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This information is current as of December 5, 2010

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J Immunol; Prepublished online 3 December 2010;
 doi:10.4049/jimmunol.1001348
<http://www.jimmunol.org/content/early/2010/12/01/jimmunol.1001348>

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The Journal of Immunology is published twice each month by
 The American Association of Immunologists, Inc.,
 9650 Rockville Pike, Bethesda, MD 20814-3994.
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 Print ISSN: 0022-1767 Online ISSN: 1550-6606.



The TLR7 Agonist R848 Alleviates Allergic Inflammation by Targeting Invariant NKT Cells to Produce IFN- γ

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It has been documented that TLR7 stimulation triggers not only antiviral responses, but also alleviates experimental asthma. Considering the implication of invariant NKT (iNKT) cells in both situations, we postulated that they might contribute to the anti-inflammatory effect of TLR7 ligands. We show in this study that spleen cells activated by the TLR7 agonist resiquimod (R848) attenuate allergic inflammation upon adoptive transfer when they are recovered from wild-type, but not from iNKT cell-deficient $\text{J}\alpha 18^{-/-}$ mice, which proves the specific involvement of this regulatory population. Furthermore, we provide evidence that IFN- γ is critical for the protective effect, which is lost when transferred iNKT cells are sorted from IFN- γ -deficient mice. In support of a direct activation of iNKT cells through TLR7 signaling in vivo, we observed a prompt increase of serum IFN- γ levels, associated with upregulation of CD69 expression on iNKT cells. Moreover, we demonstrate that iNKT cells effectively express TLR7 and respond to R848 in vitro by producing high levels of IFN- γ in the presence of IL-12, consistent with the conclusion that their contribution to the alleviation of allergic inflammation upon treatment with TLR7 ligands is mediated through IFN- γ . *The Journal of Immunology*, 2011, 186: 000–000.

Asthma is one of the most common chronic diseases in children in developed countries and has become a major public health issue. The hygiene hypothesis formulated by Strachan in 1989 claims that the decrease of infections during early childhood is responsible for the increased prevalence of allergic diseases (1). However, even though some epidemiological data support this assumption, it has not been definitively proven to date. Substantial positive evidence has been provided by the observation that infections and nonpathogenic agents such as lactobacilli reduced the frequency of atopy (2, 3). Furthermore, in experimental animal models it has been demonstrated that deliberate

infections can prevent the onset of allergic diseases. Initially, it was argued that in developed countries the lack of microbial burden in early childhood, which normally favors a strong Th1-biased immunity, redirects the immune response toward a Th2 phenotype and therefore predisposes the host to allergic responses. However, more recent studies revealed that the mechanisms are actually far more complex, highlighting the role of specialized regulatory T cell subsets in the control of the Th1/Th2 balance.

TLRs recognize pathogen-derived molecular patterns of infectious agents, which trigger particular signaling pathways that influence the orientation of the immune response. Among these, TLR7 acts as a sensor of ssRNAs and imidazoquinolin family compounds, such as R848, also called resiquimod (4, 5). This compound has already been shown to protect mice from asthma, by decreasing its typical symptoms, namely airway hyperresponsiveness (AHR), eosinophil recruitment to the lung, and Th2 responses. It has also been reported for its ability to abrogate airway remodeling in a chronic asthma model (6). The efficacy of R848 in preventing the development of allergic inflammation in rodent models has been well established, and some cellular and molecular mechanisms have been described. For example, it has been reported that the TLR7 agonist inhibits the production of OVA-specific IgE and IgG1 Abs and abolishes all hallmarks of experimental asthma, including AHR and allergic airway inflammation. The protective effect persisted, at least partially, in IL-12p35 $^{-/-}$ mice, disappearing only when IL-10 was likewise deficient (7). A recent study suggested that IFN- γ -producing CD8 $^{+}$ T cells may contribute to the anti-allergic effect of R848, even though it was not abolished in IFN- γ -deficient mice (8). Camateros et al. (9) reported that the expression profile of genes involved in NK cell-mediated cytotoxicity and NK cell recruitment to the lung was altered following R848 treatment, but could not establish a causal relationship between NK cell recruitment/activation and treatment efficiency.

Because invariant NKT (iNKT) cells, which express both NK markers and an invariant $\text{V}\alpha 14\text{-J}\alpha 18$ TCR chain, play a key role in the development of asthma, we hypothesized that they might

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Received for publication April 27, 2010. Accepted for publication October 27, 2010.

This work was supported by Centre National de la Recherche Scientifique, Université Paris Descartes, grants from the Fondation pour la Recherche Médicale (DAL20041203162), and by institute funds from la Chancellerie des Universités de Paris (Legs Poix), Fondation pour la Recherche Médicale (to F.G.), Association Française des Diabétiques (to A.A.), and the Vietnamese government (to L.P.V.).

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Abbreviations used in this paper: AHR, airway hyperactivity; BALF, bronchoalveolar lavage fluid; DC, dendritic cell; α -GalCer, α -galactosylceramide; iNKT, invariant NKT; Iono, ionomycin; *Pef*, peak expiratory flow; *Penh*, enhanced pause; *Pif*, peak inspiratory flow; *Te*, expiratory time; *Tr*, relaxation time; TT, tetramer; WT, wild-type.

