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IL-9 Expression by Invariant NKT Cells Is Not Imprinted during Thymic Development

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Invariant NKT (iNKT) cell thymic development can lead to distinct committed effector lineages, namely NKT1, NKT2, and NKT17. However, following identification of IL-9–producing iNKT cells involved in mucosal inflammation, their development remains unaddressed. In this study, we report that although thymic iNKT cells from naive mice do not express IL-9, iNKT cell activation in the presence of TGF-β and IL-4 induces IL-9 secretion in murine and human iNKT cells. Acquisition of IL-9 production was observed in different iNKT subsets defined by CD4, NK1.1, and neuropilin-1, indicating that distinct functional subpopulations are receptive to IL-9 polarization. Transcription factor expression kinetics suggest that regulatory mechanisms of IL-9 expression are shared by iNKT and CD4 T cells, with *Irf4* and *Batf* deficiency deeply affecting IL-9 production. Importantly, adoptive transfer of an enriched IL-9$^+$ iNKT cell population leads to exacerbated allergic inflammation in the airways upon intranasal immunization with house dust mite, confirming the ability of IL-9–producing iNKT cells to mediate proinflammatory effects in vivo, as previously reported. Taken together, our data show that peripheral iNKT cells retain the capacity of shaping their function in response to environmental cues, namely TGF-β and IL-4, adopting an IL-9–producing NKT cell phenotype able to mediate proinflammatory effects in vivo, namely granulocyte and mast cell recruitment to the lungs. ***The Journal of Immunology*****, 2015, 195: 3463–3471.**

Natural killer T cells comprise a minor subset of T lymphocytes reactive to lipid Ags presented by CD1d on the surface of APCs and other cell types (1, 2). Ag specificity is determined by the TCR repertoire, which allows the subdivision of NKT cells into two categories: type I and type II. Type I NKT cells express a semi-invariant TCR formed by a restricted number of TCR β-chains associated with an invariant α-chain (Vα14-Jα18 in mice, Vα24-Jα18 in humans). For this reason, type I NKT cells are frequently named invariant NKT (iNKT) cells. They specifically recognize α-galactosylceramide (α-GalCer), a glycolipid that has been widely used for their reliable identification and stimulation. Type II NKT cells have a broader TCR repertoire, enabling the recognition of other lipid Ags, and they are unable to bind α-GalCer (3–5).

Despite being a minor population of lymphocytes, NKT cells are important early orchestrators of immune responses due to their remarkable capacity of secreting high levels of cytokines rapidly after their activation, including IFN-γ, IL-4, and IL-17 (6–8). Such fast production of cytokines contributes to the initial regulation of several inflammatory responses, such as the ones associated with allergic and autoimmune diseases, antitumor and mucosal-protective responses, and infection (9–13).

During thymic development, NKT cells receive signals inducing the expression of lineage-specific transcription factors, which determine their competence to become IFN-γ, IL-4, or IL-17 secretors (6–8) (referred to as NKT1, NKT2, and NKT17, respectively; reviewed in Ref. 14). Hence, NKT1 cells express T-bet, NKT2 cells express GATA-3, and NKT17-committed cells express retinoic acid–related orphan receptor γt (15).

NKT cells expressing non-classical functions have also been documented, including Foxp3$^+$ regulatory NKT cells (16, 17), follicular helper NKT cells (18–21), and NKT cells secreting further types of cytokines, such as IL-10 (22) and IL-9 (8, 23–25). Being the archetype cytokine of Th9 responses, IL-9 secretion by invariant (iNKT) cells has been investigated (25). Not only were IL-9–producing iNKT cells identified in vivo, but also these cells were reported to confer protection against DSS-induced colitis (25). However, the development of IL-9–secreting iNKT cells has never been addressed.

The ability of peripheral NKT cells to specialize in distinct functional subsets has been controversial (26, 27). However, it is clear that, in addition to thymic generation of NKT1, NKT2, and NKT17 subsets, some iNKT cells can leave the thymus uncommitted to a particular functional phenotype, namely NKT17, and adopt that phenotype when exposed to adequate inflammatory mediators (14, 28, 29). We now establish that IL-9–producing NKT cells (NKT9) do not acquire this function in the thymus, but are readily generated in mice and humans upon activation in presence of TGF-β and IL-4. Distinct functional iNKT cell subsets could become IL-9 secretors, indicating a shared responsiveness to signals instructing the acquisition of NKT9 fate. Furthermore,

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Abbreviations used in this article: BAL, bronchoalveolar lavage; α-GalCer, α-galactosylceramide; HDM, house dust mite; iNKT, invariant NKT; IRF, IFN regulatory factor; i.t., intratracheal(ly); NKT9, IL-9–producing NKT cell; Nrp-1, neuropilin-1; TSLP, thymic stromal lymphopoietin.

IL-9 production by iNKT cells aggravated allergic inflammation in mice exposed to intranasal house dust mite (HDM). Collectively, our data show that IL-9 expression is imprinted in iNKT cells only after peripheral activation under adequate inflammatory signals (IL-4 and TGF-β) and likely contributes to the initial Th9 response.

Materials and Methods

Mice

C57BL/6J (H-2^b), BALB/c (H-2^d), B6.129S-*Batf*$^{tm1.1Kmm}$/J (H-2^b, *Batf*$^{-/-}$), B6.129S1-*Irf4*^{tm1Rdf}/J (H-2b, *Irf4*^{fl}), and B6.Cg-Tg(Vav1-cre)A2Kio/J (H-2^b, Vav-iCre) mice obtained from The Jackson Laboratory (Bar Harbor, ME) and *Foxp3*^{gfp} knock-in mice (H-2^b) obtained from University of Washington (Seattle, WA) were bred and maintained under specified pathogen-free conditions at the Instituto Gulbenkian de Ciência and Instituto de Medicina Molecular. For each experiment animals were sex and age matched. All animal work complied with the European Directive 63/2010/EU, the guidelines of the Federation for the European Laboratory Animal Science Associations, and regulations of Institutional Ethical Comities.

Isolation of human peripheral blood cells

Heparinized venous blood samples were obtained from healthy volunteers of both sexes after informed consent. The procedures were reviewed and approved by the Ethical Board of the Faculty of Medicine, University of Lisbon, Portugal. PBMCs were isolated by centrifugation on a Histopaque-1077 Hybri-Max density gradient (Sigma-Aldrich) and T cells were enriched as described elsewhere (16).

Flow cytometry and cell sorting

Mouse CD1d tetramers coupled to PE and loaded with PBS57 (an iNKT cell ligand analog to α-GalCer) were supplied by the National Institutes of Health Tetramer Facility. Fluorochrome-labeled mAbs specific to mouse TCRβ (H57-597), NK1.1 (PK136), CD4 (RM4-5), CD3 (145-2C11), CCR3 (83103), Gr-1 (RB68C5), MHC class II (M5/114.15.2), c-Kit (2B8), CD19 (eBio1D3 or MB19-1), CD24 (M1/69), CD44 (IM7), IL-4 (11B11), IL-9 (RM9A4), IFN-γ (XMG1.2), and streptavidin were purchased from eBioscience, BD Biosciences, or BioLegend. IL-9 polyclonal Ab conjugated with fluorescein was provided by J. Van Snick (Ludwig Institute for Cancer Research, Brussels Branch, Cellular Genetics Unit and Experimental Medicine Unit, Christian de Duve Institute of Cellular Pathology, Université de Louvain). For intracellular cytokine detection, cells were incubated for 2 h with 50 ng/ml PMA, 500 ng/ml ionomycin, 10 μg/ml brefeldin A (Sigma-Aldrich), and BD GolgiStop (BD Biosciences). After Ab surface staining, cells were fixed and permeabilized using BD Cytofix/Cytoperm Plus (BD Biosciences) and then incubated with unconjugated anti-CD16/32 (clone 2.4G2) rat Ab and with anti-cytokine Abs. Data were collected in a BD LSRFortessa (BD Biosciences) and analyzed using FlowJo (Tree Star). For cell sorting, murine iNKT cells were first enriched by magnetic separation, as described elsewhere (16), and samples were sorted on FACSAria I (BD Biosciences).

