

Poly(I:C) promotes the production of IL-17A by murine CD1d-driven invariant NKT cells in airway inflammation

A. Vultaggio^{1,*}, F. Nencini^{2,3,*}, S. Pratesi^{2,3}, G. Petroni^{2,3}, S. Romagnani^{2,3} & E. Maggi^{2,3}

¹Immunoallergy Unit, Azienda Ospedaliero-Universitaria Careggi, University of Florence; ²Centre of Research, Transfer and High Education DENOTHE, University of Florence; ³Department of Internal Medicine, University of Florence, Florence, Italy

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Keywords

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Correspondence

Dr. Enrico Maggi, MD, Immunoallergy Unit, Centre for Research, Transfer and High Education 'DENOTHE', University of Florence, Policlinico di Careggi, Viale Morgagni, 85, 50134 Firenze, Italy.
Tel.: +39-055-4271049
Fax: +39-055-4271028
E-mail: e.maggi@dmf.unifi.it

*A.V. and F.N. equally contributed to the paper.

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Abstract

Background: IL-17A is associated with different asthma phenotypes as virus-associated or steroid-resistant asthma. Invariant natural killer T (iNKT) cells play an important role in the pathogenesis of asthma. The aim of the study was to evaluate the activity of polyinosinic-polycytidylic acid [poly(I:C)] on IL-17A production by CD1d-activated iNKT cells.

Methods: We analysed the *in vitro* effect of poly(I:C) on the release of IL-17A by spleen and lung CD1d-activated iNKT cells with α -galactosylceramide (α -GalCer). Its activity was also investigated in an α -GalCer-induced murine models, including lung inflammation. The inhibition of IL-17A by Toll-like receptor (TLR) 7 agonists in the same *in vitro* and *in vivo* models has been analysed.

Results: Poly(I:C) upregulated the *in vitro* IL-17A production by CD1d-activated NK1.1–CD4– iNKT subset, without modifying type 1 and type 2 cytokines. The two stimuli selectively upregulated IL-17A serum levels *in vivo*. Their intratracheal administration resulted in increased airway hyper-reactivity (AHR), neutrophilia in bronchoalveolar lavage and airway inflammation, which were inhibited by anti-IL-17A antibody. Poly(I:C) effects were attributable to IL1 β and IL-23 release from dendritic cells, as showed by inhibition with neutralizing antibodies. TLR7 agonists inhibited the IL-17A production by poly(I:C) plus α -GalCer in the same models. Such effect was associated with the increased production by DC of IL-17A-inhibiting cytokines and the dampening of IL-1 β and IL-23.

Conclusions: Synthetic dsRNA selectively expand a CD1d-driven IL-17A-producing iNKT cell subset, thus explaining the worsening of airway inflammation by some viral infections. TLR3- and TLR7-triggering viral sequences can exert variable and opposite effects on adaptive immune response.

Allergic asthma is a common respiratory disease associated with a Th2-oriented response (1), but recently Th17 cells have been shown to play a role in the pathogenesis of asthma, favouring the recruitment of neutrophils and the evolution to chronicity (2–4). Th17 have been associated with different asthma phenotypes, such as severe-, steroid-resistant and virus-associated asthma (5, 6). A relevant role for invariant natural killer T (iNKT) cells has been shown in murine allergic asthma (7–9). Type I iNKT cells bear a semi-invariant TCR that recognizes a variety of glycolipid microbial antigens presented by CD1d as well as self-lipid-based antigens (10). Human iNKT cells can also be activated by allergens in a CD1d-restricted/TCR-dependent manner (11). Upon

activation, iNKT cells fastly produce IFN- γ , IL-4, IL-13 and IL-17A (7, 9).

The infection by airway-targeted viruses may exacerbate bronchial asthma. The majority of viral dsRNA trigger endosomal Toll-like receptor (TLR) 3 and cytosolic receptors (MDA-5 and RIG-I helicase) which, besides lymphocytes and plasmacytoid dendritic cells (DC), are also expressed on lung epithelial cells, fibroblasts and alveolar macrophages.

In this study, we showed that the dsRNA polyinosinic-polycytidylic acid [poly(I:C)] increased the *in vitro* and *in vivo* IL-17A production by iNKT cells stimulated in an α -galactosylceramide (α -GalCer)-mediated manner. Poly(I:C) expands an IL-17A-producing iNKT cell subset, without

modifying type 1 and type 2 cytokines. In addition, synthetic TLR7 agonists strongly downregulated the α -GalCer/poly(I:C)-mediated IL-17A production. These data suggest that TLR3 agonists may selectively expand an IL-17A-producing iNKT cell subset, thus explaining the worsening of airway inflammation and asthma following viral infections.

Materials and methods

Reagents

The *in vitro* cultures were performed in RPMI 1640 (Biochrom AG, Berlin, Germany), supplemented with 2 mM L-glutamine, 2 mM 2-mercaptoethanol, 100 U/ml penicillin and 100 μ g/ml streptomycin (complete medium) (all from Invitrogen, Milan, Italy) and 5% FCS (Thermo Scientific, Milan, Italy). 2-Butoxy-8-hydroxy-9-benzyl adenine, SA-2, and its inactive analogue (2-butoxy adenine, SA-1) were obtained as described (12, 13). Resiquimod (R848) and poly(I:C) were purchased from InvivoGen (San Diego, CA, USA). Lipopolysaccharide (LPS), polydeoxyadenylic acid–polythymidylic acid sodium salt (polydTA), phorbol 12-myristate 13-acetate, ionomycin, polymyxin B and chloroquine were purchased from Sigma-Aldrich (Milan, Italy), while α -GalCer from Alexis Biochemicals (San Diego, CA, USA). Anti-murine CD11c-APC, anti-murine CD1d (1B1) and rat IgG2b isotype control mAbs were purchased from Becton-Dickinson (Mountain View, CA, USA). Anti-murine CD4-FITC, B220-APC mAbs and anti-fluorochrome microbeads were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Anti-murine IL-17A, anti-IL23R and anti-IL1 β R1 were purchased from R&D Systems (Minneapolis, MN, USA). Goat and hamster IgG isotype controls were purchased from SouthernBiotech (Birmingham, AL, USA). Endotoxin levels in poly(I:C), polydTA and α -GalCer were below 0.003 EU/ml as measured by LAL test (Pyrotell-Associated of Cape Cod Incorporated, E. Falmouth, MA).