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take part in R848-induced asthma protection (10, 11). iNKT cells exert deleterious or beneficial effects, depending on the stage of the immune response in which they are activated. For example, they can aggravate allergic asthma by enhancing AHR and eosinophilia during the effector phase, but confer protection at disease onset, once they have been activated by their specific ligand α -galactosylceramide (α -GalCer) (12–14).

In our present study, we addressed the potential role of iNKT cells in the alleviation of allergic inflammation by TLR7 stimulation. We

provide evidence that these regulatory cells are activated *in vivo* by R848, as evidenced by upregulation of their CD69 surface expression and prompt IFN- γ production. Using adoptive transfer experiments, we demonstrate that iNKT cells are responsible for asthma protection by donor cells from wild-type (WT) mice having received a treatment with R848, as their counterpart from iNKT cell-deficient $\text{J}\alpha 18^{-/-}$ mice had no such effect.

Materials and Methods

Animals

Six- to 8-wk-old specific pathogen-free, C57BL/6J mice were purchased from Janvier (Les Genest St. Isle, France). TLR7 $^{-/-}$ and $\text{J}\alpha 18^{-/-}$ (provided by S. Akira [Osaka, Japan] and T. Taniguchi [Yokohama, Japan], respectively) and IFN- $\gamma^{-/-}$ (The Jackson Laboratory, Bar Harbor, ME) were bred in our animal facilities under specific pathogen-free conditions. Animal experiments were performed according to the recommendations of the French Institutional Committee.

Reagents

R848 was purchased from Alexis Biochemicals (Paris, France). PMA and ionomycin (Iono) were from Sigma-Aldrich (Saint-Quentin Fallavier, France). Murine rIL-12 was from R&D Systems (Lille, France). Fluorochrome-conjugated anti-CD5 (clone 55-7.3), anti-NK1.1 (clone PK136), anti-TCR β (clone H57-597), and anti-CD69 (clone H1.2F3) were from BD Pharmingen (Le Pont de Claix, France). Allophycocyanin-conjugated CD1d/ α -GalCer-loaded tetramers were prepared in our laboratory from

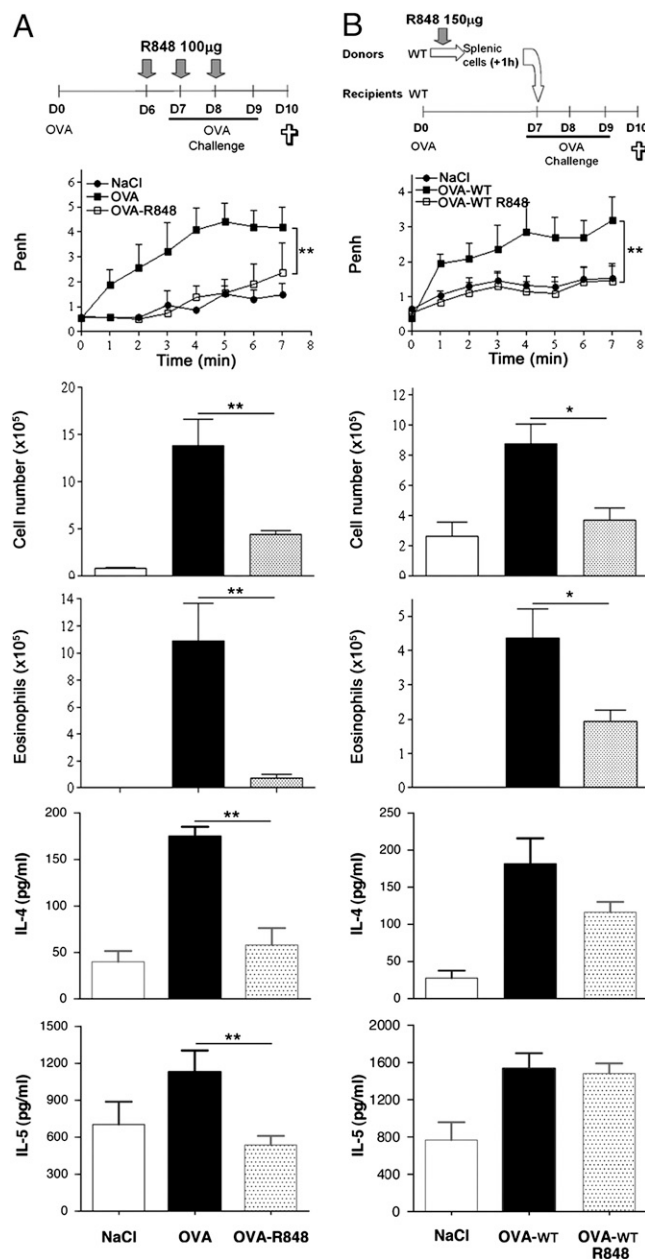


FIGURE 1. Protection against allergic inflammation can be transferred by splenocytes from mice having received R848. **A**, Mice immunized with OVA were treated on 3 consecutive days with R848 and challenged with OVA. AHR to metacholine was measured 24 h after the last challenge with OVA or NaCl. OVA-challenged mice were treated (OVA-R848; $n = 5$) or not (OVA; $n = 5$) with R848. Total cell and eosinophil recruitment was measured in BALF. **B**, WT OVA-immunized recipient mice were transferred with splenocytes from PBS (OVA-WT; $n = 5$) or R848-treated WT donor mice (OVA-WT R848). AHR, total cell, and eosinophil recruitment were evaluated 1 d after the last challenge. Each panel represents one of at least three independent experiments. * $p < 0.05$; ** $p < 0.01$.

