Invariant NKT cells inhibit development of the Th_{17} lineage

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T cells differentiate into functionally distinct effector subsets in response to pathogen encounter. Cells of the innate immune system direct this process; CD1d-restricted invariant natural killer T (iNKT) cells, for example, can either promote or inhibit Th_1 and Th_2 responses. Recently, a new subset of $CD4^+$ T helper cells, called Th_{17}, was identified that is implicated in mucosal immunity and autoimmune disorders. To investigate the influence of iNKT cells on the differentiation of naïve T cells we used an adoptive transfer model of traceable antigen-specific $CD4^+$ T cells. Transferred naïve $CD25^-CD62L^+$ $CD4^+$ T cells were primed by antigen immunization of the recipient mice, permitting their expansion and Th_{17} differentiation. This study establishes that in vivo activation of iNKT cells during T-cell priming impedes the commitment of naïve T cells to the Th_{17} lineage. In vivo cytokine neutralization experiments revealed a role for IL-4, IL-10, and IFN-γ in the iNKT-cell-mediated regulation of T-cell lineage development. Moreover, by comparing IL-17 production by antigen-experienced T cells from unmanipulated wild-type mice and iNKT-cell-deficient mice, we demonstrate an enhanced Th_{17} response in mice lacking iNKT cells. This invigorated Th_{17} response reverts to physiological levels when iNKT cells are introduced into $J\alpha18^{-/-}$ mice by adoptive transfer, indicating that iNKT cells control the Th_{17} compartment at steady state. We conclude that iNKT cells play an important role in limiting development of the Th_{17} lineage and suggest that iNKT cells provide a natural barrier against Th_{17} responses.

autoimmune encephalomyelitis | immune regulation | iNKT cells | multiple sclerosis

To respond to diverse microbial infections, T cells differentiate into functionally distinct subsets that secrete unique combinations of cytokines (1). Recently, a novel subset of T helper (Th) cells was identified—called Th_{17} cells—that produce IL-17A, IL-17F, and IL-22 (2). Th_{17} cells protect the host against extracellular pathogens encountered at mucosal surfaces, but they also play a detrimental role in experimental models of multiple sclerosis, as well as in human inflammatory bowel disease and psoriasis (2). In mice, cytokines TGF-β and IL-6 initiate the differentiation of T cells into the Th_{17} lineage by inducing expression of a transcription factor called retinoic acid receptor-related orphan nuclear receptor (ROR)-γt and of the IL-23 receptor (3–6). IL-21 and IL-23 further support the differentiation of Th_{17} cells, permitting IL-22 expression (6–10). In addition to providing autocrine support to the cells that produce them, some lineage-specific cytokines can impede the development of other Th lineages. For example, IL-21 secretion by Th_{17} cells inhibits Th_1 cytokines (11), whereas the reciprocal regulation of Th_1 and Th_2 responses is mediated by IL-4 and IFN-γ (12), both of which antagonize Th_{17} differentiation (13, 14). Upon pathogen encounter, innate immune cells critically influence Th differentiation. For example, triggering of Toll-like receptors (TLRs) on dendritic cells (DCs) drives IL-12 production, which favors Th_1 differentiation. Similarly, upon engagement of their receptors dectin-1 or nucleotide oligomerization domain 2 (Nod2), DCs produce IL-6 and IL-23, which orientate T cells to the Th_{17} lineage (15–17). Other cells of the innate immune system that influence Th differentiation include invariant natural killer T cells (iNKT), a CD1d-restricted T-cell population that expresses an invariant T-cell receptor α-chain by using a Vα14-Jα18 rearrangement in mice and a Vα24-Jα18 rearrangement in humans. Like conventional T cells, iNKT cells comprise distinct functional subsets. Notably, $NK1.1^{pos}$ iNKT cells produce IL-4 and IFN-γ, whereas $NK1.1^{neg}$ iNKT cells produce IL-17 and IL-21 (18–21). The iNKT cell response is elicited by the presentation of pathogen-derived or endogenous glycolipid antigens by CD1d-expressing antigen-presenting cells (APCs) (22). iNKT cells can support and sustain Th_1 responses by activating NK cells, which secrete IFN-γ, thus facilitating DC maturation and IL-12 production (22). Under different inflammatory conditions, iNKT cell activation favors Th_2 differentiation by producing IL-4, thereby conferring clinical benefit in animal models of organ-specific autoimmune diseases (23–27).

The ability of iNKT cells to regulate the differentiation of conventional Th_1 and Th_2 cells raises the question of whether they might also influence the Th_{17} lineage. On one hand, the $NK1.1^{neg}$ iNKT cell subset that produces IL-17 and IL-21 might support and sustain Th_{17} responses (18–21); on the other hand, the clinical benefit provided by the activation or enrichment of iNKT cells in Th_{17}-driven models of organ-specific autoimmune diseases suggests that iNKT cells might control pathogenic Th_{17} responses (23–26, 28, 29). This study investigated the influence of iNKT cells on the Th_{17} response. We found that activation of iNKT cells in vivo impedes commitment of naïve $CD4^+$ T cells to the Th_{17} lineage. Moreover, when comparing the Th_{17} compartment between wild-type and iNKT-cell-deficient mice, we found that antigen (Ag)-experienced $CD4^+$ T cells produce more IL-17 when they are isolated from iNKT-cell-deficient mice ($J\alpha18^{-/-}$). This invigorated Th_{17} response in the $J\alpha18^{-/-}$ mice can be corrected by the adoptive transfer of iNKT cells. Consequently, our data reveal that iNKT cells control the Th_{17} lineage in healthy, unmanipulated mice and illustrate the importance of iNKT cells both in regulating Th_{17} lineage development and in the persistence of the Th_{17} population.