Murine models

Pathogen-free 7-week-old C57BL/6 female mice were purchased from Charles River Laboratories (Calco, Italy) and kept under standard housing conditions. All animal studies were performed according to Institutional National guidelines and local animal ethics regulations.

Mice were treated with a single i.p., i.v. or i.t. administration of 2 μ g/mice α -GalCer plus different doses of poly(I:C) or polydTA. In some experiments, mice were i.p.-treated with neutralizing anti-IL17A mAb (25 μ g/mice) 6 h before the i.v. administration of α -GalCer/poly(I:C). In other experiments, SA-2 (50 μ g) were i.p. administered 4 h before the i.v. treatment with α -GalCer/poly(I:C) (or α -GalCer/polydTA as control). For the analysis, mice were killed by i.p. injection of pentobarbitone (Sigma-Aldrich) 6 h later.

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed and BAL fluid (BALF) analysed as described (13).

Evaluation of AHR

Airway hyper-reactivity (AHR) in response to increasing doses of inhaled methacholine (Sigma-Aldrich) was measured as described (14).

Lung histological analysis

Lungs were dissected after perfusion with saline *via* the heart. H&E staining and lung inflammation were performed as described (13).

Lung and spleen mononuclear cells' preparation

Spleen and lung mononuclear cells (MNC) were prepared as previously described (15), modified by the use of Gentle-MACS from Miltenyi Biotec.

Cell isolation and co-cultures

CD11c+ cells were purified (>98%) from spleens of C57BL/6 mice by positive selection with MACS Microbeads (Miltenyi Biotec) according to the manufacturer's instructions. Bone marrow DC (BMDC), obtained as previously described (15), were pulsed with medium or poly(I:C) (2.5 μ g/ml) for 2 h, washed and cultured for 3 h in a complete medium. α -GalCer-activated spleen MNC were cultured with these supernatants, and cultured cells were analysed at the 3-day culture supernatants for cytokines levels. In some experiments, we obtained NK1.1+ and NK1.1– cells after the depletion of CD8+, NKp46+, CD115+, TCR γ / δ +, CD45R+ cells from spleen MNC. Subsequently, we purified NK1.1– CD4– CD3+ cells using sequential immunomagnetic selection with mAbs bound to MACS Microbeads (Miltenyi Biotec) according to the manufacturer's instructions.

Measurement of cytokines

Cytokine levels in the serum and cell culture supernatants were measured by ELISA (R&D Systems, Minneapolis, MN, USA).

Transwell experiments

Transwell experiments were performed as previously described (16).

Quantitative mRNA analysis

Total RNA from snap-frozen mouse lungs were extracted using Trizol reagent (RNAwiz; Invitrogen, Milan, Italy), whereas total RNA from MNC or purified cell subsets were extracted using RNeasy mini kit (Qiagen, Milan, Italy). Real-time quantitative PCR (RT-PCR) was performed as described by using commercial primers (13).

ELISPOT assay

IL-17A production by spleen MNC was performed using mouse IL-17A Development Module (R&D Systems). Image

analysis of ELISPOT assays was performed using AID ELISpot reader (AID Autoimmun Diagnostika GmbH, Strassberg, Germany).

Statistical analysis

Results are expressed as mean values $\pm$ SEM. Statistical analysis was performed using the Student's *t* test. *P* values <0.05 were considered as significant.

Results

Poly(I:C) promotes IL-17A production by CD1d-driven iNKT cells *in vitro* and *in vivo*

The *in vitro* effect of poly(I:C) on the activity of CD1d-driven iNKT cells was firstly evaluated. Poly(I:C), but not polydTA, synergized with α -GalCer in a dose-dependent manner for the mRNA expression and production of IL-17A, but not IFN- γ , IL-4 and IL-13 by spleen and lung cells. When used alone, α -GalCer and poly(I:C) induced poor or no IL-17A production (Fig. 1A and data not shown). Poly(I:C) maintained its effects also in polymyxin B-pretreated spleen cells, in which the LPS uptake to TLR4/CD14 receptor was inhibited (17) (data not shown).

The activity of α -GalCer/poly(I:C) on Th17 response was also confirmed by the upregulation of ROR γ t and IL-23R mRNA expression (Fig. 1B, left panel). miRNA-326 expression was significantly higher in α -GalCer/poly(I:C)-stimulated spleen cells than in those cultured with α -GalCer/polydTA or α -GalCer, poly(I:C) and polydTA alone (Fig. 1B, right panel).

Previous reports indicated that α -GalCer alone induces IL-17A, IL-4, IL-13 and IFN- γ production by lung and spleen CD1d-driven iNKT cells independently of the administration routes (18, 19). We then assessed the effects of poly(I:C) in *in vivo* models by using different routes of administration of α -GalCer/poly(I:C). Mice were i.p.- or i.v.- treated with α -GalCer plus increasing doses of poly(I:C) (10, 50 and 250 μ g/mice) [α -GalCer/poly(I:C) mice] or polydTA (α -GalCer/polydTA mice). Serum levels of IL-17A, but not IFN- γ , IL-4 and IL-13, were increased in a dose-dependent fashion in α -GalCer/poly(I:C) mice compared to controls (Fig. 2A). Accordingly, IL-17A mRNA expression, as well as the proportion of IL-17A-producing spleen cells, was significantly increased in α -GalCer/poly(I:C) i.v.- or i.p.-treated mice, but not in controls (Fig. 2B,C).