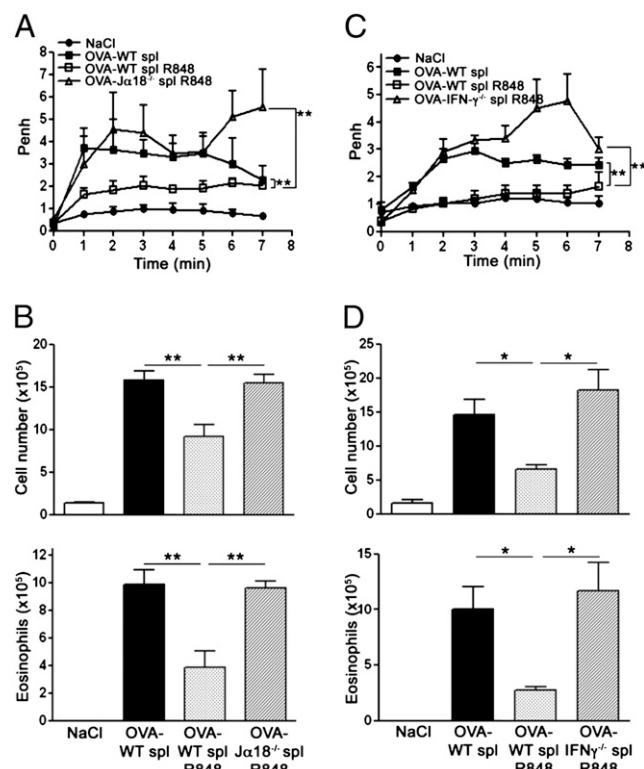


FIGURE 2. iNKT cells and IFN- γ are implicated in R848-induced protection against allergic inflammation. **A**, Mice received PBS- or R848-treated WT splenocytes (OVA-WT or OVA-WT R848, respectively; $n = 5$ per group) or R848-stimulated $\text{J}\alpha 18^{-/-}$ splenocytes (OVA- $\text{J}\alpha 18^{-/-}$ R848; $n = 5$). AHR was measured 24 h after the last challenge. **B**, Total cell and eosinophil recruitment in BALF were no longer modulated in the OVA- $\text{J}\alpha 18^{-/-}$ R848 group. **C**, AHR was measured in mice injected with WT or IFN- $\gamma^{-/-}$ splenocytes treated or not with R848: OVA-WT, OVA-WT R848, and OVA-IFN- $\gamma^{-/-}$ R848 ($n = 5$ per group). **D**, R848-stimulated IFN- $\gamma^{-/-}$ splenocytes failed to decrease total cell and eosinophil numbers in BALF. Th2-type cytokine modulation was observed in neither experiment. Each panel represents one of at least three independent experiments. * $p < 0.05$; ** $p < 0.01$.

the murine CD1d/ β_2 -microglobulin expression vector (provided by Sidobre and Kronenberg, La Jolla, CA) or provided by the National Institutes of Health tetramer core facility. Unloaded CD1d-tetramer-allophycocyanin was used as control.

Protocol of immunization and challenge

Mice were immunized by i.p. injection of 100 μ g OVA (Sigma-Aldrich) adsorbed onto 1.6 mg alum adjuvant (Merck, Fontenay-sous-Bois, France) in 0.4 ml saline solution. Seven days later, mice were challenged on 3 consecutive days (D7, D8, D9) with aerosolized OVA (5 ml of an OVA solution at 50 mg/ml) or saline alone for 20 min.

In vivo treatment and iNKT cell analysis

WT and $J\alpha 18^{-/-}$ mice were injected i.p. with 10 μ g R848, followed by collection of blood and removal of spleens 2 h later. Splenocytes were stained with CD1d/ α -GalCer-APC or unloaded CD1d/ α -tetramer-APC together with fluorochrome-conjugated anti- $\alpha\beta$ -TCR and anti-CD69 Abs, followed by flow cytometry analysis. IFN- γ and IL-4 concentrations in sera were quantified by ELISA (R&D Systems) and expressed as pg/ml based on a regression curve established for each assay with recombinant murine cytokines.

Flow cytometry

After Fc γ R blockade with 2.4G2 mAb, splenocytes were labeled with anti-CD5-APC and anti-NK1.1-PE, CD1d/ α -GalCer-tetramer-APC, anti-TCR β -FITC, and anti-CD69-PE at appropriate dilutions. For intracellular TLR7 staining, cells were fixed and permeabilized using a Cytofix/Cytoperm kit, according to the manufacturer's instructions (BD Biosciences, San

Jose, CA), followed by incubation with rabbit polyclonal anti-TLR7 Ab (Alexis Biochemicals) and goat PE-conjugated anti-rabbit IgG (BD Pharmingen). Normal rabbit polyclonal IgG was used as negative control. For intracellular IFN- γ staining, cells were prepared, as previously described (15), and labeled with PE-conjugated mAb anti-IFN- γ or control rat IgG1 mAb (BD Pharmingen). Cells were acquired on a FACSCantoII flow cytometer (BD Biosciences) and analyzed in the lymphocyte gate defined by forward- and side-scatter properties, using FACSDiva Software.