Results and Discussion

iNKT Cells Regulate IL-17 Production by $CD4^+$ T Cells. To evaluate the influence of iNKT cells on Th_{17} cells, we first compared the capacity of $CD4^+$ T cells from wild-type and iNKT-cell-deficient

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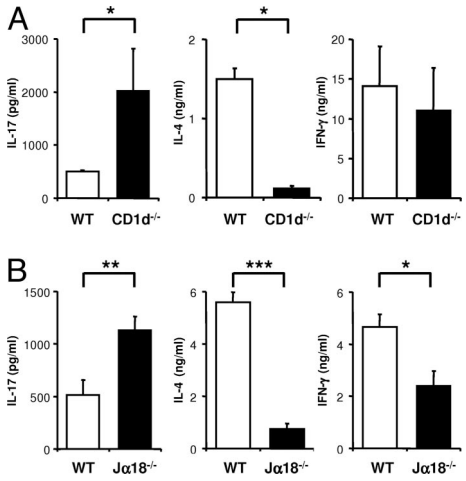

Fig. 1. iNKT cells negatively regulate IL-17 production by $CD4^+$ T cells. Purified $CD4^+$ T cells were stimulated for 72 h with plate-bound anti-CD3 and anti-CD28 mAbs and IL-23. (*A*) Release of IL-17 (*Left*), IL-4 (*Middle*), and IFN-γ (*Right*) by $CD4^+$ T cells from (*A*) wild-type (WT) or $CD1d^{-/-}$ C57BL/6 mice or (*B*) wild-type (WT) or $J\alpha18^{-/-}$ C57BL/6 mice was assayed by ELISA. Each data point represents the mean ± SEM of 2 (*A*) or 3 (*B*) experiments.

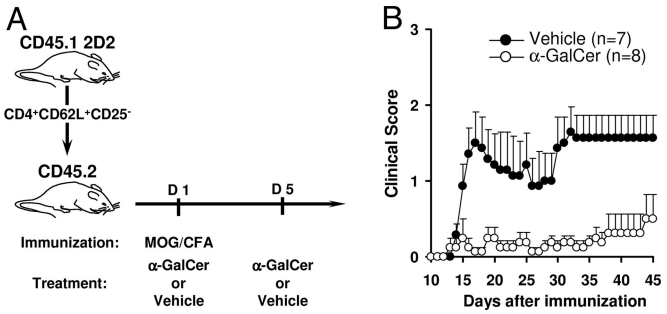

Fig. 2. In vivo treatment with α-GalCer prevents EAE. (*A*) The 2D2 transfer model uses traceable MOG-specific naïve $CD4^+$ T cells purified from CD45.1 congenic 2D2 mice. Twenty-four hours after transfer of 10^5 $CD62L^+CD25^-$ $CD4^+$ T cells, the recipient CD45.2 C57BL/6 mice were immunized with MOG_{35-55} in the presence either of α-GalCer or the PBS/DMSO solvent (vehicle). (*B*) The average clinical score from α-GalCer-treated mice (open circles, $n = 8$) and vehicle-treated controls (filled circles, $n = 7$) from 2 independent experiments is presented.

($CD1d^{-/-}$, $J\alpha18^{-/-}$) animals to produce IL-17. Splenic $CD4^+$ T cells purified from unmanipulated C57BL/6 mice were stimulated for 3 days with plate-bound anti-CD3 and anti-CD28 mAbs in the presence of IL-23. Under such APC-free conditions, IL-23 promoted the expansion of preexisting IL-17-producing cells, but did not permit de novo Th_{17} differentiation of naïve $CD4^+$ T cells (4, 6, 30). $CD4^+$ T cells from the iNKT-cell-deficient $CD1d^{-/-}$ mice produced up to 3-fold more IL-17 than $CD4^+$ T cells from wild-type mice (Fig. 1*A*), suggesting that a CD1d-dependent T-cell population controls the Th_{17} lineage. By contrast, the production of IL-4 was significantly reduced in $CD4^+$ T-cell cultures from $CD1d^{-/-}$ mice, indicative of the absence of iNKT cells, which produce large amounts of IL-4 in response to TCR stimulation (31). To unequivocally assess the implication of CD1d-restricted iNKT cells we determined the cytokine production of stimulated $CD4^+$ T cells from $J\alpha18^{-/-}$ mice that selectively lack iNKT cells. Similar to the phenotype observed with $CD1d^{-/-}$ mice, $CD4^+$ T cells from $J\alpha18^{-/-}$ mice produced more IL-17 as compared with $CD4^+$ T cells from wild-type mice (Fig. 1*B*), confirming that iNKT cells limit IL-17 production in vitro. Stimulated $CD4^+$ T cells from $J\alpha18^{-/-}$ mice also produced less IL-4 than their wild-type counterparts (Fig. 1*B*) compatible with the absence of iNKT cells. A less pronounced reduction in IFN-γ secretion was equally observed. To assess if the impact of iNKT cells on the Th_{17} lineage extends to other mouse strains we compared the cytokine profile of stimulated $CD4^+$ T cells from wild-type and $J\alpha18^{-/-}$ NOD mice, and observed a comparable inhibition of IL-17 secretion in the presence of iNKT cells (NOD: 864 ± 110 pg/ml vs. $J\alpha18^{-/-}$: 2707 ± 379 pg/ml; $P = 0.03$). Interestingly, the $CD4^+$ T-cell cultures from NOD mice produced more IL-17 than C57BL/6-derived cultures, potentially reflecting the autoimmune process developing in NOD mice.