Twenty-four hours after the i.t. treatment, AHR was significantly higher in α -GalCer/poly(I:C) mice than in α -GalCer/polydTA mice or mice treated with poly(I:C) alone (Fig. 3A). α -GalCer/poly(I:C) mice also showed higher mRNA expression of IL-17A (not IL-13 and IFN- γ) and recruitment

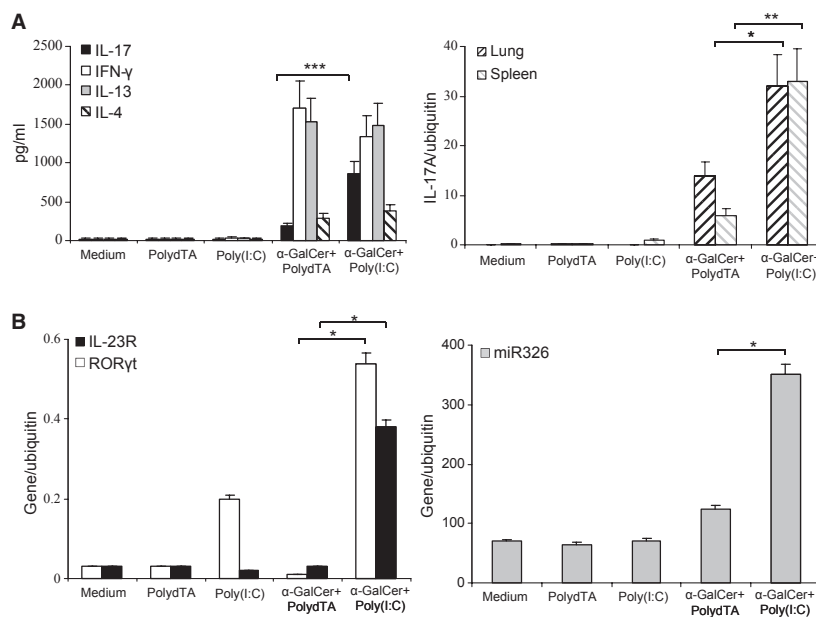


Figure 1 Poly(I:C) upregulates CD1d-driven IL-17A production by spleen and lung mononuclear cells (MNC) *in vitro*. (A) Spleen MNC (2×10^6 /ml) from C57BL/6 mice were stimulated *in vitro* with α -GalCer (10 ng/ml) in the presence of poly(I:C) or polydTA (2.5 μ g/ml). Three-day culture supernatants from spleen cells were assessed for IL-17A, IFN- γ , IL-13 and IL-4 production by ELISA. Data expressed as mean values ($\pm$ SEM) from six separate experiments are reported (left panel). Six-hour cultured spleen and lung

cells, stimulated as above, were analysed for IL-17A mRNA expression by using RT-PCR. Results are expressed as gene/ubiquitin ratio (mean values $\pm$ SEM) of eight separate experiments (right panel). (B) Six-hour cultured spleen cells (as in A) were analysed for ROR γ t and IL-23R(p19) (left panel) and miR326 (right panel) expression by using RT-PCR. Data are expressed as gene/ubiquitin ratio (mean values $\pm$ SEM) of four separate experiments. **P* < 0.05 ; ***P* < 0.01 ; ****P* < 0.001 .