Sorting of NKT cells

Splenocytes from WT or IFN- $\gamma^{-/-}$ mice were depleted of CD8 $^{+}$, CD62L $^{+}$, CD11b $^{+}$, and CD19 $^{+}$ cells after labeling with the corresponding mAbs (clone 53.67, Mel14, M1/70, 1D3, respectively) using anti-rat Ig-coated magnetic beads (Invitrogen Dynal, Cergy-Pontoise, France), and negatively enriched cells were stained with anti-NK1.1 and anti-CD5 mAbs (BD Pharmingen) before cell sorting. Alternatively, cells were labeled with CD1d/ α -GalCer-tetramer-APC and $\alpha\beta$ -TCR mAb. CD5 $^{+}$ NK1.1 $^{+}$ or CD1d/ α -GalCer-tetramer $^{+}$ TCR ^{int} cells were sorted on a FACS Vantage cell sorter (BD Biosciences).

Adoptive transfer

A total of 5×10^6 splenocytes or 5×10^4 sorted CD5 $^{+}$ NK1.1 $^{+}$ NKT cells from WT, $J\alpha 18^{-/-}$, or IFN- $\gamma^{-/-}$ C57BL/6 mice having received either PBS or 150 μ g R848 was injected i.v. into C57BL/6 mice immunized with OVA, 1 h before the first challenge on D7.

Measurement of AHR

AHR was assessed 24 h after the last challenge by delivering an aerosol of methacholine (Sigma-Aldrich) for 1 min at 150 mM to unrestrained, conscious mice placed in a plethysmographic chamber (EMKA Technologies, Paris, France), as previously described (16). The index of airflow obstruction was expressed as enhanced pause (*Penh*), calculated as fol-

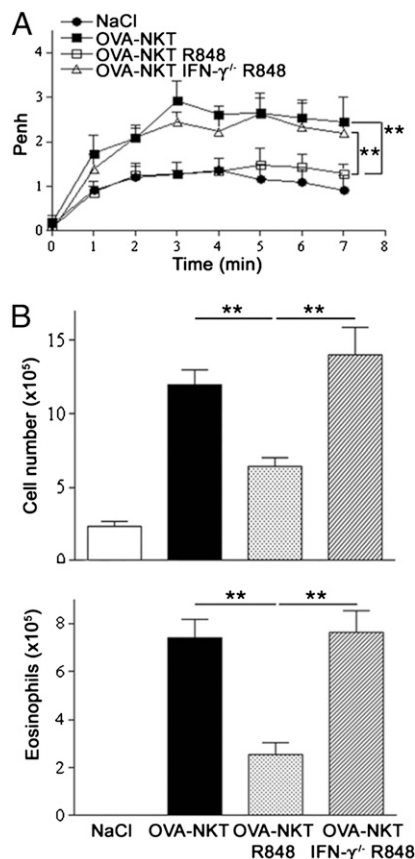


FIGURE 3. IFN- γ -producing iNKT cells account for R848-induced protection in experimental allergic inflammation. Sorted CD5 $^{+}$ NK1.1 $^{+}$ iNKT cells from WT or IFN- $\gamma^{-/-}$ R848-treated mice (OVA-NKT R848 and OVA-NKT IFN- $\gamma^{-/-}$ R848, respectively; $n = 5$ per group) or WT PBS-treated mice (OVA-NKT; $n = 5$) were transferred into OVA-immunized WT recipients. R848-activated NKT cells reduced AHR (A), total cell, and eosinophil recruitment (B), conversely to their IFN- γ -deficient counterpart. Each panel represents one of at least two independent experiments. $**p < 0.01$.