Activating iNKT Cells in Vivo Inhibits Autoantigen-Specific Th_1 and Th_{17} Responses. To investigate the influence of iNKT cells on the Th_{17} lineage in vivo, we used the mouse model of experimental autoimmune encephalomyelitis (EAE), in which immunization with a peptide fragment of myelin oligodendrocyte glycoprotein (MOG) induces myelin-specific Th_1 and Th_{17} cells that synergistically contribute to the demyelinating lesions in the CNS (32, 33). In this model, activation of iNKT cells ameliorates the disease by reducing the intensity of the pathogenic T-cell response (24–26). To study the differentiation of autoreactive T cells at the single-cell level, we introduced a traceable population of naïve MOG_{35-55}-specific $CD4^+$ T cells into the mice before inducing EAE (Fig. 2*A*). These $CD62L^+CD25^-$ autoreactive $CD4^+$ T cells were purified from CD45.1 congenic, 2D2 transgenic mice expressing an $I\text{-}A^b$-restricted TCR ($V\alpha3.2\text{-}V\beta11$) specific for MOG_{35-55} on $CD4^+$ T cells. These transferred MOG-specific T cells underwent an expansion of up to 3,700-fold, 9 days after immunization with MOG_{35-55} (Fig. S1). Analysis of the intracellular cytokine profile of the congenic ($CD45.1^+$) MOG-specific T cells by FACS enabled us to quantify IFN-γ-producing Th_1 and IL-17-producing Th_{17} cells (Fig. S1*A*). Both the Th_1 and Th_{17} responses peaked 9 days after immunization in the spleen (Fig. S1*B*) and draining lymph nodes (LNs; data not shown), but the number of Th_{17} cells was significantly inferior to that of Th_1 cells. These mice developed moderate clinical signs of EAE, on average, 17 days after immunization (Fig. 2).

To validate the regulatory capacity of iNKT cells in this model, we treated immunized mice with 2 injections of α-GalCer (Fig. 2*A*), an exogenous glycolipid that closely resembles the microbial glycosphingolipids that are agonists of iNKT cells (34). These injections significantly reduced the severity of EAE when compared with the vehicle-injected control mice (Fig. 2*B*). In vitro recall responses to MOG_{35-55} revealed that α-GalCer injections inhibited the antigen-specific secretion of IL-17 (Fig. 3*A*), IFN-γ, and TNF-α (data not shown) by splenocytes. This was not due to loss of MOG-specific $CD45.1^+$ $CD4^+$ T cells, which were only slightly reduced after α-GalCer treatment (Fig. 3*B*). Instead, activation of iNKT cells with α-GalCer markedly reduced both the frequency (Fig. 3 *C* and *D*) and the absolute numbers (vehicle: $17 \pm 6 \times 10^3$ vs. α-GalCer: $2 \pm 1 \times 10^3$; $P = 0.001$) of Th_{17} cells as assessed by analysis of intracellular cytokines in the transferred $CD45.1^+$ $TCRV\alpha3.2^+$ $CD4^+$ T cells. A similar inhibition of Th_{17} differentiation was observed amongst endogenous $CD45.1^-$ $CD4^+$ T cells (data not shown). Interestingly, although the activation of iNKT cells also reduced the frequency (Fig. 3*E*) and slightly reduced the absolute numbers (vehicle: $34 \pm 12 \times 10^3$ vs. α-GalCer: $8 \pm 2 \times 10^3$; $P = 0.1$) of Th_1 cells, its impact on the Th_1 response appeared more modest than on the Th_{17} response. This inhibition of the MOG-specific Th_{17} and Th_1 responses is attributable to iNKT cells because α-GalCer treatment of $J\alpha18^{-/-}$ mice had no significant influence on the expansion and differentiation of autoreactive T cells (Fig. 3 *B*, *D*, and *E*). Similar results were obtained with cells from the draining LNs, where α-GalCer