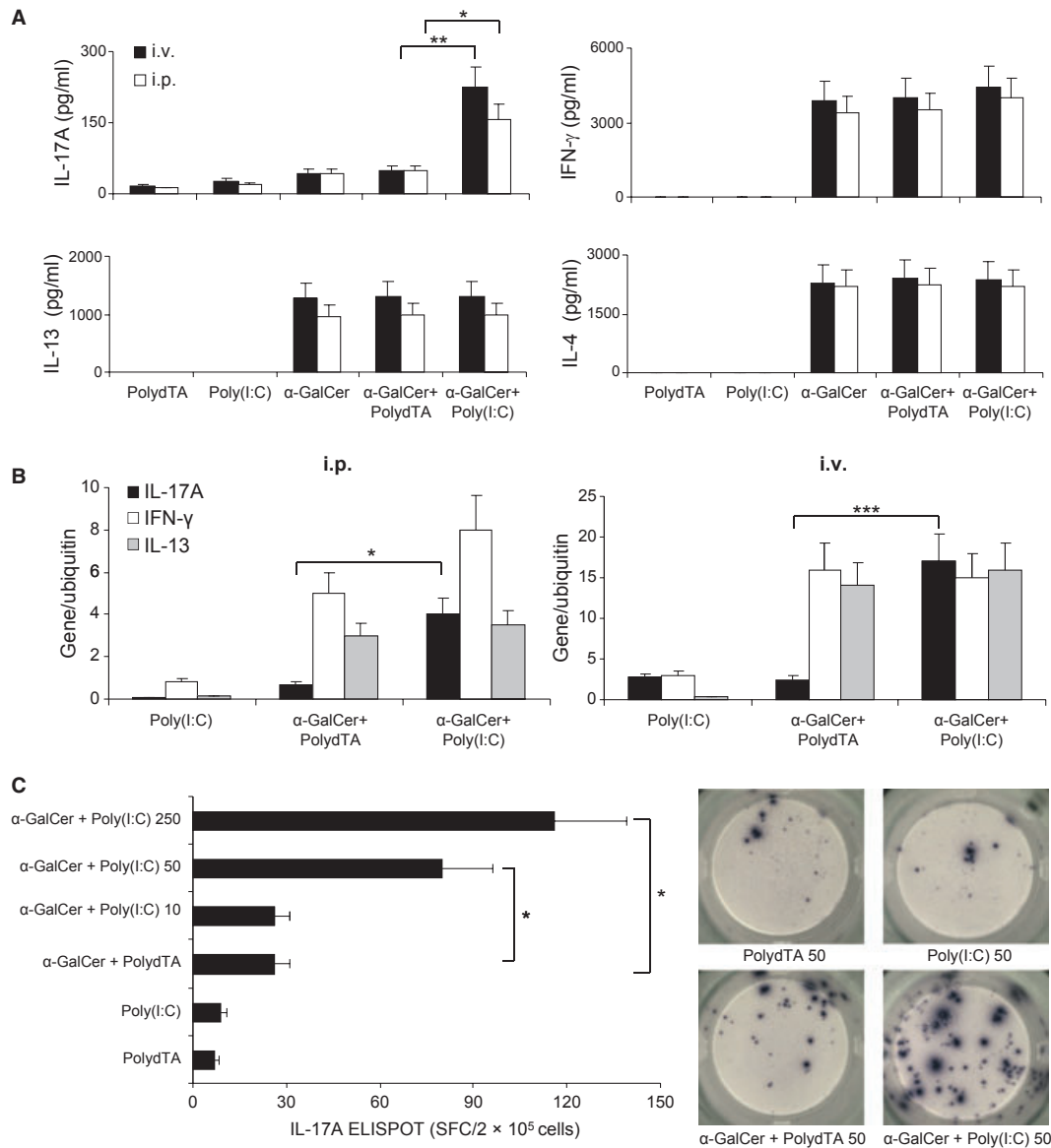


Figure 2 Poly(I:C) synergizes with α -GalCer for the upregulation of IL-17A *in vivo*. (A) Cytokine (IL-17A, IFN- γ , IL-13 and IL-4) serum levels were measured by ELISA 6 h after the i.p. or 4 h after the i.v. administration of poly(I:C) (250 μ g/mice) plus α -GalCer (2 μ g/mice) or polydTA plus α -GalCer or poly(I:C) alone. Data are expressed as mean values ($\pm$ SEM) from five separate experiments (five mice/group/experiment). (B) C57BL/6 mice were treated as in (A) and the spleen IL-17A, IL-13 and IFN- γ mRNA expression was analysed 6 h after the

i.p. (left panel) or the i.v. (right panel) treatment by RT-PCR. Results are expressed as gene/ubiquitin ratio (mean values $\pm$ SEM) of four separate experiments (five mice/group/experiment). (C) The number of IL-17A-producing cells was analysed in the spleen of C57BL/6 mice i.p.-treated with α -GalCer plus poly(I:C) or polydTA (as in A) by calibrated ELISPOT assay. Data are expressed as mean of spot-forming cells (SFC)/2 $\times$ 10⁵ cells ($\pm$ SEM) from three separate experiments. * P < 0.05, ** P < 0.02, *** P < 0.001.

of neutrophils in BALF as well as an enhanced lung perivascular (3.25 ± 0.07 vs 2.06 ± 0.39 , $P = 0.076$) and peribronchial (2.7 ± 0.14 vs 1.58 ± 0.6 , $P = 0.064$) inflammation scores than α -GalCer/polydTA mice (Fig. 3B,C and data not shown).

The treatment with neutralizing anti-IL-17A, but not the isotype control mAb, resulted in a significant reduction in Penh values and neutrophils in BALF (Fig. 3D).

Poly(I:C)-stimulated DC selectively produce molecules essential for the upgrowth of IL-17A-producing iNKT cells

Firstly, we investigated whether poly(I:C)-stimulated IL-17A production through the engagement of endosomal (TLR3) or cytosolic (MDA5 and RIG-I) receptors, by adding chloroquine to α -GalCer/poly(I:C)-stimulated spleen cells. Chloroquine inhibited in a dose-dependent

