tissues may become rapidly activated after infection, but it probably takes about at least 5 d before substantial numbers of activated MHC class I– and class II–restricted T cells are present in the liver and spleen. In contrast, CD1d-restricted T cells represent a substantial fraction of the T cell compartment in the liver, spleen and intestinal intraepithelial lymphocyte populations of mice[14,31]. Our studies have shown that the amplification of their self-reactivity by IL-12 is sufficient for the secretion of large amounts of IFN-γ by CD1d-restricted T cells, indicating that clonal expansion is not necessary before their activation.

Many studies have shown that NKT cells become undetectable *in vivo* quickly after stimulation with anti-CD3, αGC or IL-12, a process that is thought to be due to apoptosis[31–33]. This activation-induced cell death of NKT cells is followed by renewal from a pool of dividing cells[32]. Our studies show that total numbers of CD1d-restricted T cells detected by αGC–CD1d tetramers in the first 3 d after *S. typhimurium* infection in liver and spleen remain largely unchanged. The extent to

which this is due to the recruitment of CD1d-restricted T cells to these organs or their proliferation and the fate of CD1d-restricted T cells later during infection remains to be determined.

In our studies of the activation of CD1d-restricted T cells during *S. typhimurium* infection, a notably high proportion of CD1d tetramer–positive T cells became activated very early during infection, compared with NK cells and CD1d tetramer–negative T cells. However, the CD1d tetramer–positive cells represent only a relatively small fraction of the total pool of IFN-γ-producing cells. Most IFN-γ-positive cells were $CD3^-$, and were likely to be NK cells, but most of the IFN-γ-positive T cells were also CD1d tetramer–negative. The nature of the CD1d tetramer–negative T cells remains undefined, but they could be CD1d-restricted T cells that are not detected by αGC-loaded CD1d tetramers, such as those with diverse TCRα receptors. They also could be γδ T cells, T cells restricted by nonclassical MHC molecules such as TL or H-2M, or MHC class I– or class II–restricted cross-reactive memory T cells, as the mice were not previously exposed to *S. typhimurium* and therefore are unlikely to have a memory T cell repertoire specific for *S. typhimurium*. It is unclear whether the activation of NK cells and non-CD1d-restricted T cells at these early time points during infection occurs before, during or after the activation of CD1d-restricted T cells, as occurs after pharmacological administration of αGC[34].

The IFN-γ secreted by CD1d-restricted T cells after activation by microbial products could serve to activate cell-mediated effector functions of macrophages or could modulate antibody production by B cells[35]. Furthermore, CD1d-restricted T cells can directly mediate cytolysis, indicating that they could function as cytolytic effectors that contribute to bacterial killing by lysis of infected cells. This possibility is supported by the observation that $CD4^-$ CD1d-restricted T cells in human peripheral blood mononuclear cells (PBMC) samples upregulate perforin after exposure to IL-12 (ref. 36). Additionally, early IFN-γ and IL-12 production may affect the magnitude of an antigen-specific CD4 and CD8 T cell response and the extent and durability of memory.

In summary, our results indicate that CD1d-restricted T cells may use a strategy for their activation by microbial products and during infection whereby weak responses to self antigens are amplified by proinflammatory cytokines. This mechanism might explain the rapid activation of CD1d-restricted T cells in a variety of microbial infections without dependence on TCR-specific foreign antigen recognition, and has no clear counterpart among MHC-restricted T cells.

METHODS

T cell clones, monocyte-derived DCs, and CD1d transfectants. CD1d-restricted T cell clones were derived from human PBMCs by staining with αGC-loaded CD1d tetrameric complexes[36] and subsequent cloning by single-cell flow cytometry cell sorting followed by culture in the presence of irradiated allogeneic PBMCs as feeders, phytohemagglutinin (Sigma-Aldrich) and IL-2 (2 nM; Chiron). Tetanus toxoid–specific MHC class II–restricted clones were derived from PBMCs stimulated with tetanus toxoid (0.01 limit of flocculation units/ml; Massachusetts Biological Laboratories). After two rounds of antigen stimulation, tetanus toxoid–specific lines were determined by [^{3}H]thymidine incorporation. Positive lines were 'single-cell cloned' by limiting dilution at a density of 0.3 cells/well on tetanus toxoid–pulsed autologous, irradiated feeders. Positive clones were characterized by split-well assay, [^{3}H]thymidine incorporation, and cell surface marker flow cytometry. Immature monocyte-derived DCs were prepared from fresh leukapheresis mononuclear cells or PBMCs. Mononuclear cell populations isolated by Ficoll-Hypaque density centrifugation (Amersham Biotech) were enriched for monocytes by plastic adherence, and immature monocyte-derived DCs were derived by culture for 3 d in granulocyte-monocyte colony-stimulating factor (300 U/ml; Sargramostim; Immunex) and IL-4 (200 U/ml; PeproTech) in complete RPMI medium (RPMI supplemented with L-glutamine and penicillin/streptomycin; Life Technologies) and 10% FBS (Hyclone Laboratories). For the generation of CD1d-transfected cell lines, a cDNA construct of human CD1d in the pSRα-neo expression vector was generated[37,38]. The 721.221 lymphoblastoid cell line[39] and K562 myelomonocytic cell line[40] were transfected with the human CD1d cDNA construct by electroporation, grown under G418 (Life Technologies) selection, then sorted by flow cytometry for cells expressing CD1d.