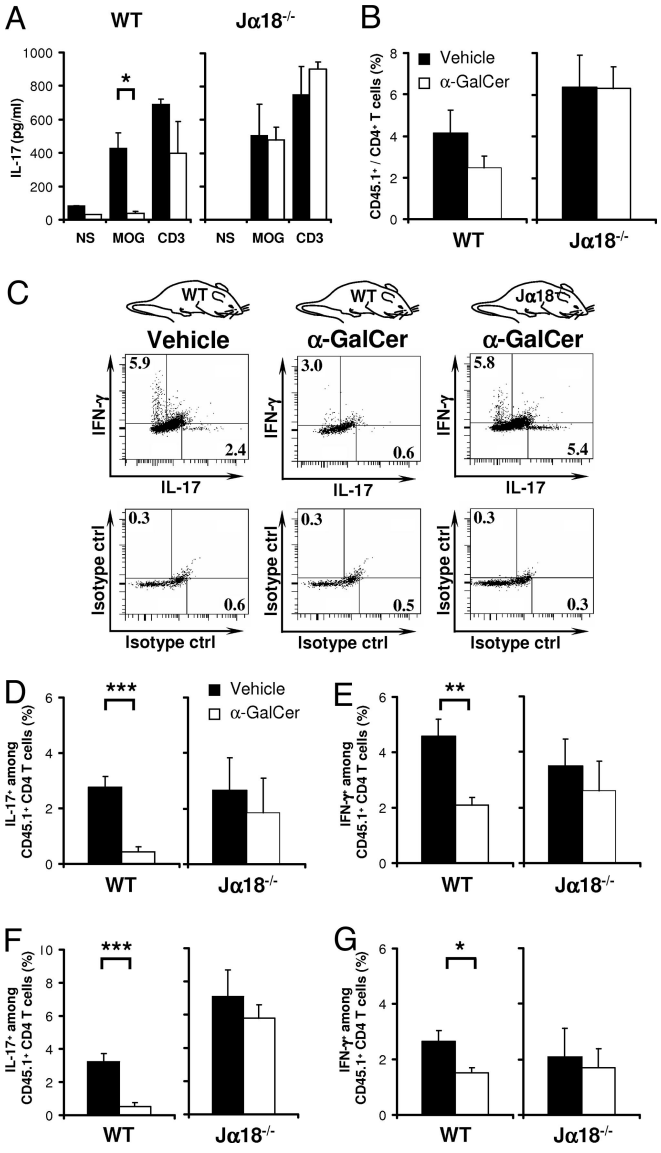

Fig. 3. Activation of iNKT cells by α-GalCer blocks the commitment of naïve MOG-specific T cells to the Th_1 and Th_{17} lineages. By using the 2D2 transfer model described in Fig. 2, we assessed MOG-specific T-cell differentiation in immunized C57BL/6 (WT) and $J\alpha18^{-/-}$ recipient mice treated with α-GalCer or vehicle. (*A*) Nine days after immunization, we assessed the in vitro recall response by splenocytes from α-GalCer-treated recipients (white bars; WT, $n = 4$; $J\alpha18^{-/-}$, $n = 4$) or vehicle-treated recipients (black bars; WT, $n = 4$; $J\alpha18^{-/-}$, $n = 4$). IL-17 release was measured from unstimulated (NS), MOG_{35-55}-stimulated (MOG; 100 μg/ml), and anti-CD3 mAb-stimulated (CD3) cultures after 72 h. (*B*) The number of $CD45.1^+TCRV\alpha3.2^+$ $CD4^+$ T cells was established in mice treated with α-GalCer (white bars; WT, $n = 11$; $J\alpha18^{-/-}$, $n = 4$) or vehicle (black bars; WT, $n = 11$; $J\alpha18^{-/-}$, $n = 4$). α-GalCer slightly reduced the proportion of 2D2 $CD45.1^+TCRV\alpha3.2^+$ $CD4^+$ T cells in the spleens of WT but not $J\alpha18^{-/-}$ mice. (*C–G*) The MOG-specific Th_{17} and Th_1 differentiation was analyzed by establishing the intracellular IL-17 and IFN-γ production of $CD45.1^+TCRV\alpha3.2^+$ $CD4^+$ T cells from WT and $J\alpha18^{-/-}$ recipient mice treated with α-GalCer or vehicle. Th_{17} differentiation is indicated by the frequency of IL-17-producing 2D2 T cells in the spleen (*C* and *D*) and draining LNs (*F*). Th_1 differentiation is indicated by the frequency of IFN-γ-producing 2D2 T cells in the spleen (*C* and *E*) and draining LNs (*G*). Intracellular cytokine analyses were also performed after antigen-specific restimulation with MOG_{35-55} and revealed a similar inhibition of Th_1 and Th_{17} responses by α-GalCer treatment, although the number of cytokine-positive cells was generally lower. Cytokine secretion in (*A*) presents 1 representative experiment of 3 performed. Intracellular cytokine analyses in (*B–G*) present 3 independent experiments for the WT mice and 1 experiment for $J\alpha18^{-/-}$ mice.