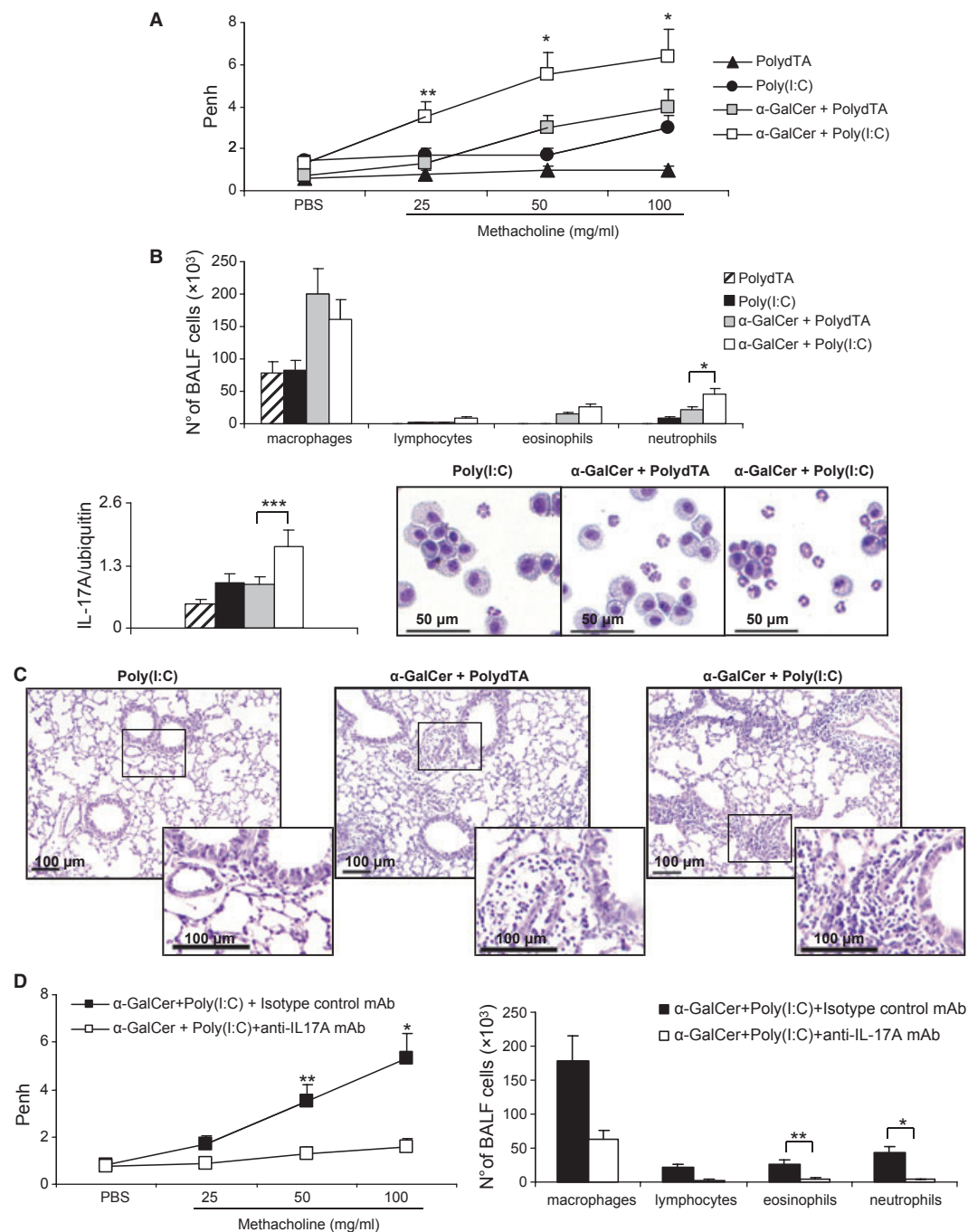


Figure 3 Poly(I:C) upregulates the α -GalCer-induced lung inflammation. (A) Airway hyper-reactivity (AHR) was measured 24 h after the i.t. administration of α -GalCer (2 μ g/mice) plus poly(I:C) or polydTA (250 μ g/mice). Control mice received poly(I:C) or polydTA alone. Penh = Enhanced pause. Values are expressed as mean Penh ($\pm$ SEM) of three separate experiments (six mice/group). *P* values refer to values of α -GalCer/poly(I:C) group vs α -GalCer/polydTA. (B) Forty-eight hours after the i.t. administration of poly(I:C) plus α -GalCer as in (A), Bronchoalveolar lavage (BAL) was performed and BAL fluid (BALF) was taken for cellular analysis. Absolute values ($\pm$ SEM) of cells in BAL (upper panel) and IL-17A mRNA expression in BAL

cells (lower left panel), obtained from three experiments (six mice/group), are reported. The morphological aspects of spun- and Diff-Quick-stained cells in a representative experiment are reported (lower right panel). (C) Representative photomicrographs of H&E-stained lung tissue sections ($\times 10$) and inlets ($\times 100$). (D) AHR and BALF analysis performed as in (A) in mice treated with anti-IL-17A or isotype control mAbs, as described in Methods. Values of Penh are expressed as mean ($\pm$ SEM) of three separate experiments (six mice/group) (left panel). Absolute values ($\pm$ SEM) of cells in BAL are reported (right panel). **P* < 0.05; ***P* < 0.02, ****P* < 0.01.

manner the production of IL-17A, thus suggesting that poly(I:C) effect was attributable to TLR3 triggering (Fig. 4A).

We then studied the activity of supernatants obtained from poly(I:C)-pulsed BMDC. We found that they were able to induce a dose-related increase in IL-17A (not IL-13 and