Cell culture

FACS-sorted populations of mouse iNKT or CD4 T cells were cultured in 96-well round-bottom plates previously coated with anti-CD3 (clone 145-2C11, eBioscience) at 3 μg/ml. Culture medium was RPMI 1640 with GlutaMAX, supplemented with 10% FBS, 1% HEPES, 1% penicillin/streptomycin, 1% sodium pyruvate, 0.1% 2-ME, and 0.05% gentamicin (all from Life Technologies). For Th9 differentiation, cultures were supplemented with TGF-β (5 ng/ml, R&D Systems), IL-2 (5 ng/ml, eBioscience), IL-4 (20 ng/ml, eBioscience), and anti-CD28 (2 μg/ml, eBioscience). In some conditions, in-house–produced anti–IL-4 (11B11) or anti–TGF-β (1D11) was added to the culture. Human cells were cultured in 96-well flat-bottom plates previously coated with anti-CD3 (clone OKT3, BD Biosciences) at 1 μg/ml. Culture medium was RPMI 1640 with GlutaMAX, supplemented with 10% FBS, 1% HEPES, 1% penicillin/streptomycin, and 0.05% gentamicin (all from Life Technologies). Th9 differentiation of human cells was induced by adding TGF-β (5 ng/ml, R&D Systems), IL-4 (25 ng/ml, eBioscience), IL-2 (50 U/ml, eBioscience), anti–IL-12/23, and anti–IFN-γ (10 μg/ml, eBioscience) and anti-CD28 (1 μg/ml, eBioscience).

Cytokine detection assays

Measurement of IL-9 in culture supernatants was performed using the mouse IL-9 Flex Set (BD Biosciences). IL-4 was detected in MaxiSorp plates (Nunc) using a murine IL-4 ELISA kit (PeproTech). All assays were performed according to the manufacturers' instructions.

Adoptive cell transfer and allergic airways disease induction

Wild-type C57BL/6J mice were anesthetized, the trachea was surgically exposed, and 1×10^4 iNKT cells were injected. Mice were challenged i.n. with 25 μl PBS containing 25 μg crude extract of HDM (Greer, Lenoir, NC) plus 500 ng thymic stromal lymphopoietin (TSLP; PeproTech) daily for 5 d and sacrificed 24 h after the last challenge. Some mice received i.v. 1 mg anti–IL-9 mAb (clone 9C1, Bio X Cell) 1 d before and after iNKT cell transfer. To assess airway infiltration, the trachea was cannulated, the airways were lavaged three times with 1 ml PBS, and the bronchoalveolar lavage (BAL) was collected. Cell populations were assessed by flow cytometry. Lungs were perfused with 4% formalin solution (Sigma-Aldrich) and fixed at 4°C. Tissue samples were embedded in paraffin, sectioned, and stained with H&E. Images were taken using a Leica DM2500 microscope and a Leica DFC420 camera.

RNA extraction and RT-PCR

RNA was extracted from 1,000 to 50,000 FACS-sorted cells with an RNeasy Micro kit (Qiagen) following the manufacturer's instructions, with cells being directly sorted into RLT buffer. cDNA synthesis was performed using random primers and SuperScript III reverse transcriptase (Life Technologies). Transcripts were detected with the following primers: *Eif1a*, forward, 5′-ACACGTAGATTCCGGCAAGT-3′, reverse, 5′-AGGAGCCCTTTCCCATCTC-3′; *Il9*, forward, 5′-GACCAGCTGCTTGTGTCTCT-3′, reverse, 5′-AAGGACGGACACGTGATGTT-3′; *Gata3*, forward, 5′-CACTACCTTTGCAATGCCTG-3′, reverse, 5′-AGCTTGTAGTACAGCCCACA-3′; *Spi1* (PU.1), forward, 5′-CCAACGTCCAATGCATGACT-3′, reverse, 5′-GCATGTAGGAAACCTGGTGA-3′; *Batf*, forward, 5′-TTAGAACCATGCCTCACAGC-3′, reverse, 5′-AGCTGCGTTCTGTTTCTCCA-3′; *Irf4*, forward, 5′-AGCCAGCTGGATATCTCTGA-3′, reverse, 5′-ATAATCCCTCCAGCTCCTGT-3′. Real-time PCRs were performed using Power SYBR Green PCR master mix and the RT-PCR ViiA 7 sequence detection system (Life Technologies).

Statistical analysis

Data were analyzed using GraphPad Prism 5. The p values were calculated by a nonparametric unpaired Mann–Whitney U test, and p values < 0.05 were deemed significant.

Results

Expression of IL-9 mRNA and protein are undetectable in thymic iNKT cells

Although IL-9–producing iNKT cells were identified in the gut (25), a putative thymic commitment of these cells was not addressed. To investigate whether the genetic program leading to IL-9 expression in iNKT cells is initiated during development, we isolated thymic iNKT cells from naive C57BL/6J mice and assessed intracellular expression of cytokines ex vivo. As shown in Fig. 1A, ∼30% of iNKT cells expressed IL-4, 5% expressed IFN-γ, and 5% coexpressed both cytokines. IL-9 expression, however, was undetectable.

To increase the detection limit, we assessed by real-time RT-PCR the presence of *Il9* transcripts in thymic iNKT cells sorted either in bulk or subdivided according to their developmental stage defined by CD24 and CD44 coexpression: CD24^{hi}CD44^{lo} (stage 0), CD24^{lo}CD44^{lo} (stage 1), and CD24^{lo}CD44^{hi} (stage 2). The later subset was analyzed both unfractionated and subdivided according to CD4 and NK1.1 expression (Fig. 1B). In contrast with the predictable expression of *Il4*, *Gata3*, *Ifng*, and *Tbx21*, which were present in total iNKT cells and in the most mature subsets, expression of *Il9*, similar to *Foxp3*, was undetectable in all thymic iNKT subpopulations (Fig. 1C and data not shown).

These data indicate that IL-9 expression in iNKT cells is induced outside the thymus.

iNKT cells can be polarized toward IL-9 production

Although undetectable in naive mice, Foxp3$^+$ iNKT cells can be identified in vivo after immunization in several models, including 1) in cervical lymph nodes in experimental autoimmune

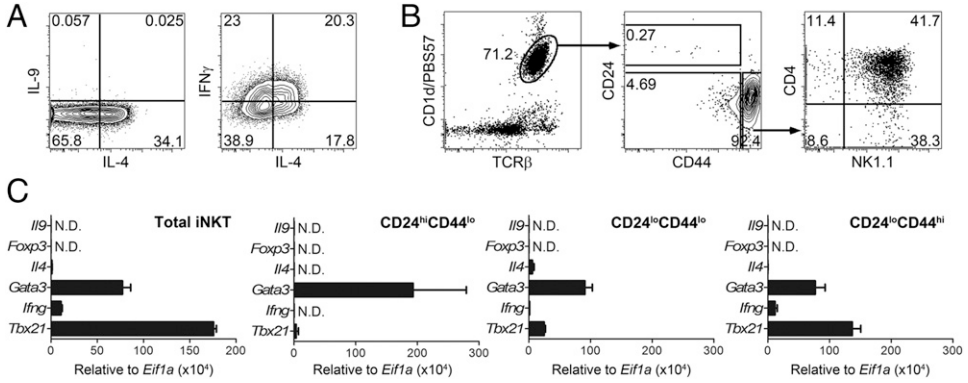

FIGURE 1. IL-9 expression by iNKT cells is undetectable in the thymus. Thymic iNKT cells were isolated from naive C57BL/6J mice, and expression of cytokines and transcription factors was assessed by intracytoplasmic staining and quantitative RT-PCR. (**A**) Intracytoplasmic cytokine expression by thymic iNKT cells following stimulation with PMA/ionomycin in presence of protein transport inhibitors. Isotype control for IL-4 and IFN-γ stainings is shown in the *left panel*. IL-9 was stained using a polyclonal Ab. (**B**) Identification of iNKT cell thymic developmental stages based on CD24 and CD44 expression after gating of CD1d/PBS57 tetramer$^+$ cells. CD24^{lo}CD44^{hi} iNKT cells were further subdivided according to expression of CD4 and NK1.1. (**C**) Relative expression of *Il9*, *Foxp3*, *Il4*, *Gata3*, *Ifng*, and *Tbx21* transcripts in unfractionated thymic iNKT cells and in iNKT cells from the distinct thymic developmental stages as defined in (B). Samples were sorted by FACS and analyzed in triplicate. Expression levels are relative to eukaryotic translation initiation factor 1A (*Eif1A*) mRNA. Error bars represent SE. Data are representative of three experiments performed independently. N.D., not detected.