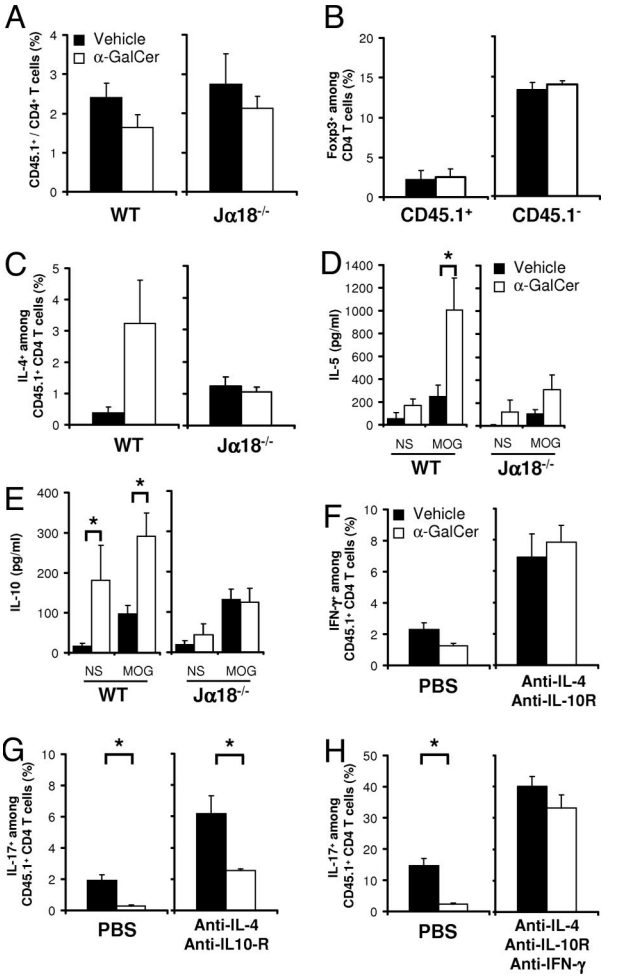

Fig. 4. Activation of iNKT cells by α-GalCer regulates the Th_1 and Th_{17} responses by immune deviation and IL-10 production. By using the 2D2 transfer model described in Fig. 2, we assessed MOG-specific T-cell differentiation in the draining LNs of immunized C57BL/6 (WT) and $J\alpha18^{-/-}$ mice. Nine days after immunization, the intracellular IFN-γ, IL-17, IL-4, and IL-10 expression by $CD45.1^+TCRV\alpha3.2^+$ $CD4^+$ T cells was assessed in α-GalCer-treated mice (white bars; WT, $n = 11$; $J\alpha18^{-/-}$, $n = 4$) and vehicle-treated mice (black bars; WT, $n = 11$; $J\alpha18^{-/-}$, $n = 4$). (*A*) The effect of α-GalCer on the proportion of 2D2 $CD45.1^+TCRV\alpha3.2^+$ $CD4^+$ T cells in the draining LNs of recipient mice. (*B*) The frequency of Foxp3-positive cells among 2D2 $CD45.1^+TCRV\alpha3.2^+$ and endogenous $CD45.1^-$ $CD4^+$ T cells. (*C*) The proportion of IL-4-positive cells among 2D2 $CD45.1^+TCRV\alpha3.2^+$ $CD4^+$ T cells increased upon α-GalCer treatment of WT, but not $J\alpha18^{-/-}$ mice, indicating an enhanced Th_2 differentiation in the presence of iNKT cells but not in their absence. (*D* and *E*) The release of IL-5 (D) and IL-10 (*E*) during the in vitro recall response was assessed in α-GalCer-treated mice (white bars; WT, $n = 4$; $J\alpha18^{-/-}$, $n = 4$) or vehicle-treated mice (black bars; WT, $n = 4$; KO, $n = 4$). Cytokine release in unstimulated (NS) and MOG_{35-55}-stimulated (100 μg/ml) cultures was measured after 72 h by Luminex immunoassay. (*F–H*) The role of IL-4, IL-10, and IFN-γ in regulating T-cell differentiation was evaluated in vivo by injecting neutralizing mAbs. Th_1 (*F*) and Th_{17} (G-H) commitment was assessed in mice treated with vehicle or α-GalCer that had received mAbs neutralizing IL-4 and IL-10R (*F* and *G*), or IL-4, IL-10R, and IFN-γ (*H*). The single-cell analysis presents 3 separate experiments for the WT mice (*A* and *C*) and a single experiment for the $J\alpha18^{-/-}$ mice (*A* and *C*). For the cytokine secretion (*D* and *E*), 1 representative experiment of 2 is shown. The cytokine neutralization data (*F–H*) are from 1 experiment involving 4 individual mice per group for each neutralization protocol.

treatment influenced expansion only modestly (Fig. 4*A*), but blunted IFN-γ and IL-17 production by MOG-specific $CD4^+$ T cells in an iNKT-cell-dependent manner (Fig. 3 *F* and *G* and Fig. S2). Taken together, our data show that activation of iNKT cells

in vivo inhibits differentiation of naïve autoreactive $CD4^+$ T cells into pathogenic Th_1 and Th_{17} effector cells, and that the inhibitory effect is most pronounced on the Th_{17} lineage.

iNKT Cell-Induced IL-4 and IL-10 Inhibit the Th_1 Response, and IL-4, IL-10, and IFN-γ Are Required to Control the Th_{17} Response. The therapeutic efficacy of α-GalCer in EAE depends on the production of anti-inflammatory cytokines IL-4 and IL-10 (24, 25). To assess whether α-GalCer promotes the commitment of $CD4^+$ T cells to the IL-4-producing Th_2 or IL-10-producing Tr_1 lineages, we analyzed the intracellular cytokine-profile of the MOG-specific T-cell response. In addition, given the reciprocal relationship between Th_{17} and inducible regulatory T cells (3, 4, 35), we enumerated the frequency of $Foxp3^+$ $CD4^+$ T cells. No variation in the proportion of $Foxp3^+$ $CD4^+$ T cells was observed among congenic 2D2 T cells and in the endogenous $CD4^+$ T-cell pool (Fig. 4*B*). Evidence of immune deviation was obtained, as the proportion of IL-4-producing congenic 2D2 $CD4^+$ T cells (and endogenous Th_2 cells; data not shown) augmented significantly in α-GalCer-treated wild-type mice when compared with the vehicle-treated mice (Fig. 4*C* and Fig. S2), and their restimulation in vitro is associated with elevated levels of the Th_2-associated cytokine IL-5 (Fig. 4*D*). By contrast, α-GalCer treatment of J$\alpha18^{-/-}$ mice had no effect on the frequency of Th_2 cells and their associated cytokines (Fig. 4 *C* and *D*), indicating that the Th_2 bias seen in wild-type mice depended on iNKT cells. Interestingly, an enhanced production of IL-10 was observed in cultures from α-GalCer-treated mice (Fig. 4*E*) irrespective of $MOG_{35–55}$ restimulation. In addition, no detectable increase in the frequency of IL-10-producing MOG-specific 2D2 T cells (Fig. S2) was observed, suggesting that iNKT cells can promote IL-10 production by non-T cells.

To assess whether IL-4 and IL-10 contributed to the inhibition of Th_1 and Th_{17} differentiation by the in vivo α-GalCer treatment, we neutralized the effects of both IL-4 and IL-10 with mAbs injected i.p. Under these cytokine-blocking conditions, the proportion of Th_1 2D2 cells tripled and the regulatory effect of iNKT cell activation by α-GalCer on Th_1 cells was fully blocked (Fig. 4*F* and Fig. S3*A*). Concomitantly, the proportion of Th_{17} cells increased. Nevertheless, α-GalCer treatment still significantly limited the Th_{17} response (Fig. 4*G* and Fig. S3*A*). Given the capacity of iNKT cells to produce copious amounts of IFN-γ (22), the increase in IFN-γ-producing Th_1 cells after IL-4 and IL-10R neutralization, and the capacity of IFN-γ to antagonize Th_{17} differentiation (13, 14), we asked whether IFN-γ could be an additional factor involved in the regulation of Th_{17} commitment by iNKT cells. Neutralizing IFN-γ in vivo doubled the proportion of Th_{17} 2D2 $CD4^+$ T cells in both PBS and α-GalCer-treated mice (Fig. S3*B*). Nevertheless, α-GalCer remained efficient in reducing the frequency of Th_{17} cells, indicating that IFN-γ is necessary but not sufficient for the iNKT-cell-mediated regulation of the Th_{17} response (Fig. S3*A*). Importantly, neutralizing IL-4, IL-10, and IFN-γ simultaneously tripled the proportion of Th_{17} cells and fully abrogated the capacity of α-GalCer to inhibit Th_{17} commitment (Fig. 4*H* and Fig. S3*A*). Blocking IL-4 + IL-10R and/or IFN-γ also prevented the EAE-protective effect of α-GalCer, rendering these mice susceptible to severe disease (Table S1). We conclude that iNKT-cell-induced IL-4 and IL-10 production mediates the inhibition of the Th_1 response, whereas the regulation of Th_{17} commitment by iNKT cells requires IL-4, IL-10, and IFN-γ.