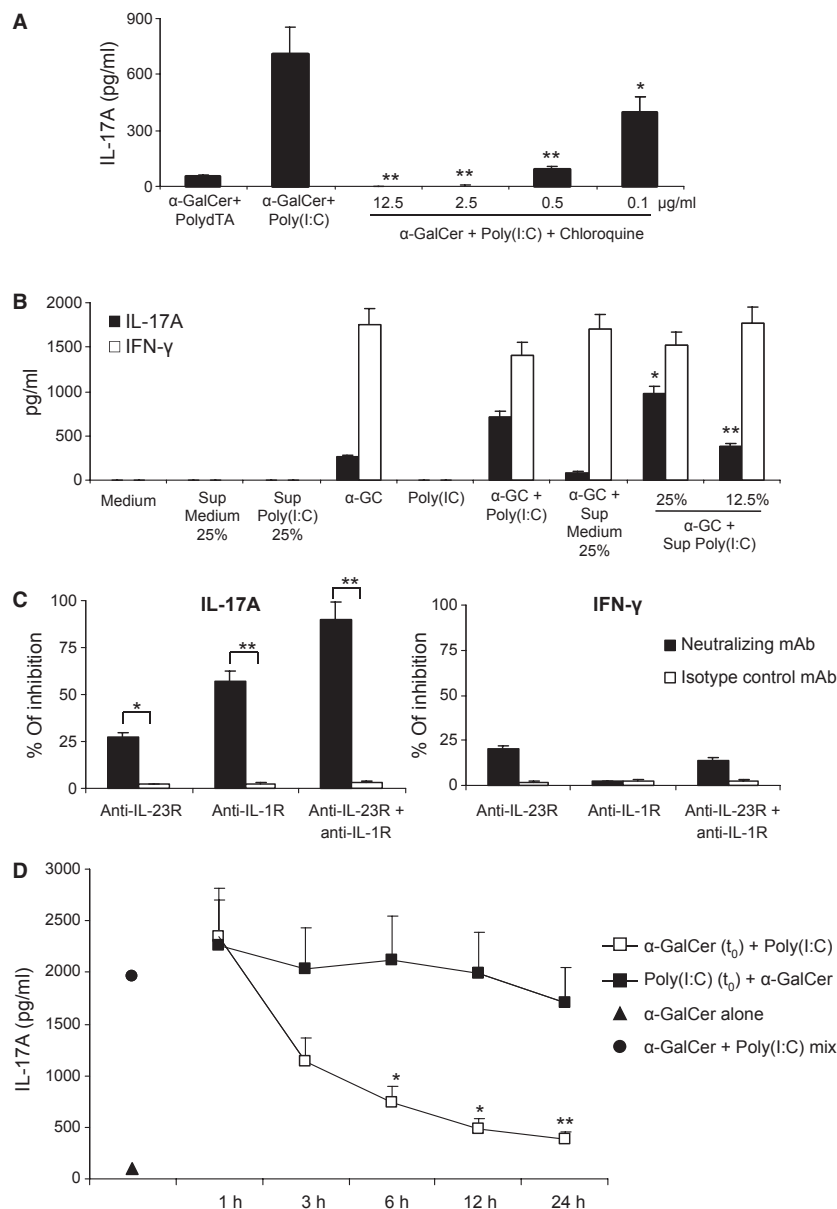


Figure 4 Poly(I:C) stimulates bone marrow dendritic cells (BMDC) to produce IL-17A-inducing molecules. (A) Spleen mononuclear cells (MNC) from C57BL/6 mice were stimulated with α -GalCer plus poly(I:C) or polydTA (as in Fig. 1A) in the presence of different doses of chloroquine. Three-day culture supernatants were assessed for IL-17A production by ELISA. Results are expressed as mean values ($\pm$ SEM) from three separate experiments. (B) α -GalCer-activated spleen cells (2×10^6 /ml) from C57BL/6 mice were cultured in the presence of different supernatants from unstimulated (Sup medium) or poly(I:C)-stimulated [Sup poly(I:C)] BMDC, as described in Methods. Three-day culture supernatants were assessed for IL-17A and IFN- γ production by ELISA. Data

are expressed as mean values ($\pm$ SEM) from three separate experiments. (C) Spleen MNC from C57BL/6 mice were stimulated with α -GalCer plus poly(I:C) or polydTA in the presence of anti-IL-23R (10 μ g/ml), anti-IL-1 β R (50 ng/ml) or the mixture of two mAbs. Three-day culture supernatants were assessed for IL-17A and IFN- γ production by ELISA. Data are expressed as mean values ($\pm$ SEM) of per cent inhibition of cytokines' production from three separate experiments. (D) Spleen cells were stimulated with α -GalCer or poly(I:C) and at different time poly(I:C) or α -GalCer was added. Respectively, 3-day culture supernatants from these cells were assessed for IL-17A production by ELISA as described in Methods. Data are expressed as mean values ($\pm$ SEM) from three separate experiments. * $P < 0.01$; ** $P < 0.001$.

IFN- γ) by α -GalCer-stimulated spleen MNC. Transwell experiments, performed by seeding poly (I:C)-pulsed BMDC in the upper chamber and the α -GalCer-pulsed DC plus CD4 + T cells in the lower chamber, confirmed that IL-17A was upregulated by soluble molecules secreted in the upper chamber (Fig. 4B and data not shown).

Poly(I:C)- but not polydTA-loaded CD11c+ and BMDC exhibited a slight upregulation of the IL-1 β and IL-23 mRNA expression, without any effect on IL-6, IL-10 and IL-12, which, in contrast, were strongly increased by LPS (data not shown). To confirm this finding, we tested the effects of neutralizing anti-IL-1 β R and anti-IL-23R mAbs on α -GalCer/poly(I:C)-stimulated spleen MNC. The IL-17A production was partially inhibited by each mAb and almost abrogated by their mixture, whereas their isotype controls did not exert any effect (Fig. 4C).

Finally, we assessed the influence of the two stimuli on the IL-17A kinetics *in vitro*. High IL-17A levels were observed in 3-day supernatants when α -GalCer was added later (from 1 to 24 h) to the cultures of the poly(I:C)-stimulated cells, whereas they progressively decreased in 3-day supernatants when poly(I:C) was added later (from 1 to 24 h) to the cultures of α -GalCer-stimulated cells (Fig. 4D).

NK1.1- CD4- iNKT cells are the major source of IL-17A upon α -GalCer plus poly(I:C) stimulation

To confirm the role of iNKT cells in the amplification of IL-17A response induced by poly(I:C), the neutralizing anti-CD1d mAb was added to α -GalCer/poly(I:C)-stimulated spleen MNC. The IL-17A production was reduced in a dose-dependent manner by the anti-CD1d but not the isotype control mAbs (Fig. 5A).

Taking into account that iNKT cells consist of functionally distinct subsets (20), we next evaluated whether the IL-17A induced by α -GalCer/poly(I:C) was preferentially produced by a defined iNKT cell subset. After initial depletion of spleen MNC from NK, B, pDC, CD8a and γ/δ T cells, we purified two (NK1.1+ CD3+ and NK1.1- CD4- CD3+) iNKT cell subsets. Upon α -GalCer stimulation, IL-17A and IL-13 (but not IFN- γ and IL-4) were detected in supernatants from NK1.1- CD4- iNKT cells, whereas NK1.1+ iNKT cells produced high levels of IFN- γ and IL-4, low levels of IL-13 and no IL-17A. More importantly, the addition of poly(I:C) strongly upregulated the IL-17A only from NK1.1- CD4- iNKT subset, with no effect on type 1 and type 2 cytokines produced by both CD1d-driven iNKT subsets (Fig. 5B).