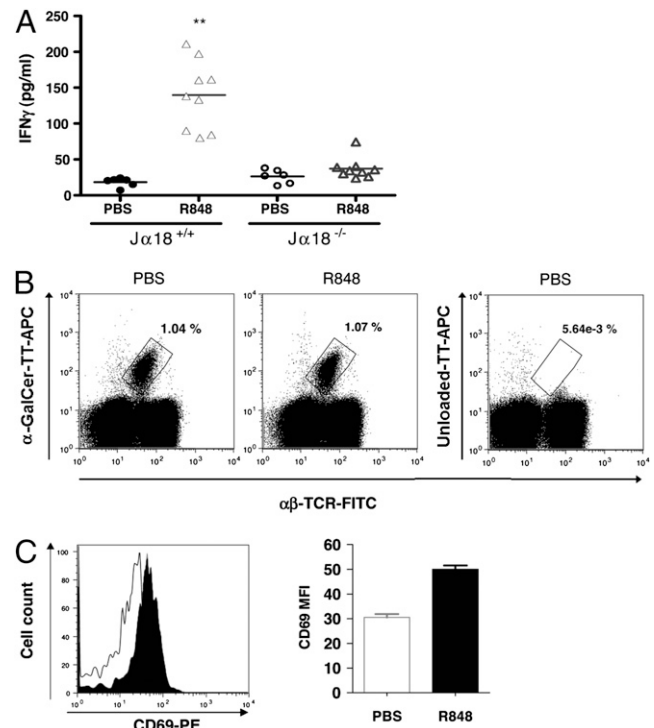


FIGURE 4. R848 induces iNKT cell activation and rapid IFN- γ production. A, WT and $J\alpha 18^{-/-}$ mice were injected i.p. with 10 μ g R848 or PBS. Sera were collected 2 h after injection when IFN- γ levels were measured by ELISA. B and C, Splenocytes from R848- or PBS-injected mice were stained with loaded CD1d/ α -GalCer-tetramer-allophycocyanin (α -GalCer-TT-APC) or CD1d/unloaded tetramer-allophycocyanin (unloaded-TT-APC) and anti- $\alpha\beta$ -TCR and anti-CD69 Abs. Cell surface expression of CD69 was increased on iNKT cells from R848-treated mice (shaded histogram) compared with PBS-injected mice (blank histogram). The CD69 mean fluorescence intensity was significantly increased in the R848-treated group ($n = 4$). Each panel represents one of at least two independent experiments.

lows: $Penh = (Te - Tr)/Tr \times (Pef/Pif)$, where Te is the expiratory time, Tr is the relaxation time, Pef is the peak expiratory flow, and Pif is the peak inspiratory flow. The values of $Penh$ were expressed per minute and are the average of three determinations recorded every 20 s. For reasons of simplification, the area under the curve was calculated for 7 min after methacholine inhalation. The resulting area under the curve values are indicated in graphs and represent the quantitative expression of AHR.

Bronchoalveolar lavage fluid and lung homogenates

Immediately after evaluating AHR, mice were anesthetized by an i.p. injection of urethane (2 g/kg body mass; Sigma Aldrich). A tracheal canula was inserted, and bronchoalveolar lavage was performed by instillation of sterile PBS. Lungs were then perfused with a saline solution through the pulmonary artery and homogenized in sterile PBS. Differential cell counts were determined in bronchoalveolar lavage fluid (BALF) after May-Grünwald-Giemsa (Merck) staining of cells on cytospin slides, and IL-4 and IL-5 levels were measured in lung homogenates by ELISA (R&D Systems).

In vitro iNKT cell stimulation assays

A total of 2×10^4 purified cells (purity >99%) per well was cultured in 200 μ l RPMI 1640 medium supplemented with 10% inactivated FCS, 1% penicillin-streptomycin, and 5×10^{-5} M 2-ME. Cells were then stimulated with murine rIL-12 and R848, PMA plus Iono, or medium alone. IFN- γ levels in supernatants were measured by ELISA (R&D Systems) after 48 h of stimulation.

RT-PCR analysis

RNA from splenocytes or sorted CD5⁺NK1.1⁺iNKT cells was extracted with TRIzol reagent (Invitrogen). A total of 10 μ g tRNA was added prior to RNA extraction, and reverse transcription was performed with total RNA. TLR7 expression was determined by RT-PCR analysis (MWG AG Biotech [Ebersberg, Germany] Primus 96 apparatus) using the sense 5'-TTCCGATACGATGAATATGCACG-3' and antisense 5'-TGAGTTTGTC-CAGAAGCCGTAAT-3' primers (Sigma Genosys, Saint-Quentin Fallavier, France) under the following cycling conditions: denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. Double amplification of 35 cycles each was performed.

Statistical analysis

In the allergic model, the difference between $Penh$ values of each group during the period of measurement was calculated using the Mann-Whitney U test for unpaired data. Some results are presented as mean values $\pm$ SEM, and statistical significance was established by the log rank test. The p values <0.05 were regarded as significant (* p < 0.05; ** p < 0.01). All statistical analyses were performed with GraphPad (San Diego, CA) Prism 4.0 software.

Results

R848-induced protection against allergic inflammation can be transferred to immunized recipient mice

We set out to confirm the protective effect of R848 against OVA-induced symptoms of allergic inflammation in our experimental protocol. As expected, OVA-immunized C57BL/6 mice treated with R848 1 h prior to OVA challenge versus saline controls developed less AHR, recruited fewer infiltrating cells to the lung, especially eosinophils, and produced less Th2 cytokines such as IL-4, IL-5, IL-10, and IL-13, as well as chemokines like eotaxin-1 (Fig. 1A and data not shown).

We then devised an adoptive transfer protocol to evaluate the potential role of iNKT cells in the protective effect. To this end, we transferred splenocytes from donors having previously received a treatment with R848 (OVA-WT R848) relative to saline controls (OVA-WT) before the first OVA challenge of the recipients. We observed a significant decrease in AHR, total cell, and eosinophil recruitment, as compared with mice injected with splenocytes from PBS-treated mice (Fig. 1B). This result validated the experimental approach proving that R848-targeted immune cells can alleviate allergic inflammation in recipient mice, even though no significant downmodulation of IL-4 and IL-5 occurred in these conditions (Fig. 1B).