iNKT Cells Regulate the Th_{17} Compartment at Steady State. Having established that iNKT cells control Th_{17} differentiation, we aimed to assess whether this cellular mechanism is of importance in controlling the Th_{17} compartment under steady-state conditions in vivo. To this end, we compared IL-17 production by Ag-experienced $CD4^+$ T cells from wild-type and J$\alpha18^{-/-}$ mice in vitro. Splenic $CD62L^-$ $CD4^+$ T cells were purified by FACS sorting. In addition, using the CD1d-αGalCer tetramer we eliminated iNKT cells to exclude their potential in vitro effects. Polyclonal activation in vitro revealed a 2-fold enhancement of IL-17 production when the Ag-experienced T cells were isolated from J$\alpha18^{-/-}$ mice when compared with those isolated from wild-type mice (Fig. 5*A*). No influence on the IFN-γ response was observed (cells from wild-type mice produced 3,150 pg/ml IFN-γ, those from J$\alpha18^{-/-}$ mice produced 3,155 pg/ml IFN-γ). IL-17 production by Ag-experienced $CD4^+$ T cells was not affected by neutralization of IL-4 and IFN-γ in vitro (Fig. 5*B*), consistent with the reported resistance of committed Th_{17} cells to IL-4- and IFN-γ-mediated regulation (13, 14). These findings suggest that iNKT cells control the Th_{17} compartment in vivo at steady state as they limit the IL-17-producing capacity of Ag-experienced $CD4^+$ T cells.

To investigate this novel function of iNKT cells in vivo, we reconstituted iNKT-deficient nonirradiated J$\alpha18^{-/-}$ mice with iNKT cells by adoptive transfer of 10^6 CD1d:α-GalCer tetramer-positive TCR-$\alpha\beta$ thymocytes from wild-type mice. We assessed the impact of this iNKT cell reconstitution on the Th_{17} population after 21 days, a period that is probably insufficient for the Th17 compartment to be replenished by de novo activated naïve T cells at steady state. Thus, the data likely reflect the impact of the introduced iNKT cells on preexisting Ag-experienced cells. CD1d:α-GalCer tetramer staining revealed efficient grafting of wild-type iNKT cells in J$\alpha18^{-/-}$ mice as indicated by the 4% frequency of CD1d:α-GalCer tetramer-positive cells among liver mononuclear cells (Fig. 5*C*). Strikingly, this grafting of wild-type iNKT cells fully reversed the enhanced IL-17 production by $CD4^+$ T cells from J$\alpha18^{-/-}$ mice (Fig. 5*D*), demonstrating that iNKT cells control the Th_{17} compartment at steady state. This effect was specific for the Th_{17} lineage because we observed no alteration in IFN-γ production by the Th_1 compartment (Fig. 5*E*). No reciprocal induction of $Foxp3^+$ regulatory T cells was observed after iNKT-cell reconstitution (data not shown). To address whether the transferred iNKT cells impacted the frequency of Th_{17} cells in vivo we enumerated the proportion of IL-17-producing $CD4^+$ T cells using intracellular cytokine staining. The reconstitution of iNKT cells reduced the frequency of Th_{17} cells almost to the level observed in unmanipulated WT mice (Fig. 5*F*). These splenic $CD4^+$ T-cell cultures from reconstituted mice express on average 0.05% of iNKT cells (Fig. 5*C*), making an in vitro effect by iNKT cells unlikely. As such, our findings reveal a previously undescribed function for iNKT cells in regulating the size of the Th_{17} pool in vivo at steady state.

Conclusions. Th_{17} cells contribute to the host defense against extracellular pathogens, but excessive Th_{17} responses or those targeting self-antigens can provoke severe tissue injury (2, 36). Mechanisms controlling the Th_{17} subset might therefore be important to preserve the integrity of tissues. Our study reveals the importance of iNKT cells in regulating Th_{17} lineage commitment and persistence. We show that activation of iNKT cells with α-GalCer during priming of the $CD4^+$ T-cell response prevents the differentiation of naïve $CD4^+$ T cells toward the Th_{17} lineage without impairing T-cell expansion. The mechanism used by iNKT cells to abrogate Th_{17} commitment by naïve $CD4^+$ T cells requires IL-4, IL-10, and IFN-γ. No reciprocal influence on $Foxp3^+$ T cells was observed. After α-GalCer treatment IL-4 expression is likely initiated by iNKT cells and sustained by autoreactive Th_2 cells that regulated the Th_{17} response. α-GalCer activation of iNKT cells and the ensuing NK response are expected sources of IFN-γ, with a moderate impact of IFN-γ by the conventional Th_1 response due to its efficient inhibition by α-GalCer. Our data, therefore, suggest an important role for innate IFN-γ in regulating Th_{17} development. These mechanisms were effective in limiting CNS tissue damage in the paralytic mouse model of EAE, in which autoimmune Th_1 and