encephalomyelitis, 2) in mesenteric lymph nodes following glycolipid ingestion, and 3) in the lungs during acute and chronic allergic airways disease (M. Monteiro and L. Graca, unpublished results and Refs. 14, 16). TGF-β is critical for upregulation of Foxp3 in iNKT cells and also for Th9 differentiation of conventional naive CD4 T cells. We have therefore investigated the impact of TGF-β on IL-9 expression by iNKT cells activated in vitro (Fig. 2, Supplemental Fig. 1). Purified splenic iNKT cells stimulated in the presence of IL-2 alone did not show significant expression of IL-9, but exogenous addition of TGF-β promoted massive IL-9 intracellular

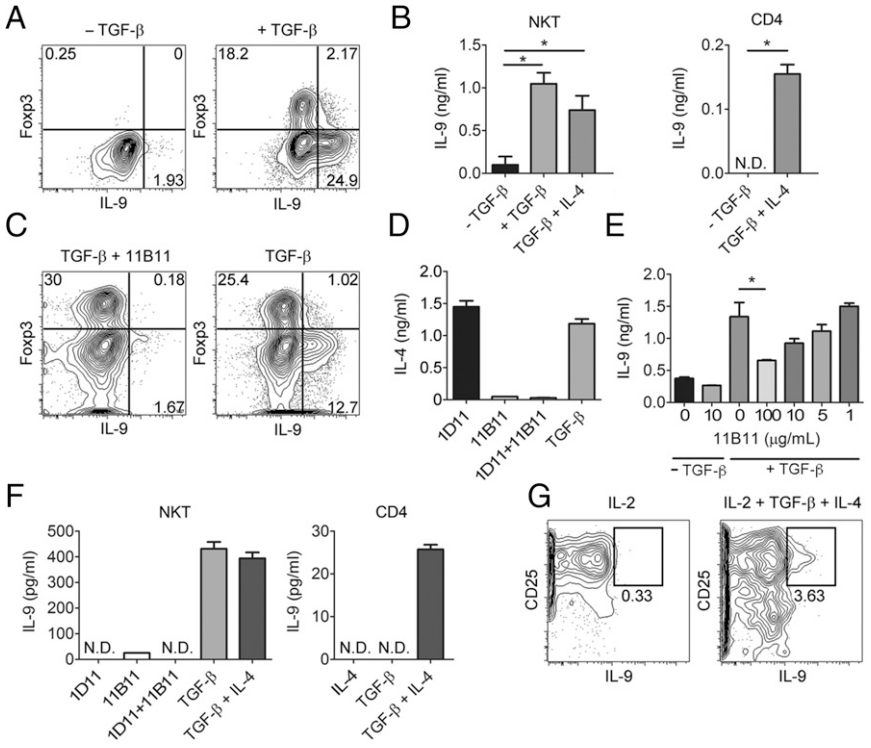

FIGURE 2. IL-9 production is induced in iNKT cells following stimulation in presence of TGF-β and IL-4. Splenic iNKT and CD25$^-$ CD4 T cells from C57BL/6 mice were purified by FACS and stimulated in vitro in the presence or absence of TGF-β and IL-4, as well as IL-4 (11B11) or TGF-β (1D11) blocking Abs, as indicated. Culture media in all conditions were supplemented with IL-2, and supernatants were harvested after 72 h of culture. (**A**) Intracellular expression of IL-9 and Foxp3 in iNKT cells cultured in the absence (*left*) or presence (*right*) of TGF-β, using an anti–IL-9 polyclonal Ab. (**B**) IL-9 concentration in supernatants of iNKT (*left*) and CD4 T cell (*right*) cultures supplemented with the indicated cytokines. (**C**) Intracellular expression of IL-9 and Foxp3 in iNKT cells stimulated under the indicated conditions, using a commercial anti–IL-9 mAb (RM9A4). (**D**) Concentration of IL-4 in the supernatant of iNKT cell cultures. (**E**) IL-9 concentration in iNKT cell culture supernatants upon stimulation in the presence or absence of TGF-β and various concentrations of neutralizing anti–IL-4 Ab (11B11). (**F**) IL-9 concentration in iNKT (*left*) and CD4 T cell (*right*) culture supernatants upon stimulation in the presence of the indicated cytokines and neutralizing Abs. (**G**) Intracellular expression of IL-9 in human CD1d/PBS57$^+$ iNKT cells after 5 d of stimulation in the presence or absence of the IL-9–inducing cytokine mixture, as indicated. Bars graphs represent the average of triplicate or quadruplicate cell cultures per assay and error bars indicate SE. Data are representative of three experiments performed independently. *$p < 0.05$. N.D., not detected.

expression and secretion (Fig. 2A–C). Notably, iNKT cells isolated from thymus and liver also responded to stimulation in presence of TGF-β with significant upregulation of *Il9* mRNA and potent IL-9 secretion (Supplemental Fig. 1).

In contrast to CD4 T cells, which required exogenous addition of IL-4 to undergo Th9 polarization, as described (30, 31) (Fig. 2B, 2F, Supplemental Fig. 1), stimulation of iNKT cells in the presence of TGF-β alone was sufficient to induce high IL-9 secretion, and exogenous IL-4 addition did not increase IL-9 levels in supernatants (Fig. 2B). However, unlike CD4 T lymphocytes, iNKT cells produce significant amounts of IL-4 upon activation (Fig. 2D). To understand whether endogenous IL-4 had an impact on IL-9 induction in iNKT cells, we added to the cultures a IL-4–blocking mAb (11B11; Fig. 2E, 2F). In these conditions, IL-9 secretion by iNKT cells decreased inversely to the concentration of anti–IL-4, confirming that IL-4 is critical for the induction of a Th9-like program in iNKT cells (Fig. 2E). Low levels of secreted IL-9 could also be detected in cultures set without exogenous TGF-β, suggesting that the medium could contain traces of TGF-β. Indeed, addition of neutralizing anti–TGF-β mAb (1D11) completely abrogated IL-9 secretion by iNKT cells (Fig. 2F). Finally, we observed that human iNKT cells also induce IL-9 production when activated under NKT9-promoting conditions (Fig. 2G).

These observations show that both mouse and human iNKT cells are able to respond to the same stimuli promoting Th9 polarization in conventional CD4 T cells, thus supporting that developmentally mature iNKT cells retain the capacity of initiating a Th9-like program in response to the inflammatory context of their activation.

Expression of IL-9 can be induced in different iNKT cell subpopulations

Subdivision of iNKT cells into distinct subsets according to CD4 and NK1.1 expression has been claimed to relate to predominant cell functions (8, 15, 32). In particular, IL-4 secretion has been associated with $CD4^+$ iNKT cells, whereas an IFN-γ–secreting phenotype has been attributed to $NK1.1^+$ iNKT cells, and IL-17 secretion to the $CD4^-NK1.1^-$ subset. We have therefore investigated whether different iNKT cell subsets defined by the expression of CD4 and NK1.1 have any restriction in their capacity of acquiring IL-9 expression (Fig. 3). The four corresponding iNKT subpopulations ($NK1.1^-CD4^+$, $NK1.1^+CD4^+$, $NK1.1^+CD4^-$, and $NK1.1^-CD4^-$) were sorted and IL-9 expression was assessed following 72 h of stimulation under NKT9-polarizing conditions (Fig. 3A–C). Both IL-9 and Foxp3 were detectable in all iNKT cell subsets stimulated in the presence of TGF-β, with $NK1.1^+CD4^+$ and $NK1.1^+CD4^-$ subpopulations presenting the highest frequency of IL-9–expressing cells and amount of IL-9 secretion (Fig. 3B, 3C).