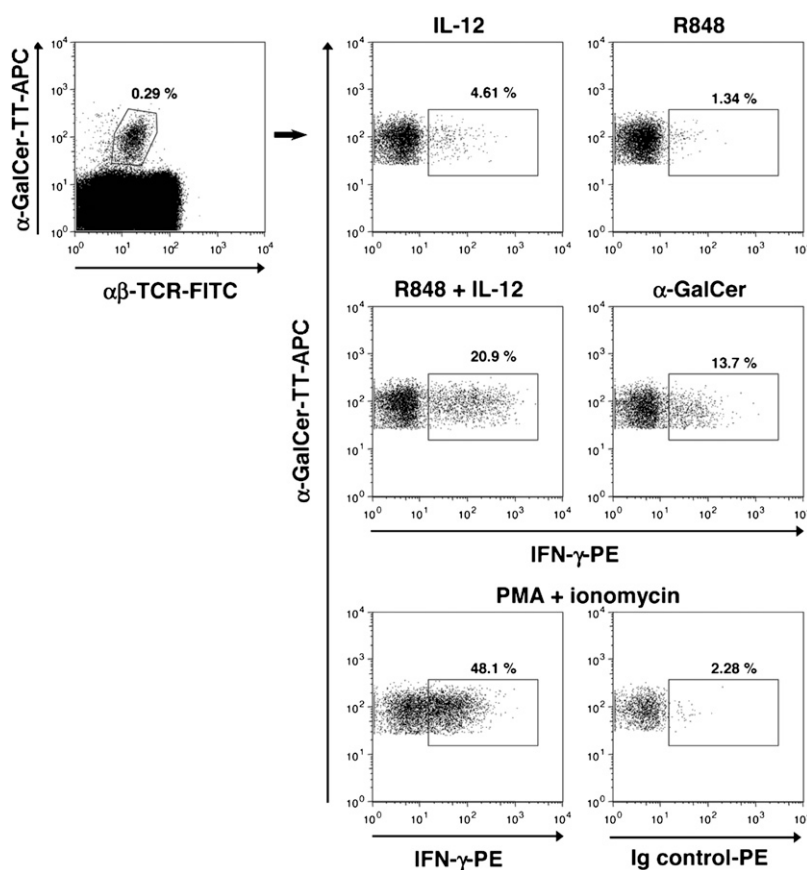


FIGURE 5. Spleen iNKT cells produce IFN- γ in response to R848 and IL-12. Splenocytes from WT mice (2.5×10^6 cells/ml) were stimulated with IL-12 (10 ng/ml), R848 (1 μ g/ml), R848 + IL-12, α -GalCer (100 ng/ml), or PMA (5×10^{-9} M) + Iono (10^{-6} M) for 4 h in the presence of brefeldin A (10 μ g/ml). Cells were then stained with CD1d/ α -GalCer-tetramer-APC and anti- $\alpha\beta$ -TCR mAb, followed by intracellular staining with anti-IFN- γ or isotype control mAb. Representative FACS profile of IFN- γ expression (right panels) in gated CD1d/tetramer⁺TCR $\alpha\beta$ ⁺ iNKT cells (right panel). Percentages are indicated. Data are representative of two independent experiments.

IFN- γ -producing iNKT cells account for R848-induced protection in allergic inflammation

To assess the role of iNKT cells in the protection conferred by spleen cells from R848-treated mice, we compared donor cells from WT and iNKT cell-deficient $\text{J}\alpha 18^{-/-}$ C57BL/6 mice for their anti-allergic action. We found that AHR as well as total cell and eosinophil infiltration in BALF were no longer attenuated when splenocytes from R848-treated $\text{J}\alpha 18^{-/-}$ mice were transferred (Fig. 2A, 2B).

Because it has been reported that protection in allergic inflammation models mediated by iNKT cells is IFN- γ dependent (12–14), we examined whether this cytokine was also involved in our model. Hence, we transferred splenocytes from IFN- γ -deficient R848-treated mice and found that, like their iNKT cell-deficient counterpart, they failed to reduce AHR and eosinophilia in BALF (Fig. 2C, 2D), supporting the conclusion that alleviation of allergic inflammation induced by TLR7 activation depended on both iNKT cells and IFN- γ production.

To verify whether iNKT cells can provide active protection on their own, we injected sorted $\text{CD}8^{-}\text{CD}62\text{L}^{-}\text{CD}11\text{b}^{-}\text{CD}19^{-}\text{NK}1.1^{+}\text{CD}5^{+}$ iNKT cells from R848-treated WT donors (OVA-NKT R848). These cells suppressed allergic inflammation as efficiently as total splenocytes, whereas iNKT cells from untreated donors had no such effect in OVA-challenged mice (OVA-NKT; Fig. 3). The key role of IFN- γ in this process was established by transferring iNKT cells from R848-treated IFN- γ -deficient mice (Fig. 3). The lack of effect proved that the treatment with R848 alleviated allergic syndromes through iNKT cell-derived IFN- γ .

TLR7 stimulation activates iNKT cells to produce IFN- γ both in vivo and in vitro

We investigated the ability of the TLR7 agonist R848 to activate iNKT cells in vivo by measuring the production of IFN- γ and the expression of the activation marker CD69 on the cell surface. A strong increase of IFN- γ levels was detected in sera of R848-treated mice only 2 h after i.p. injection, as compared with controls having received PBS (Fig. 4A). This response was iNKT cell dependent because it failed to occur in the circulation of $\text{J}\alpha 18^{-/-}$ mice having received the TLR7 agonist. The proportion of iNKT cells, identified as $\text{CD}1\text{d}/\alpha\text{-GalCer-tetramer}^{+}\alpha\beta\text{-TCR}^{+}$ splenocytes, was not modified by the treatment that induced a strong upregulation of the mean fluorescence intensity of the early activation marker CD69 relative to control mice (Fig. 4B).