TLR7 agonists inhibit IL-17A induced by α -GalCer plus poly (I:C)

We asked whether TLR7 agonists exerted some activity on IL-17A driven by α -GalCer/poly(I:C)-stimulated cells. In a time course experiment, we showed that SA-2 and R848, but not SA-1, significantly inhibited IL-17A by α -GalCer/poly(I:

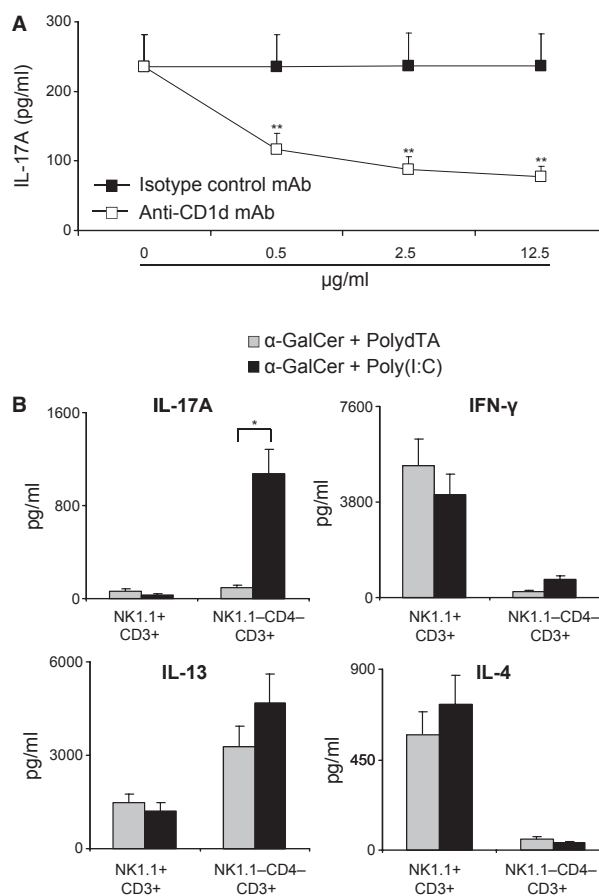


Figure 5 IL-17A production by NK1.1- CD4- iNKT cells upon α -GalCer plus poly(I:C) stimulation. (A) Spleen mononuclear cells from C57BL/6 mice were stimulated with α -GalCer plus poly(I:C) in the presence of increased dose of neutralizing anti-CD1d (1B1) or isotype control mAbs. Three-day culture supernatants were assessed for IL-17A level by ELISA. Results, expressed as mean values ($\pm$ SEM) from three separate experiments, are reported. (B) NK1.1- CD4- and NK1.1+ iNKT cells (1.5×10^6 /ml) were co-cultured with CD11c+ (0.5×10^6 /ml) in the presence of α -GalCer plus poly(I:C) or polydTA. Three-day culture supernatants were assessed for IL-17A, IFN- γ , IL-13 and IL4 production by ELISA. Data are expressed as mean values ($\pm$ SEM) from three separate experiments. * $P < 0.05$; ** $P < 0.01$.

C)-stimulated spleen MNC and upregulated IL-27 and IL-10 in the same culture supernatants (Fig. 6A). They inhibited mRNA expression of IL-1 β and IL-23, but increased that of IL-10 and IL-27, on poly(I:C)-stimulated CD11c+ cells or BMDC (data not shown).

The i.p. administration of SA-2 before the i.v. treatment with α -GalCer/poly(I:C) decreased the IL-17A serum levels without affecting IL-13 and IFN- γ (Fig. 6B and data not shown). Finally, i.p.-administered SA-2 decreased the mRNA expression of IL-17A (but not of IL-13 and IFN- γ) and increased that of IL-10 and IL-27 (Fig. 6C and data not shown), in spleen and lung MNC of i.p. α -GalCer/poly(I:C)-treated mice.