We have also assessed the capacity of IL-9 induction in iNKT cells subdivided according to neuropilin-1 (Nrp-1) expression, a receptor expressed by recent thymic emigant iNKT cells that are enriched in NKT17 committed cells (Fig. 3D). After 72 h of stimulation in presence of TGF-β, significant amounts of IL-9 were detected in culture supernatants of both $Nrp\text{-}1^-$ and $Nrp\text{-}1^+$ iNKT cells (Fig. 3E).

Collectively, these results show that an NKT9 functional program can be induced in all iNKT cell subsets commonly associated with IL-4, IFN-γ, or IL-17 secretion.

Transcriptional control of Il9 *in iNKT cells*

Transcriptional regulation of Th9 differentiation in conventional CD4 T cells is complex, as it relies on several transcription factors, including PU.1, GATA-3, BATF, and IFN regulatory factor (IRF)-4 (31, 33–35). To identify the controlling factors of IL-9 gene expression in iNKT cells, we first defined the kinetics of IL-9 expression at protein and mRNA levels to determine the best time point for analysis. For that purpose, we purified and stimulated in vitro splenic iNKT cells in the presence of TGF-β and analyzed

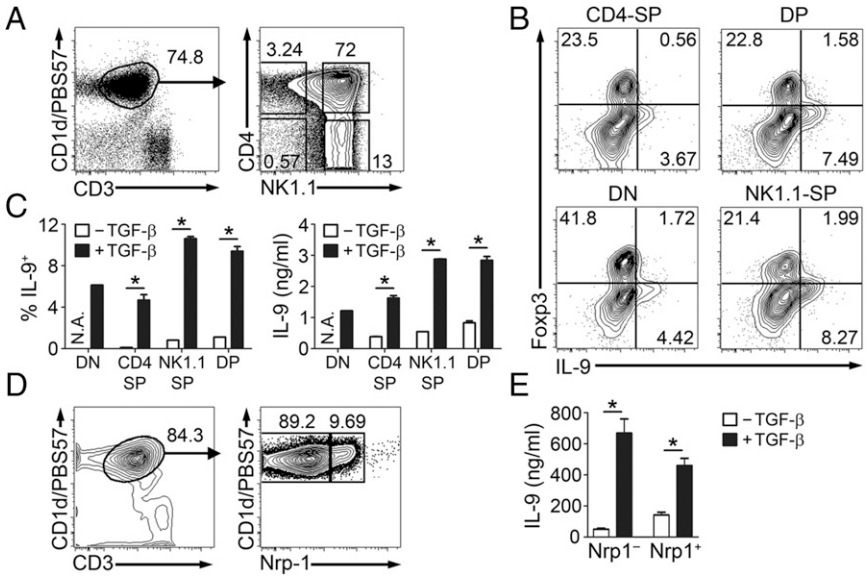

FIGURE 3. Expression of IL-9 can be induced in iNKT cell subpopulations defined by expression of NK1.1 and CD4 or Nrp-1. (**A**–**C**) Splenic iNKT cells from nonmanipulated C57BL/6 mice were sorted into four populations, according to expression of CD4 and NK1.1, and stimulated in vitro in the presence of IL-2 and TGF-β. (A) FACS plots showing the gating strategy for cell sorting. (B) Intracellular Foxp3 or IL-9 expression evaluated after 3 d of culture. (C) Frequency of $IL\text{-}9^+$ cells (*left*) and IL-9 concentration in culture supernatants (*right*). Results are representative of three independent experiments. (**D** and **E**) $Nrp\text{-}1^+$ and $Nrp\text{-}1^-$ iNKT cells were purified by FACS and stimulated in vitro in the presence of IL-2 with or without TGF-β. (D) FACS plots showing the gating strategy for cell sorting. (E) Concentration of IL-9 in culture supernatants of $Nrp\text{-}1^+$ and $Nrp\text{-}1^-$ iNKT cells after 3 d of stimulation. Bar graphs represent the average of triplicate cell cultures per assay (with the exception of $NK1.1^-CD4^-$ cultures, which had no replicates within each experiment) and error bars indicate SE. Data are representative of two (D) or three (A–C) experiments performed independently. CD4-SP, $NK1.1^-CD4^+$ (CD4 single positive); DN, $NK1.1^-CD4^-$ (double-negative); DP, $NK1.1^+CD4^+$ (double-positive); NK1.1-SP, $NK1.1^+CD4^-$ (NK1.1 single positive). $^*p < 0.05$. N.A., not assessed.

Il9 expression and IL-9 concentration in the supernatant after 12, 36, 60, and 84 h. Whereas IL-9 concentration in supernatants progressively accumulated over time in iNKT cell cultures, being detectable as soon as 36 h, *Il9* mRNA was already detectable after 12 h of culture, peaked at 36 h, and continuously decreased thereafter (Fig. 4A, 4B, *left panels*). Kinetics of *Il9* expression in CD4 T cells cultured in presence of TGF-β and IL-4 were distinct, with ∼24 h of delay at both mRNA and protein levels when compared with iNKT cells. Hence, IL-9 in CD4 T cell culture supernatants was detectable only after 60 h of stimulation, and *Il9* mRNA could be detected at 36 h, reaching the highest expression between 60 and 84 h of culture (Fig. 4A, 4B, *right panels*). Of note, both iNKT and CD4 T cells did not express *Il9* mRNA at the beginning of cultures.

iNKT cells isolated from different organs, namely thymus, spleen, and liver, upregulated *Il9* mRNA expression and secreted IL-9 to the culture supernatant in response to TGF-β with different kinetics (Supplemental Fig. 1).

We then assessed the expression of transcription factors relevant for Th9 differentiation at the peak of the *Il9* mRNA response, which corresponds to 36 and 60 h, respectively, for iNKT and CD4 T cells (Fig. 4C). After calculating their relative expression (using *Eif1a* as internal reference), the fold expression of *Sfpi1* (the gene coding for PU.1), *Batf*, *Irf4*, and *Gata3* was normalized to their corresponding expression ex vivo, that is, at the culture onset. Notably, with exception of *Gata3*, which was downregulated in iNKT cells, the expression profile of the different genes was qualitatively similar between iNKT and CD4 T cells, suggesting that *Il9* expression is regulated by the same factors in the two lymphocyte populations (Fig. 4C). Because *Irf4* was the gene showing the highest level of upregulation at the peak of *Il9* response, we studied mice with a deletion of the *Irf4* gene in the hematopoietic compartment to address the importance of IRF-4 for the induction of *Il9* gene expression in iNKT cells. These mice were generated by crossing Vav-iCre with $Irf4^{fl/fl}$ mice, in which *Irf4* deletion could be tracked through expression of enhanced GFP. Purified splenic