We used intracellular staining to establish that iNKT cells were the source of IFN- γ among stimulated splenocytes. As shown in Fig. 5, the cytokine was clearly detected after exposure to R848, provided that IL-12 was added during incubation. This combination was even more effective than the cognate ligand $\alpha\text{-GalCer}$, whereas the TLR7 agonist had no effect on its own and the percentage of positive cells was only slightly increased in response to IL-12 alone. IFN- γ production induced by PMA plus Iono is shown as a positive control (Fig. 5).

R848 targets iNKT cells to promote IFN- γ production in combination with IL-12

We next addressed the question whether R848 targeted iNKT cells directly to induce IFN- γ production in vitro. Such a mechanism was plausible, as TLR7 was expressed on both transcriptional and protein levels in sorted cells, using RT-PCR analysis and intracellular staining (Fig. 6A, 6B).

In accordance with the results of intracellular staining, R848 alone did not induce IFN- γ production by purified $\text{CD}5^{+}\text{NK}1.1^{+}$ iNKT cells. This is consistent with previous evidence showing that inflammatory signals, which are effectively generated in response

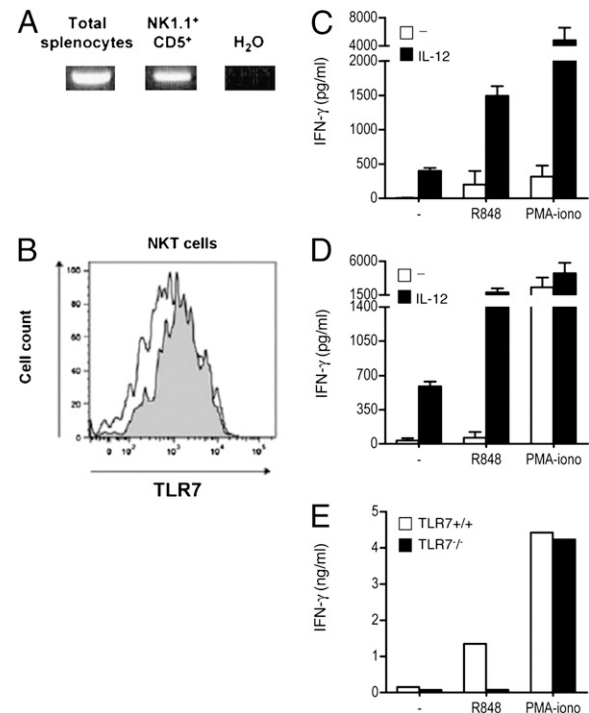


FIGURE 6. iNKT cells respond to R848 by producing IFN- γ in a TLR7-dependent manner. *A*, mRNA expression of TLR7 by sorted $\text{CD}5^{+}\text{NK}1.1^{+}$ iNKT cells was evaluated by RT-PCR. Total splenocytes and H_2O were used as positive and negative control, respectively. *B*, Intracellular TLR7 expression was evaluated in iNKT cells by flow cytometry using rabbit polyclonal Ab against TLR7 and goat PE-labeled anti-rabbit IgG (shaded histogram). Normal rabbit polyclonal IgG (white histogram) was used as negative control. *C*, Sorted $\text{CD}5^{+}\text{NK}1.1^{+}$ iNKT cells (2×10^4 /well; purity >98%) were stimulated with R848 ($1 \mu\text{g/ml}$) or 10^{-8} M PMA + 10^{-6} M Iono with or without 10 ng/ml IL-12. IFN- γ production was evaluated in supernatants by ELISA 48 h after stimulation. Data represent means $\pm$ SEM of seven individual experiments. *D*, Sorted $\text{CD}1\text{d}/\alpha\text{-GalCer-tetramer}^{+}$ iNKT cells (2×10^4 /well; purity >99%) were incubated in the presence of R848 + IL-12, and the biological activity was quantified, as described in *C*. Data represent means $\pm$ SEM of three individual experiments. *E*, Sorted $\text{CD}5^{+}\text{NK}1.1^{+}$ iNKT cells (2×10^4 /well; purity >99%) from TLR7^{+/+} or TLR7^{-/-} mice were incubated in the presence of 10 ng/ml IL-12, $1 \mu\text{g/ml}$ R848, or PMA + Iono, and IFN- γ was measured in supernatants 24 h later. Data represent a typical experiment of two.

to TLR7 stimulation, might be required to prime iNKT cells for IFN- γ production (17–21). Indeed, stimulation of $\text{CD}5^{+}\text{NK}1.1^{+}$ iNKT cells with R848 induced IFN- γ release in the presence of IL-12 (Fig. 6C). Sorted $\text{CD}1\text{d}/\alpha\text{-GalCer-tetramer}^{+}\alpha\beta\text{-TCR}^{+}$ cells responded similarly, with higher background values (Fig. 6D). As shown in Fig. 6E, R848-induced IFN- γ production was inhibited in iNKT cells sorted from TLR7^{-/-} mice, which proved the specific involvement of this signaling pathway. It can therefore be concluded that in an appropriate inflammatory environment, in which cytokines like IL-12 are released, iNKT cells become responsive to TLR7 stimulation by R848 and produce IFN- γ .