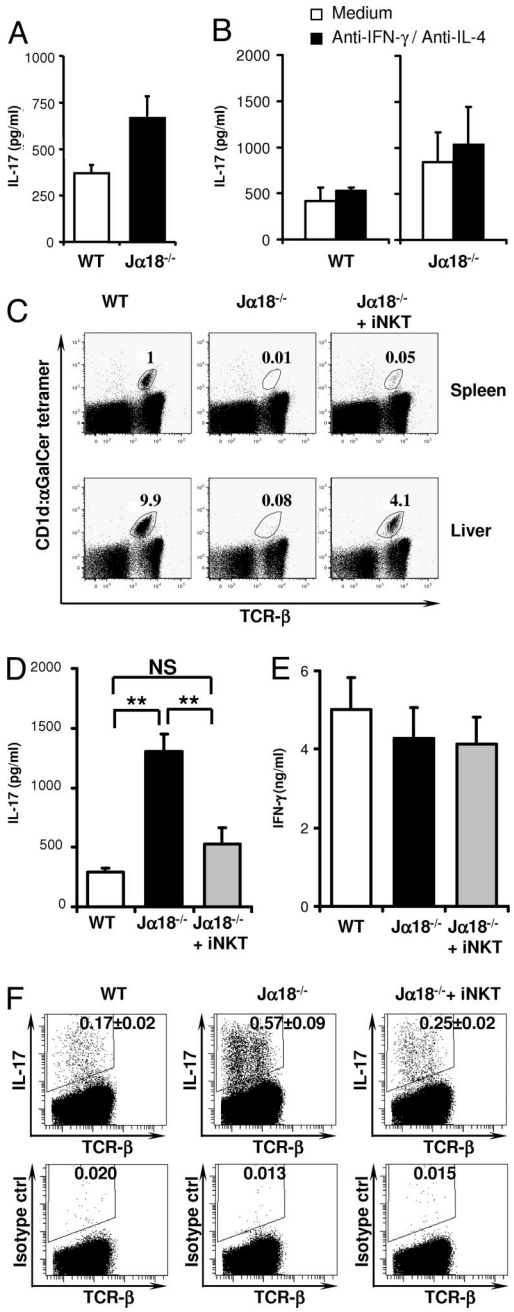

Fig. 5. iNKT cells inhibit IL-17 production by $CD4^+$ T cells at steady state. (*A*) $CD62L^-$ α-GalCer-CD1d $tetramer^{neg}$ $CD4^+$ T cells from wild-type (WT) or $J\alpha18^{-/-}$ C57BL/6 mice were sorted by FACS and stimulated with plate-bound anti-CD3 and anti-CD28 mAbs and IL-23. IL-17 levels were measured after 72 h. (*B*) As in (*A*) but in the presence or absence of neutralizing mAbs against IL-4 and IFN-γ. These mAbs failed to significantly enhance IL-17 production by Ag-experienced $CD4^+$ T cells. Data are from 3 (*A*) or 2 (*B*) individual experiments using 2–4 pooled spleens per group. (*C*) $J\alpha18^{-/-}$ recipient mice received thymic iNKT cells ($J\alpha18^{-/-}$ + iNKT), and 3 weeks later the proportion of the CD1d:α-GalCer $tetramer^+$ $TCR\beta^+$ (iNKT) cells (gated on mononuclear cells) in the spleen (*Upper*) and liver (*Lower*) of recipient mice was compared with that of the control wild-type (WT) and non-reconstituted $J\alpha18^{-/-}$ mice. A representative FACS plot is shown for each group. (*D–G*) $J\alpha18^{-/-}$ recipient mice received thymic iNKT cells as in (*C*). Three weeks later, MACS-purified splenic $CD4^+$ T cells from the recipient and control mice were cultured with plate-bound anti-CD3 and anti-CD28 mAbs and IL-23. IL-17 (*D*) and IFN-γ (*E*) secretion were measured 72 h later in cultures derived from recipient ($J\alpha18^{-/-}$ + iNKT, $n = 6$) and control (WT, $n = 6$; $J\alpha18^{-/-}$, $n = 6$) mice. The frequency of Th_{17} cells (*F*) was assessed under identical conditions in cultures derived from iNKT-reconstituted ($J\alpha18^{-/-}$ + iNKT, $n = 2$) and control (WT, $n = 4$; $J\alpha18^{-/-}$, $n = 6$) mice. Data represent the mean ± SEM of n individual mice per group.

Th_{17} responses contribute to demyelinating lesions in the CNS (32, 33). This observation might also be of relevance for microbial infections where iNKT cells are activated by either pathogen-derived or endogenous glycolipid antigens presented in the hydrophobic groove of CD1d (37–39). It is conceivable that the activation of iNKT cells during infections might similarly influence T-cell differentiation and prevent the induction of an excessive Th_{17} response, thereby limiting secondary tissue damage.

Importantly, we show that iNKT cells also impact on the Th_{17} pool in vivo in unmanipulated mice. We reveal that iNKT cells restrain IL-17 production by $CD4^+$ T cells in both C57BL/6 and autoimmune-prone NOD mice. Strikingly, the enhanced IL-17 production observed in iNKT-cell-deficient mice was fully reversed by the transfer of thymic iNKT cells. This was associated with the capacity of the transferred iNKT cells to reduce the frequency of Th_{17} cells in vivo, restoring a Th_{17} frequency similar to that observed in WT mice. Because no effect on IFN-γ production by antigen-experienced Th_1 cells was observed, it is assumed that iNKT cells specifically control the persistence of Ag-experienced Th_{17} cells. Under steady-state conditions, iNKT cells are activated and functionally engage dendritic cells and B cells (40, 41), which is thought to be of importance to prepare the host for future pathogen encounter. Our findings suggest that, in addition, iNKT cells impact on Ag-experienced $CD4^+$ T cells at steady state, implying a role for iNKT cells in shaping the T-cell response before pathogen exposure. Given the role of the Th_{17} cell subset in inflammatory tissue damage, its confinement by iNKT cells might provide a barrier that limits predisposition to inflammatory diseases and supports iNKT cells as a therapeutic target in Th_{17} mediated diseases (42).