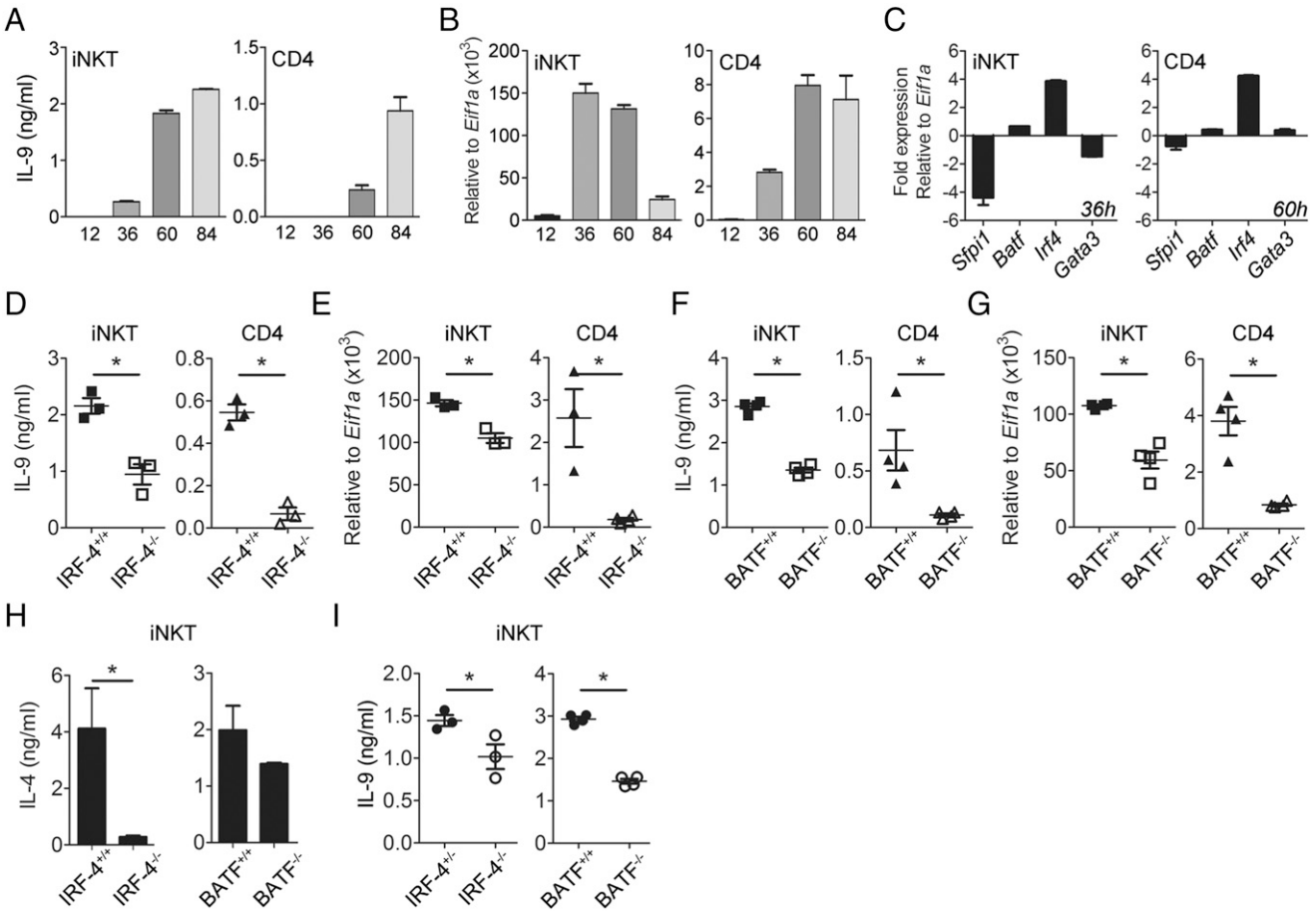

FIGURE 4. Kinetics of IL-9 expression in iNKT cells and transcription factors associated with its induction. Splenic iNKT and $CD25^-$ CD4 T cells from C57BL/6 mice were purified by FACS and stimulated in the presence of IL-2 and, respectively, TGF-β or TGF-β plus IL-4. IL-9 concentration in supernatants was measured every 24 h and cells were harvested for RNA extraction and gene expression analysis using quantitative RT-PCR. (**A**) Accumulation of IL-9 protein in the supernatant of iNKT (*left*) and CD4 (*right*) cell cultures over time. Numbers in the horizontal axes indicate hours after culture set up. (**B**) Relative amount of *Il9* transcripts in iNKT cells (*left*) and CD4 T cells (*right*) at the indicated time points. Gene expression is relative to *Eif1a* expression level. (**C**) Fold expression difference of the indicated transcription factors in iNKT and CD4 T cells at the peak of *Il9* expression in relationship to ex vivo. Gene expression was normalized to ex vivo expression levels and is relative to *Eif1a*. (**D** and **F**) Concentration of IL-9 in culture supernatants of iNKT (*left*) and CD4 T cells (*right*) isolated from IRF-$4^{+/+}$ and IRF-$4^{-/-}$ (D) or $BATF^{+/+}$ and $BATF^{-/-}$ (F) mice. Supernatants of iNKT and CD4 T cell cultures were analyzed, respectively, at 60 and 84 h. (**E** and **G**) Relative expression of *Il9* mRNA in iNKT (*left*) and CD4 T cells (*right*) isolated from IRF-$4^{+/+}$ and IRF-$4^{-/-}$ (E) or $BATF^{+/+}$ and $BATF^{-/-}$ (G) mice and analyzed, respectively, after 60 or 84 h of culture. Gene expression is relative to *Eif1a* expression level. (**H**) IL-4 concentration assessed after 60 h of culture in the supernatant of $IRF4^{+/+}$ and IRF-$4^{-/-}$ (*left*) and $BATF^{+/+}$ and $BATF^{-/-}$ (*right*) iNKT cell cultures. (**I**) IL-9 concentration assessed after 60 h of culture in supernatants of $IRF4^{+/-}$ and IRF-$4^{-/-}$ (*left*) and $BATF^{+/+}$ and $BATF^{-/-}$ (*right*) iNKT cell cultures supplemented with 10 μg/ml IL-4. Bar graphs represent the average of triplicate or quadruplicate cell cultures per assay, and error bars indicate SE. Data are representative of two (C–I) or three (A and B) independent experiments. $*p < 0.05$.

iNKT and CD4 T cells from IRF-4–sufficent and –deficient mice were cultured under Th9-polarizing conditions, as described above. Supernatants were harvested 1 d after the peak of *Il9* mRNA response. Both $Irf4^{-/-}$ iNKT and CD4 T cells had significantly lower IL-9 secretion (Fig. 4D). These results were also mirrored at mRNA level, with $Irf4^{-/-}$ cells showing fewer *Il9* transcripts (Fig. 4E).

Because *Batf* was also upregulated in iNKT cells after IL-9 induction, we assessed IL-9 expression at protein and mRNA levels in $Batf^{-/-}$ iNKT and CD4 T cells. Similarly to what was observed with *Irf4* deficiency, $Batf^{-/-}$ cells showed a marked decrease in IL-9 secretion and *Il9* mRNA expression (Fig. 4F, 4G).

To exclude that $Irf4^{-/-}$ and $Batf^{-/-}$ mice have basal defects on iNKT cell development that could bias the aforementioned results, we analyzed iNKT cells in $Irf4^{-/-}$ and $Batf^{-/-}$ naive mice (Supplemental Fig. 2). The frequency and number of iNKT cells in the spleen and liver were not significantly different in either $Irf4^{-/-}$ and $Batf^{-/-}$ mice in comparison with wild-type mice (Supplemental Fig. 2A, 2C). Furthermore, the composition of iNKT cell subsets, defined by CD4 and NK1.1 expression, was also similar in the two mouse strains (Supplemental Fig. 2B, 2D).

Because both IRF-4 and BATF are also implicated in the regulation of IL-4 expression (35, 36), the impact of *Irf4* and *Batf* deficiency on IL-9 induction could be due to an impairment of endogenous IL-4 secretion in iNKT cells. Indeed, $Irf4^{-/-}$ and $Batf^{-/-}$ iNKT cells had decreased IL-4 production (Fig. 4H). However, supplementation of culture media with exogenous IL-4 was not sufficient to rescue full secretion of IL-9, indicating that *Irf4* and *Batf* deficiency directly affect the capacity of iNKT cells to express IL-9 (Fig. 4I).

These data indicate that the genetic network controlling *Il9* expression is probably similar between iNKT and CD4 T cells.