Discussion

In the current study, we provide evidence for an IFN- γ -dependent contribution of iNKT cells to the alleviation of experimental allergic inflammation induced by in vivo treatment with the TLR7 agonist R848. This particular T cell population has been previously reported for reducing AHR and eosinophilia by inhibiting effector cells after activation by a single injection of its cognate ligand $\alpha\text{-GalCer}$ (12–14). The IFN- γ -dependent alleviation of

asthma symptoms by R848 given shortly before OVA challenge is reminiscent of this effect.

It is intriguing that i.p. injection of R848 or α -GalCer, which results in a consistent induction of IFN- γ production, is associated with asthma protection, whereas α -GalCer given intranasally triggers AHR (22). This paradox might be explained by the fact that according to the route of administration, different immunoregulatory populations are activated depending on their local distribution. Hence, systemic treatment with α -GalCer or R848 might privilege the activation of iNKT cells, whereas intranasal administration might enhance the activities of Th2 and Th17 cells already present locally, together with the production of mediators (such as thymic stromal lymphopoietin, IL-25, IL-33...) leading to asthma exacerbation.

This study demonstrates that iNKT cells are activated by TLR7 stimulation, thus providing protection against allergic inflammation. This mechanism of action is consistent with previous studies documenting the activation of multiple genes expressed by NK and iNKT cells upon TLR7 activation (9). It is also in agreement with the protective role ascribed to IFN- γ in R848-induced asthma protection in a rodent model (8). Hence, we postulate that systemic treatment with R848 activates immunoregulatory cells, such as iNKT cells, which might alleviate allergic airway disease by preventing effector Th2 and/or Th17 responses in the lung in an IFN- γ -dependent manner.

We have established the critical role of iNKT cells in the protection against allergic inflammation mediated through their prompt production of IFN- γ detected in vivo only 2 h after R848 administration and in vitro by intracellular staining 4 h after exposure to R848 together with IL-12. This combination elicits a response that is even stronger than that promoted by TCR stimulation, suggesting that iNKT cells are the prominent source of IFN- γ at the onset of the immune response. Yet, we can exclude neither the participation of other regulatory cell populations targeted by R848, nor the implication of additional immunoregulatory cytokines in the final outcome. For instance, TLR7 stimulation activates both dendritic cells (DCs) and NK cells and induces Th1 responses that generate high amounts of Th1-type cytokines such as IFN- γ , IL-10, and IL-12 to counteract the allergic Th2 cytokine profile. In favor of this hypothesis, it has been shown that the TLR3 and TLR7 agonists poly(I:C) and R848 protect mice from allergic asthma, airway inflammation, goblet cell hyperplasia, and AHR in an IL-12-dependent manner, whereas both IL-12 and IL-10 were required for the reduction of eosinophil and lymphocyte counts in BALFs (7). Other investigators have implicated IFN- γ in the anti-allergic effect of R848 following the conversion of human allergen-specific CD4⁺ Th2 lymphocytes into IFN- γ -producing cells. This effect was associated with increased production of IL-12, IFN- α , IL-18, TNF- α , IL-10, and IL-15 by CD14⁺ cells, as well as expansion of IFN- γ -producing CD3⁺CD16⁺ NK cells (23). Note that additional mechanisms of action might come into play, involving notably plasmacytoid DCs and/or suppressive regulatory T cells (24–26).

We provide evidence that iNKT cells can become functional in response to the TLR7 agonist R848 in an inflammatory environment. However, in physiological conditions, DCs are most likely coactors in iNKT cell activation by providing large amounts of proinflammatory molecules. Several reports have described a DC-dependent activation of iNKT cells by microbial products, targeting TLR4, 7, and 9 (19, 20, 27). Indeed, CpG ODNs activate iNKT cells only when plasmacytoid and myeloid DCs are present to provide TLR9-induced type I IFNs and the CD1d required for TCR engagement, respectively. In contrast, IFN- γ production by iNKT cells exposed to LPS depends on IL-12 and IL-18, generated

by DCs (19, 27). Another recent study has demonstrated an IL-12-dependent mechanism in IFN- γ production by iNKT cells (28).

R848 drives iNKT cell activation through the TLR7/MyD88 pathway, in accordance with a role during early innate immune responses to infection. Stimulation of TLR7 may enable iNKT cells to control viral replication, as already reported following exposure to viruses, like HIV-1, hepatitis C virus, hepatitis B virus, or HSV type 1 (29–32). This novel mechanism of activation may explain the response to murine cytomegalovirus infection by iNKT cells reported by Wesley et al. (33), who suggest that they sense exogenous stimuli through a mechanism that does not require CD1d.

Acknowledgments

We thank S. Akira and T. Taniguchi for providing MyD88^{−/−} and α 18^{−/−} mice, respectively. We thank S. Diem and J. M  gret for technical support in flow cytometry and cell sorting, respectively, and the National Institutes of Health tetramer core facility.

Disclosures

The authors have no financial conflicts of interest.

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