Materials and Methods

Mice, EAE Induction, and in Vivo Treatments. C57BL/6 mice (Charles River Laboratories), $CD1d^{-/-}$ mice (43), $J\alpha18^{-/-}$ mice (44), 2D2 MOG-TCR mice (45), and NOD mice were housed under specific pathogen-free conditions. All experimental protocols were approved by the local ethics committee and are in compliance with European Union guidelines. EAE was induced by s.c. immunization with 50 μg of MOG_{35-55} peptide (MEVGWYRSPFSRVVH-LYRNGK; Mimotopes) emulsified in CFA (Difco) and 2 i.v. injections of pertussis toxin (day 0, 200 ng; day 2, 400 ng; List Biological Laboratories). Disease severity was monitored daily (28). Where indicated, mice were treated with two 4-μg doses of α-GalCer (Kirin Brewery) on day 0 (emulsified in the MOG_{35-55} CFA mixture) and day 4 (i.p.) or with anti-IL-4 (11B11), anti-IL10R (1B1), or anti-IFN-γ (XMG1.2) mAbs injected i.p. at a dose of 0.5 mg/mouse on days −3, −2, 0, 2, 5, and 8 for 2D2 differentiation, or days −2, −1, 0, 3, (0.5 mg/mouse) and 5, 9, 13, 17, and 21 (0.15 mg/mouse) for EAE.

T-Cell Purification and Adoptive Transfer. Splenic $CD4^+$ T cells were isolated (>94% pure) by MACS positive selection (Miltenyi Biotec). Subsequently, conventional Ag-experienced $CD4^+$ (CD1d:α-GalCer $tetramer^{neg}$ $CD62L^-$ $CD4^+$) T cells were isolated by FACS (>97% pure). MOG-specific naïve $CD62L^+$ $CD25^-$ $CD4^+$ cells were purified from CD45.1 congenic 2D2 mice by MACS negative selection (Miltenyi Biotec) by using anti-CD8 (YTS169), anti-B220 (RA3–6B2), anti-Mac1 (CI:A3–1), and anti-CD25 (7D4) mAbs followed by positive selection with anti-CD62L-coated beads, providing >80% $CD4^+$ T cells, of which 95% expressed the transgenic 2D2 TCR. For reconstitution studies, freshly isolated thymocytes were enriched for iNKT cells by CD8 depletion (Invitrogen Life Technology); 3%−5% of recovered thymocytes were CD1d:α-GalCer $tetramer^+$ $TCR\beta^+$ iNKT cells. The equivalent of 1.0×10^6 iNKT cells was adoptively transferred i.v. into $J\alpha18^{-/-}$ mice.

Intracellular and Intranuclear Cytokine Staining and Flow Cytometry. Single-cell suspensions from LNs (0.5×10^6/well) or spleen (3×10^6/well) were stimulated for 4 h with PMA (0.5 μg/ml), ionomycin (1 μg/ml; Sigma), and GolgiPlug™ (BD Biosciences). After FcγR blockade (mAb 2.4G2), cells were stained with fluorescently labeled mAbs: anti-TCR Vα3.2 (RR3–16), anti-CD4 (RM4–5), anti-CD45.1 (A20), and anti-cytokine mAbs against IL-10 (JES5–16E3), IFN-γ (XMG1.2), IL-17 (TC11–18H10), IL-4 (BVD4–1D11), or IgG1 isotype control (A110–1) using a Cytofix/Cytoperm™ Plus Kit (BD Biosciences) or an intranuclear Foxp3 detection kit (eBiosciences). Labeled cells were analyzed with a

LSRII flow cytometer (Becton Dickinson) using the BD FACS-Diva software. Cell sorting was performed using a FACSAria Flow Cytometer (Becton Dickinson).

In Vitro Cytokine Production. 2.0×10^5 $CD4^+$ T cells or 0.5×10^5 Ag-experienced $CD4^+$ T cells were cultured with plate-bound anti-CD3 (1 μg/ml) and anti-CD28 (10 μg/ml) mAbs (BD Biosciences) and IL-23 (10 ng/ml; R&D Systems). When indicated, anti-IFN-γ (10 μg/ml) and anti-IL-4 (10 μg/ml) mAbs (BD Biosciences) were added. For in vitro recall responses, single-cell suspensions of LN cells (0.5×10^6/well) or splenocytes (1×10^6/well) were cultured with the indicated stimuli. Supernatants were analyzed for IFN-γ, IL-17A, or IL-4 by Duoset ELISA (R&D Systems) or tested for 10 different cytokines (IFN-γ, TNF-α, CXCL10, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, and IL-17A) by using LINCOplex Multiplex immunoassays and Luminex instrumentation (Millipore).

Statistical Analyses. Data are presented as means of *n* individual mice per group $\pm$ SEM, unless otherwise stated. The Mann-Whitney *U* test was used, except for cytokine production, which was analyzed using the unpaired Student *t* test. Kinetics of EAE development was analyzed using the log rank test; EAE incidence and mortality using a χ^2 test; and severity using the Mann-Whitney *U* test. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$.

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