Cultures of T cells plus APCs. T cells were cultured with CD1d transfectants or DCs in 96-well flat-bottomed plates (Costar) with APCs (5×10^4) and T cells (5×10^4) in complete RPMI medium. Bacteria or antigens were added and cultures were incubated for 16–24 h at 37 °C. Culture supernatants were analyzed by enzyme-linked immunosorbent assay (ELISA) with matched antibody pairs according to the manufacturer's instructions (Pierce Endogen). Proliferation was determined by 6 h of incorporation of [^{3}H]thymidine (PerkinElmer). For experiments with tetanus toxoid–specific clones, autologous monocyte-derived DCs were used as APCs and tetanus toxoid was used at a concentration of 0.05 limit of flocculation units/ml. After being dissolved in DMSO, αGC (provided by G. Besra, School of Biosciences, University of Birmingham, Edgbaston, Birmingham, United Kingdom) was used at a final concentration of 10 ng/ml unless stated otherwise. For mAb blocking experiments, 100 bacteria were added per DC, and CD1d and IL-12 mAbs were used at a concentration of 20 μg/ml.

Splenocyte assays. After red blood cell lysis, freshly isolated splenocytes were incubated for 18–24 h in round-bottomed 96-well plates (2×10^6 cells/well) with different concentrations of recombinant mouse IL-12 in the presence of blocking CD1d mAb (1B1) or isotype-matched rat IgG2b control antibody. Mouse IFN-γ was detected in supernatants by sandwich ELISA (BD Biosciences Pharmingen).

Cell free CD1d-Fc fusion protein plate assay. Human CD1d-Fc β_2-microglobulin single-chain fusion protein was prepared as described[36]. Then, 96-well protein G–coated plates (Pierce Biotechnology) were incubated with fusion protein (0.4 μg/well) and LFA-1 antibody (50 ng/well; Pierce Endogen) or control antibody in PBS, pH 7.2. After being diluted in PBS, αGC was added at a molar ratio of 40:1 of antigen to fusion protein and incubated for 24–48 h at 37 °C, then washed two times with PBS and once with culture medium. T cells were added at a density of 5×10^4 cells per well. The plates and cells were incubated at 37 °C for 16–20 h, and cytokine concentrations were determined in culture supernatants by ELISA.

Antibodies and recombinant proteins. Antibodies for FACS staining were cychrome-conjugated anti-mouse CD3 (clone 145-2C11), phycoerythrin-conjugated anti-mouse IFN-γ (clone XMG1.2), phycoerythrin-conjugated anti-mouse IL4 (clone 11B11), phycoerythrin-conjugated anti-mouse CD69 (clone H1.2F3) and isotype-matched controls (BD Biosciences Pharmingen). Antibodies for blocking studies were anti-human CD1d (CD1d42.1; ref 17) and anti-human IL-12 (Pierce Endogen). Recombinant human cytokines IL-1β, IL-6, IL-12, TNF-α and mouse IL-12 were from PeproTech.

Flow cytometry. Mononuclear cells were isolated from livers perfused with PBS or from spleens by Histopaque density centrifugation (Sigma-Aldrich). Splenocyte samples were depleted of B cells with anti-CD19 magnetic-activated cell separation beads according to the manufacturer's protocol (Miltenyi). Cells were stained with αGC- or DMSO-loaded CD1d tetramers that were produced from mouse CD1d-Fc fusion proteins[20] as described[36]. Intracellular cytokine staining was done after fixation and permeabilization with a Cytofix-Cytoperm kit according to the manufacturer's instructions (BD Biosciences Pharmingen). Flow cytometric data was collected on a FACSort flow cytometer (Becton Dickinson) and analyzed with FlowJo software (Treestar). Samples were gated on lymphocytes with forward- and side-scatter properties.

Cytokine determination in conditioned medium. Cytokine concentrations for human IL-1β, IL-6, IL-12p70 and TNF-α were determined by SearchLight multiplex analysis (Pierce Endogen) in supernatants from DCs incubated for 24 h with heat-inactivated bacteria at a ratio of 100 bacteria per DC.

Bacteria, mice and infections. *S. typhimurium* (MT110; ref. 41) and *S. aureus* (ATCC 25923) were grown in Luria-Bertani medium at 37 °C until mid-log

phase, then bacteria were collected and diluted according to measurements of absorbance at 600 nm, and numbers of bacteria were determined by plating of bacteria in serial dilutions on McConkey agar and Luria-Bertani plates, respectively. Bacteria were heat inactivated for 45 min at 65 °C and were washed two times with PBS. C57BL/6 mice were from Jackson Laboratories. All animal studies were approved by the Dana Faber Cancer Institute Office for the Protection of Research Subjects. Male mice 6–10 weeks old received either *S. typhimurium* (1×10^3 to 1×10^4 bacteria) in 0.9% NaCl (200 μl) or 0.9% NaCl alone intravenously. For infections, bacteria were cultured for 18 h in Luria-Bertani medium at 37 °C, collected, washed three times with sterile 0.9% NaCl and diluted according to measurements of absorbance at 600 nm. Numbers of injected bacteria were determined by plating of bacteria in serial dilutions on McConkey agar plates. For antibody-blocking experiments, mice received blocking IL-12 or CD1d mAbs (0.3 mg; clone C17.8 and clone 1B1; BD Biosciences Pharmingen) or rat IgG2a or IgG2b control mAbs in PBS (300 μl) intravenously 1 d before infection and on day 2 after infection.

Bacterial cell fractionation and lipid isolation. Heat-inactivated bacteria were disrupted by sonication in PBS. After ultracentrifugation at 100,000*g* for 45 min, supernatants were separated by organic phase separation according to a published method[23]. Phases were dried, redissolved and diluted in culture medium and were added to T cell clones and DCs. LPS from *Salmonella abortus equi* was purchased from Sigma-Aldrich.

Note: Supplementary information is available on the Nature Immunology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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