IL-9$^+$ iNKT cells can contribute to the development of allergic inflammation

It was previously reported IL-9–producing iNKT cells can confer protection against DSS-induced colitis (25). Because iNKT cells showed an outstanding capacity of secreting elevated amounts of IL-9, and conventional Th9 CD4 T cells are known to contribute to allergic inflammation by promoting proliferation of mast cells and goblet cells, as well as influx and local maturation of eosinophils (37–39), we investigated whether iNKT cells could contribute to allergic airway inflammation. Classical mouse models of allergic airways disease were not suitable for this aim because 1) they do not allow a reliable correlation between NKT9 with any disease parameter because IL-9 expression by iNKT cells occurs early after activation and is only transient, as shown in Fig. 4, requiring iNKT cells to be analyzed several days before the initial disease manifestations; and 2) other innate cells, such as innate lymphoid cells, also produce IL-9 (40), thus making it hard to discriminate the specific contribution of iNKT cells for the early IL-9 response. To circumvent these obstacles, we induced IL-9 expression in splenic iNKT cells isolated from $Foxp3^{gfp}$ reporter mice in vitro, as described above, and after 48 h of culture we sorted Foxp3$^+$ and Foxp3$^-$ iNKT cells, considering that the latter would be enriched in NKT9 cells (according to data in Fig. 2). Wild-type C57BL/6J mice received by intratracheal (i.t.) injection 5×10^4 cells Foxp3$^+$ or Foxp3$^-$ iNKT cells and, without any further immunization, mice were sacrificed 48 h after cell transfer. This strategy allowed us to control the time of IL-9 induction in iNKT cells and ensured that the main secretion of IL-9 would come from iNKT cells. Analysis of the cellular content of the BAL revealed significant infiltration of eosinophils (defined as MHC class II$^-$Gr-1^{int}) and mast cells (defined as MHC class II$^-$Gr-1$^-$c-Kit$^+$) in the airways of recipients of NKT9 cells (Fig. 5A, 5B).

In a second approach, Foxp3$^-$ iNKT cells were generated in cultures with TGF-β (IL-9$^+$ iNKT) or under conditions adverse to Th9 polarization (without TGF-β and in presence of 11B11; activated iNKT). Cells (1×10^4) of each iNKT population were transferred i.t. to wild-type recipients subsequently challenged intranasally with HDM and TSLP) for 5 d before analysis of airways inflammation. Recipients of NKT9 cells had increased inflammatory infiltrates in the BAL, particularly eosinophils and mast cells (Fig. 5C, 5D), and histological analysis of the lungs revealed higher prevalence of peribronchiolar inflammatory infiltrates (Fig. 5E). Importantly, neutralization of IL-9 prevented the accumulation of eosinophils and mast cells in the airways (Fig. 5F, 5G). This observation shows that the inflammatory infiltrates observed following adoptive transfer of IL-9–secreting iNKT cells are due to IL-9 production (Fig. 5F, 5G).

Taken together, our results show that IL-9–secreting iNKT cells can contribute to allergic airways disease.

Discussion

Functional diversification in the thymus allows NKT cells in the periphery to rapidly execute their distinct functional imprinted programs upon TCR stimulation, namely, IFN-γ, IL-4, or IL-17 secretion. Hence, it is possible to identify NKT cells expressing basal levels of mRNA molecules coding for IFN-γ, IL-4, or IL-17 in non–immunized mice, and to detect intracytoplasmic expression of the correspondent proteins after stimulation with PMA and ionomycin (Fig. 1, Ref. 41). The ability of peripheral NKT cells to specialize in distinct functional subsets has been controversial (11, 35–38). However, Foxp3 expression and acquisition of B cell helper capacity are not found in naive mice, but arise upon immunization (16–19). These observations strongly support the view that NKT cells are capable of modulating their function in response to signals received in the periphery, which is corroborated by the fact that iNKT cells escaping retinoic acid–related orphan receptor γt imprinting in the thymus can later adopt an NKT17 phenotype and produce IL-17 when activated under Th17 proinflammatory conditions (28, 29).

We report in the present study that, despite being undetectable in non–immunized mice, IL-9 expression by iNKT cells can be readily induced upon stimulation under appropriate inflammatory conditions, namely, in the presence of TGF-β and IL-4. These are also the key cytokines promoting Th9 differentiation of conventional CD4 T cells (30, 31). The regulation of Th9 differentiation is not yet fully understood, but it is thought to rely on the balance of several transcription factors, including PU.1, BATF, IRF-4, and GATA-3 (35, 42). Except for *Gata3* mRNA, which also regulates development, survival, activation, and effector function of iNKT cells (25), the gene regulatory network more specific for the Th9 program, namely PU.1, BATF, and IRF-4, displayed a similar kinetic profile between iNKT and CD4 T cells, strongly suggesting that IL-9 expression is regulated through similar mechanisms in both cell types. *Irf4* and *Batf* were the only genes exhibiting a heightened expression in iNKT cells, in comparison with ex vivo. Accordingly, IRF-4 and BATF deficiency have a significant impact on IL-9 production by iNKT cells, although redundant mechanisms are likely to operate because abrogation of IL-9 production is incomplete in both cases.

Whereas CD4 T cells require several days to undergo full Th9 differentiation and initiate IL-9 secretion (Fig. 4, Refs. 30, 31), we show that IL-9 production by iNKT cells occurs in a brief burst shortly after activation, within the first 36 h, exhibiting an explosive profile in terms of magnitude of secretion and rapid termination. Because mRNA coding for IL-9 is undetectable in iNKT cells isolated ex vivo, the mechanisms operating in iNKT cells to induce