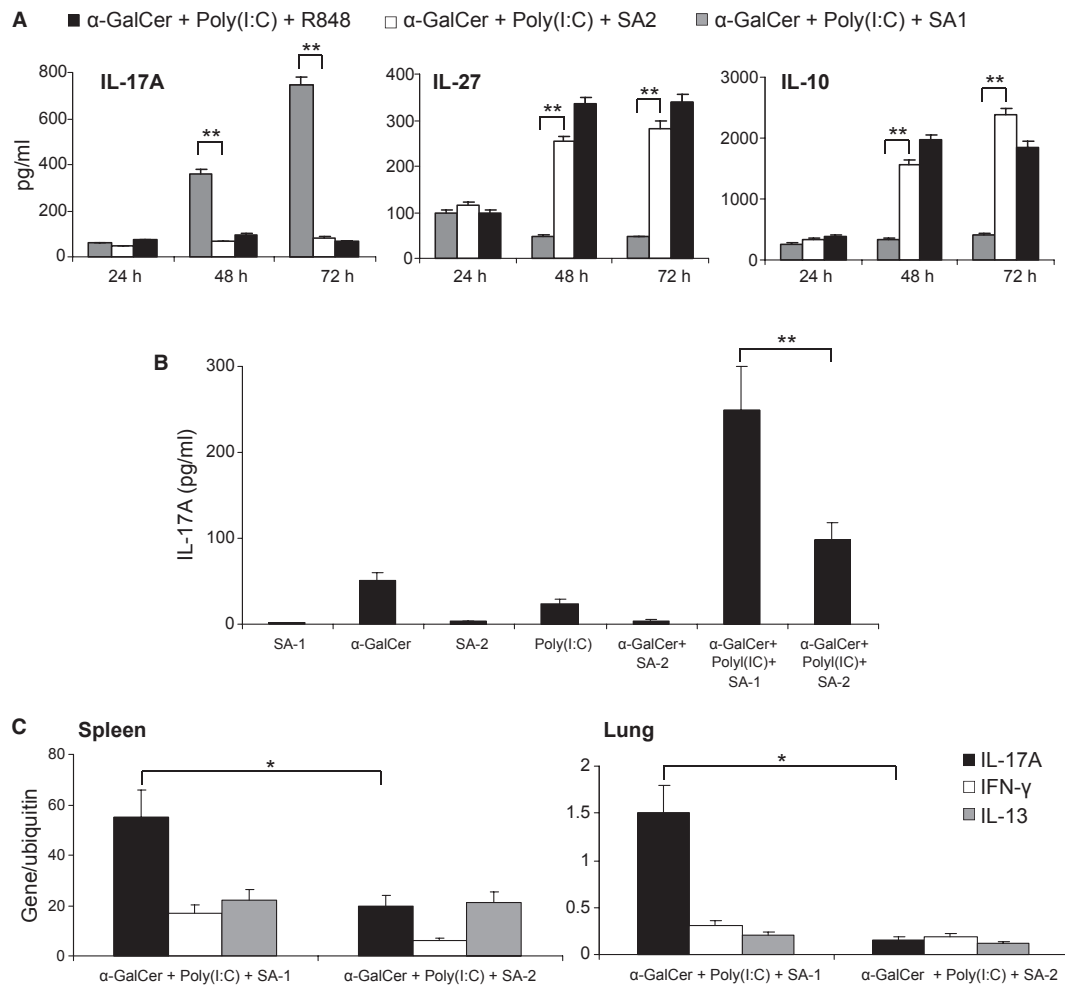


Figure 6 TLR7 agonists selectively inhibit the IL-17A production by α -GalCer/poly(I:C)-stimulated T cells. (A) Cytokine production by spleen cells (2×10^6 /ml) stimulated as in Fig. 1A for 24, 48 and 72 h, in the presence of SA-2 (10 μ g/ml) or SA-1 (10 μ g/ml) or R848 (6 μ M). Data are expressed as mean values ($\pm$ SEM) from four separate experiments. (B) SA-2 or SA-1 were i.p. administered 4 h before the i.v. treatment with poly(I:C) (250 μ g/mice) plus α -GalCer

(2 μ g/mice). Blood sera, collected 6 h later, were assessed for IL-17A by ELISA. Results are expressed as mean values ($\pm$ SEM) from three separate experiments (five mice/groups/experiment). (C) Mice were treated as in (B) and mRNA expression of cytokines was evaluated on spleen and lung tissues by RT-PCR. Data are expressed as gene/ubiquitin ratio (mean values $\pm$ SEM) of three separate experiments (five mice/group/experiment). * $P < 0.05$; ** $P < 0.01$.

Discussion

In this study, we examined the activity of the dsRNA poly(I:C) on the IL-17A production by α -GalCer-activated iNKT cells, a model mimicking the activity of both RNA viruses and microbial antigens on inflamed lung tissue (10). Because LPS contaminants are able to induce IL-17A “di per se” (21), we selected endotoxin-free reagents that were subsequently used throughout the study. Poly(I:C) synergizes *in vitro* and *in vivo* with α -GalCer for IL-17A, but not for IFN- γ and IL-4 or IL-13 production. Alpha-GalCer/poly(I:C) stimulation also upregulates the expression of Th17-related molecules (ROR γ t, IL-23R) as well as of miRNA-326, which have been associated with the activation and expansion of Th17 cells (22, 23).

In vivo α -GalCer plus poly(I:C) induced a significant increase in neutrophils in BALF and AHR, which were significantly downregulated when mice were pretreated with anti-IL-17A mAb, thus confirming a relevant role of this cytokine in lung inflammation.

Even though indirectly, data obtained in mice and humans agree with our results (24–26). However, our data are at odds with a study referring that poly(I:C) directly induces IL-17A and IL-21 by polyclonally activated human CD4 $^{+}$ T cells (27). Our experiments exclude a direct activity of poly(I:C) on CD4 $^{+}$ T cells, showing that IL-17A was induced by soluble factors produced by poly(I:C)-pulsed BMDC such as IL-1 β and IL-23. The *in vitro* production of IL-17A (but not IFN- γ) was inhibited by anti-IL-1 β R or anti-IL-23R mAbs and virtually abrogated with the mixture of the two mAbs.

However, we cannot exclude in our system a role of other molecules such as prostaglandin E2 (PGE2), which increases IL-17A through the upregulation of IL-1 β R and IL-23R on T cells (28).

We also showed that poly(I:C) triggers endosomal (TLR3) and not cytosolic (MDA5 and RIG-I helicases) receptors on APC, because IL-17A induced by α -GalCer/poly(I:C) *in vitro* was strongly inhibited by chloroquine, an antimalaric drug which enables the poly(I:C) molecular structure interfering only with endosomal receptors (29).

Finally, we showed that poly(I:C) induced a prolonged activation of DC favouring the subsequent IL-17A production by α -GalCer-induced iNKT cells. This suggests that viral infections can modify the lung milieu for long time, thus expanding pathogenic IL-17A-secreting iNKT cells when the CD1d-driven stimulation occurs.

One important issue of the paper is the demonstration that an iNKT cell subset is actually the main responsible for the production of IL-17A. We clearly showed that IL-17A was upregulated by poly(I:C) only on α -GalCer-stimulated NK1.1 $^{+}$ CD4 $^{-}$ iNKT cells, when co-cultured with purified CD11c $^{+}$ cells. Our data confirm previous results on the preferential IL-17A production by NK1.1 $^{+}$ CD4 $^{-}$ iNKT cells upon α -GalCer activation (20).

TLR7 agonists, able to induce IL-10 and IL-27 upregulation, inhibit poly(I:C) effect on IL-17A response. In addition to a cytokine-mediated regulatory mechanism, TLR7 agonists may reduce the TLR3-driven effect, also through their nucleic acid-binding ability, thus preventing the interaction of dsRNA with TLRs (18, 29). Several *in vivo* studies have

shown that TLR7 agonists reduce airway inflammation through the increased expression of type 1 cytokines (13, 30, 31). Allergic asthma is worsened by viral infections, especially sustained by rhinoviruses, which trigger TLR3, different to other respiratory viruses that stimulate both TLR3 and TLR7 receptors (influenza, respiratory syncytial) (32). It has been clearly shown a tight association of asthma with rhinovirus infection, but not other respiratory viruses (33). We can speculate that the predominant activation of TLR3 by rhinoviruses induces a strong lung inflammation, also through a selective increase in lung IL-17A response, whereas other viruses activating both TLR3 and TLR7, or TLR7 alone, induce less inflammation.

This finding associated with the described TLR7 polymorphism in asthma (34) and the bronchodilatory effects of several TLR7 ligands (35) make these compounds attractive candidates for novel vaccine formulations of respiratory allergy.

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Conflict of interest

The authors declare no conflict of interests.

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