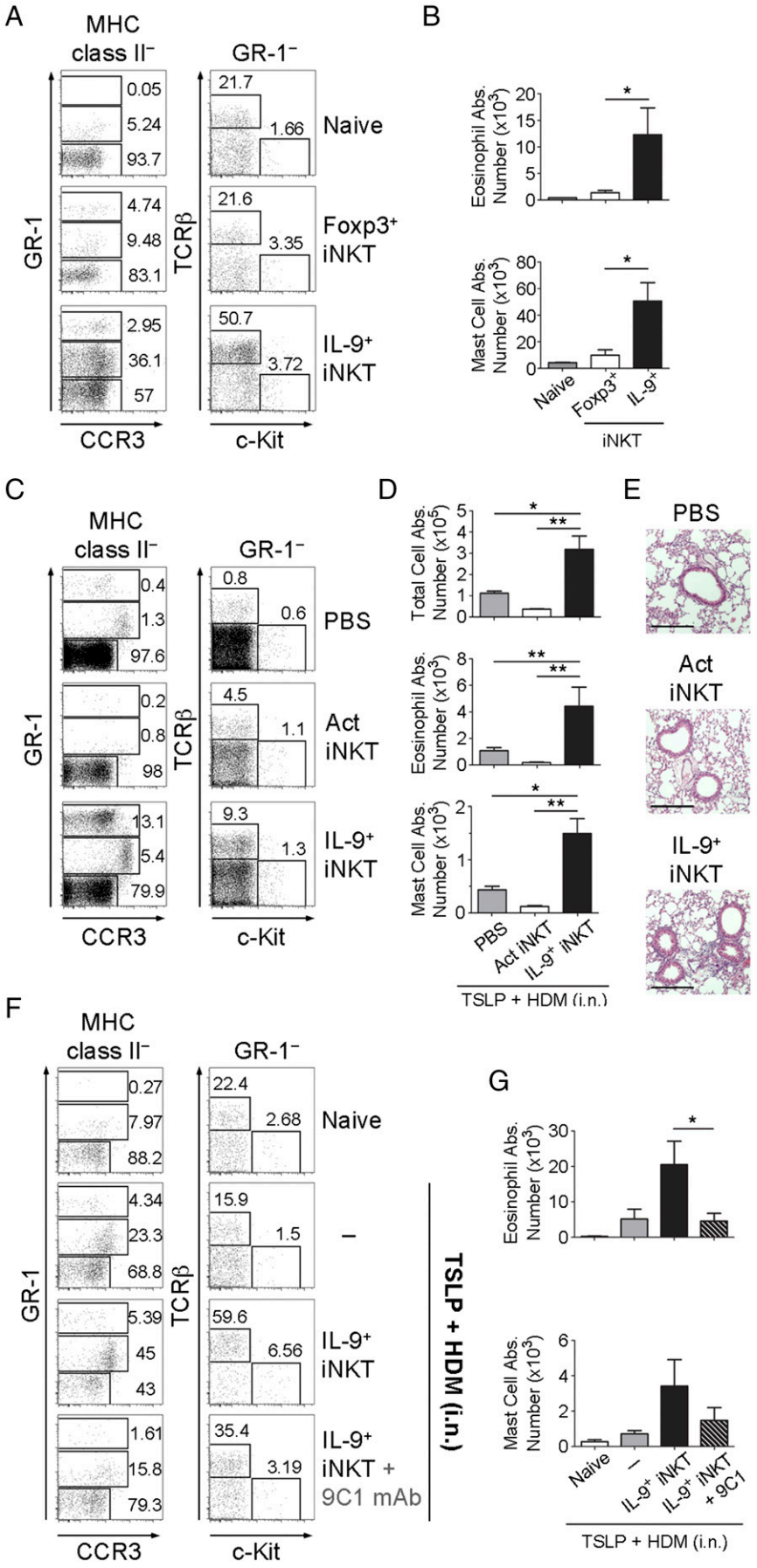

FIGURE 5. IL-9–secreting iNKT cells promote allergic airway inflammation. Splenic iNKT cells isolated from Foxp3^{gfp} reporter mice were purified by FACS and stimulated in vitro for 48 h in the presence of IL-2 and 11B11 or IL-2 and TGF-β. Cells were then sorted according to GFP expression and adoptively transferred into wild-type C57BL/6J mice. (**A** and **B**) Mice received by i.t. injection 5×10^4 Foxp3$^-$ (enriched for NKT9, IL-9$^+$ iNKT cells) or Foxp3$^+$ iNKT cells generated in cultures supplemented with TGF-β and were sacrificed 48 h following cell transfer. FACS plots show the cell content of the BAL, and bar graphs represent absolute cell numbers of eosinophils and mast cells defined, respectively, as MHC class II$^-$ Gr-1^{int} and MHC class II$^-$Gr-1$^-$c-Kit$^+$ cells. (**C**–**E**) Wild-type mice received by i.t. injection 1×10^4 Foxp3$^-$ iNKT cells from cultures set in the presence of IL-2 plus 11B11 (activated iNKT) or IL-2 plus TGF-β (enriched for NKT9, IL-9$^+$ iNKT) and were challenged daily with HDM and TSLP for 5 d. (C) Cell composition of the BAL assessed by flow cytometry. (D) Absolute cell counts of eosinophils and mast cells, as defined above. (E) Airway histology from the different experimental groups showing staining with H&E. Scale bars, 200 μm. (**F** and **G**) Wild-type mice adoptively transferred and immunized as described in (C)–(E) were injected with anti–IL-9 (9C1). (F) Cell composition of the BAL assessed by flow cytometry. (G) Absolute cell counts of eosinophils and mast cells, as defined above.

IL-9 expression are remarkably fast. This places NKT9 cells in the first line of Th9 responses, which are typically elicited in mucosal sites where NKT lymphocytes are abundant, as early contributors of the initial burst of IL-9 production, as was previously shown in the case of gut protection from induced colitis (25). Additionally, human iNKT cells conserve the same ability to respond to TGF-β and IL-4 by inducing IL-9 expression. The relative contribution of NKT9 and Th9 cells over time for IL-9–mediated immunity deserves further investigation.

We observed that addition of exogenous TGF-β alone was sufficient to trigger IL-9 production by iNKT cells, because IL-4 was provided by iNKT cells endogenously. Nevertheless, both TGF-β and IL-4 were required to drive optimal IL-9 secretion, as neutralization of either IL-4 or TGF-β would severely impair NKT9 induction. In the absence of TGF-β, low levels of IL-9 could still be detected. However, addition of a TGF-β–neutralizing Ab completely abrogated IL-9 production, suggesting the presence of residual traces of TGF-β in cultures provided either by the serum used to supplement culture media, or by NKT cells themselves (43, 44). In turn, neutralization of IL-4 did not completely abrogate IL-9 production, suggesting that IL-4 could be acting in an autocrine and immediate way on iNKT cells, thus preventing the Ab to have a complete blocking action.

The fact that iNKT cell subpopulations defined by NK1.1 and CD4, or Nrp-1 expression, which have differential tissue distributions (26), are fully competent to induce IL-9 expression suggests that NKT9 cells can be generated in several tissues when appropriate inflammatory signals are present. The highest IL-9 secretion was observed among subsets expressing NK1.1, which can secrete IL-4, but are especially enriched in IFN-γ–producing iNKT cells (45, 46). This observation is interesting in the light of recent findings showing the importance of IL-9 for antitumor responses, by promoting appropriate homing of cytotoxic lymphocytes to tumor sites and enabling them to efficiently seek out and destroy target cancer cells (47, 48). The ability of iNKT cells to boost antitumor immune responses is well known and attributed to their rapid IFN-γ secretion (49). The finding that NKT9 potential is maximal among the NK1.1$^+$ iNKT compartment, which is the one providing the most efficacious response to cancer, suggests that, in addition to IFN-γ, iNKT cell–derived IL-9 might also contribute to reinforce antitumor responses. Of note, TGF-β is commonly produced in tumor sites (50).

In addition to cancer, IL-9–dependent responses have also been associated with autoimmune and allergic diseases, which are immune responses where iNKT cell intervention has been extensively documented (42, 47, 48, 51, 52). Because the contribution of IL-9 for the development of allergic airways inflammation is well established (33, 34, 53), we investigated the impact of IL-9 produced by iNKT cells in that condition. Our results show that the presence of IL-9–secreting iNKT cells in the airways, even at low numbers, increases local infiltration by eosinophils and mast cells. Despite that iNKT cells have been reported to respond directly to TSLP by increasing IL-13 production and lead to increased eosinophilia in a mouse model of OVA-induced allergic airways disease (54), in vivo blockade of IL-9 completely prevented local accumulation of eosinophils and, to a great extent, mast cells. This outcome demonstrates that IL-9 is the main factor driving airway infiltration in this model. Taken together, these data indicate that NKT9 cells can contribute to accelerate the onset of and boost Th9 responses at mucosal sites.

In conclusion, following completion of thymic development, NKT cells remain receptive to functional polarization in the periphery. Activation in presence of TGF-β and IL-4 leads to NKT9 polarization and IL-9 production. However, IL-9 production does not appear restricted to a particular iNKT subset, corresponding rather to a functional state. In comparison with conventional Th9 cells, secretion of IL-9 by iNKT cells is faster, shorter, and higher in magnitude, favoring an innate role for NKT9 cells at the early phase of Th9 responses. Accordingly, adoptive transfer of NKT9 cells exacerbated allergic airways inflammation, thus demonstrating the impact of this functional subset in vivo.

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Disclosures

The authors have no financial conflicts of interest.

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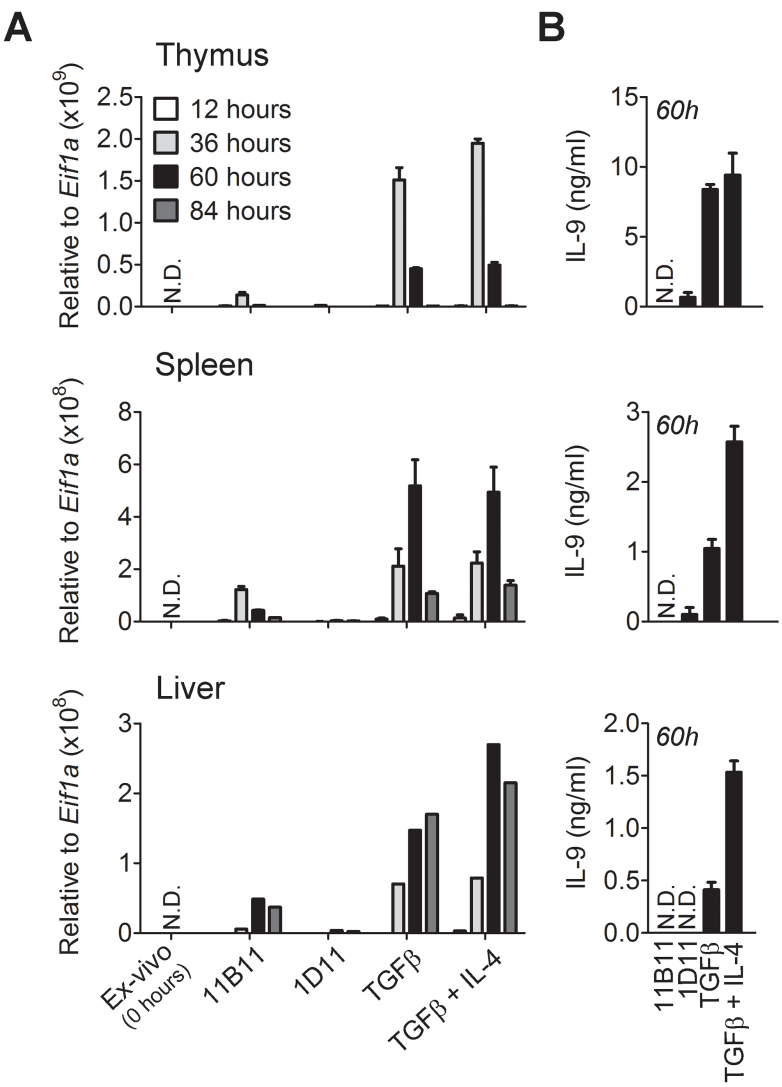
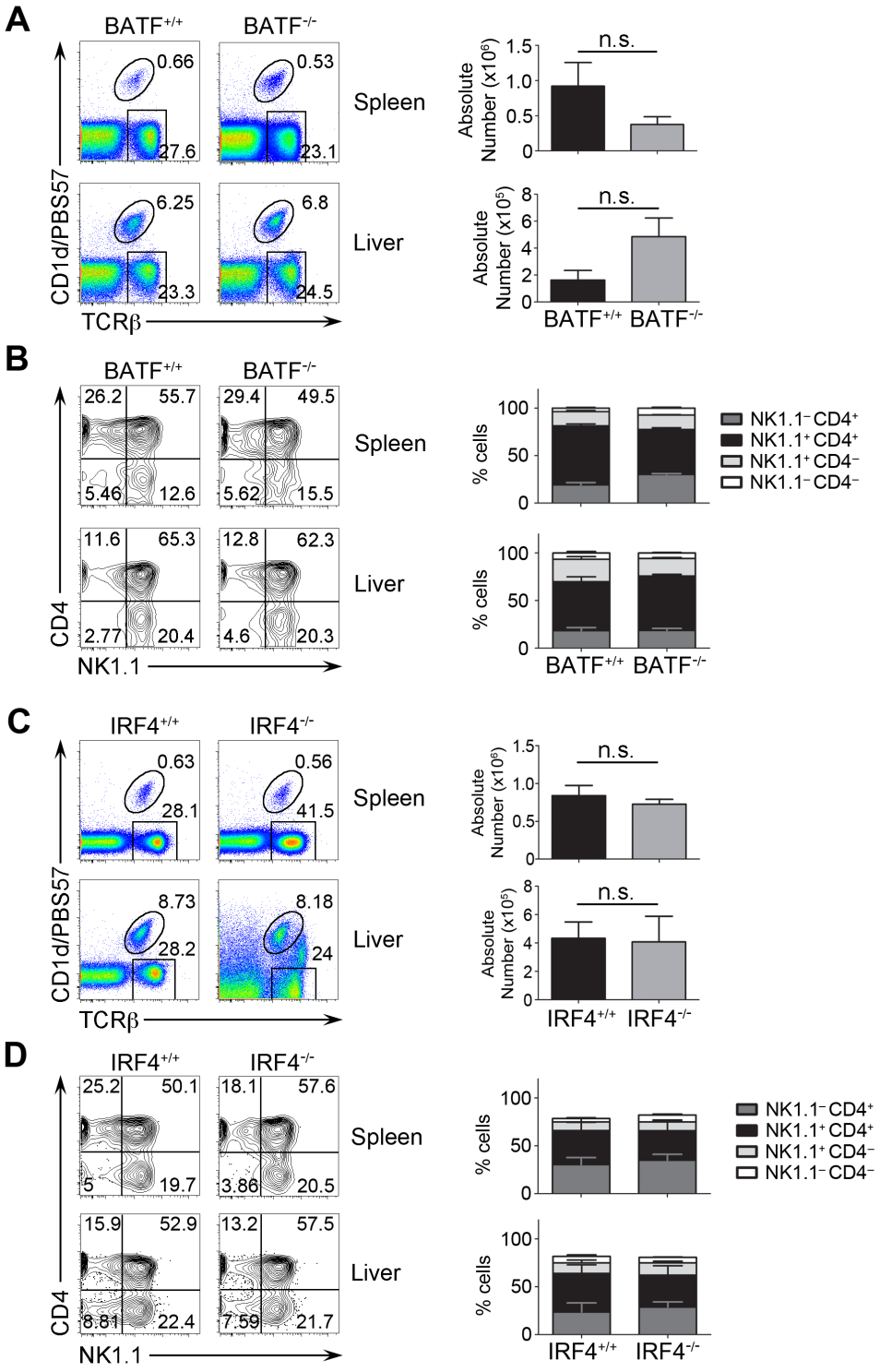


Supplementary Figure 1: Kinetics of *Il9* mRNA expression and IL-9 secretion by iNKT cells isolated from thymus, spleen and liver

Splenic iNKT cells isolated from thymus, spleen and liver of Balb/c WT mice were stimulated in presence of IL-2 the indicated antibodies or cytokines. IL-9 concentration in supernatants was measured every 24 hours and cells were harvested for RNA extraction and gene expression analysis using qRT-PCR.
(A) Relative amount of *Il9* transcripts in iNKT cells cells at the indicated time points. Gene expression is relative to *Eif1a* expression level. Il9 expression was undetectable *ex vivo* in iNKT cells isolated from all the organs (not depicted).
(B) Concentration of IL-9 protein in the supernatant of iNKT-cell culture supernatant 60 hours after set up.
N.D. = not detected. Bar graphs represent the average of triplicate cell culture wells per assay, except for mRNA expression in the liver, in which one well per assay was analyzed, and error bars indicate standard error.



Supplementary Figure 2: Analysis of iNKT cells at the steady state in *Batf*$^{-/-}$ and *Irf4*$^{-/-}$ mice.

iNKT cells were isolated from spleen and liver of *Batf*$^{-/-}$ (**A,B**) or *Irf4*$^{-/-}$ (**C,D**) unmanipulated mice, and analyzed by flow cytometry in comparison with WT age-matched controls.

(A) Flow cytometry identification of iNKT cells in the spleen and liver of WT and *Batf*$^{-/-}$ mice. Dotplots were gated in the CD19^{-} population. Quantification of iNKT-cell absolute numbers is provided on the right.
(B) Flow cytometry profile (left) and frequency of iNKT-cell subsets defined by CD4 and NK1.1 expression (right) in WT and *Batf*$^{-/-}$ mice. Dotplots were gated in TCRβ^{int}CD1d/PBS57^{+} cells.
(C) Flow cytometry identification of iNKT cells in the spleen and liver of WT and *Irf4*$^{-/-}$ mice. Dotplots were gated in the CD19^{-} population. Quantification of iNKT-cell absolute numbers is provided on the right.
(D) Flow cytometry profile (left) and frequency of iNKT-cell subsets defined by CD4 and NK1.1 expression (right) in WT and *Irf4*$^{-/-}$ mice. Dotplots were gated in TCRβ^{int}CD1d/PBS57^{+} cells.

n.s. = not significant. Results are representative of two independent experiments with three to five animals per group. Bar graphs represent the average and error bars indicate standard error.