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This information is current as of March 11, 2011

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J Immunol; Prepublished online 9 March 2011;

doi:10.4049/jimmunol.1002398

<http://www.jimmunol.org/content/early/2011/03/04/jimmunol.1002398>

Supplementary Data

<http://www.jimmunol.org/content/suppl/2011/03/07/jimmunol.1002398.DC1.html>

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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 Ligand 9-Benzyl-2-Butoxy-8-Hydroxy Adenine Inhibits IL-17 Response by Eliciting IL-10 and IL-10–Inducing Cytokines

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This study evaluates the ability of a novel TLR7 ligand (9-benzyl-2-butoxy-8-hydroxy adenine, called SA-2) to affect IL-17 response. The SA-2 activity on the expression of IL-17A and IL-17–related molecules was evaluated in acute and chronic models of asthma as well as in in vivo and in vitro α -galactosyl ceramide (α -GalCer)-driven systems. SA-2 prepriming reduced neutrophils in bronchoalveolar lavage fluid and decreased methacholine-induced airway hyperresponsiveness in murine asthma models. These results were associated with the reduction of IL-17A (and type 2 cytokines) as well as of molecules favoring Th17 (and Th2) development in lung tissue. The IL-17A production in response to α -GalCer by spleen mononuclear cells was inhibited in vitro by the presence of SA-2. Reduced IL-17A (as well as IFN- γ and IL-13) serum levels in mice treated with α -GalCer plus SA-2 were also observed. The in vitro results indicated that IL-10 produced by B cells and IL-10–promoting molecules such as IFN- α and IL-27 by dendritic cells are the major player for SA-2–driven IL-17A (and also IFN- γ and IL-13) inhibition. The in vivo experiments with anti-cytokine receptor Abs provided evidence of an early IL-17A inhibition essentially due to IL-10 produced by resident peritoneal cells and of a delayed IL-17A inhibition sustained by IFN- α and IL-27, which in turn drive effector T cells to IL-10 production. These findings suggest that such TLR7 agonist downregulating Th17 (as well as Th2) response has to be considered a valid candidate for novel vaccine formulations in allergy. *The Journal of Immunology*, 2011, 186: 000–000.

The IL-17 family includes the isoforms IL-17A and IL-17F, which share the highest degree of homology and are known to play a relevant role in the pathogenesis of autoimmune and chronic inflammatory diseases (1). Increasing interest has been recently focused on the role of IL-17A/F in acute and chronic allergic asthma and in the induction of fibrosis and tissue remodeling (2–4). IL-17A is expressed by cells in airways

of patients with moderate to severe asthma, and its level is high in the serum and sputum of patients with asthma and correlates with neutrophil infiltration and airway hyperresponsiveness (AHR) (5, 6).

Classical or type I NKT cells bear a semi-invariant TCR (invariant NKT [iNKT] cells) that recognizes a variety of glycolipid Ags presented by the CD1d molecule, including α -galactosyl ceramide (α -GalCer), derived from a marine sponge, absent in mammalian cells. When activated, iNKT cells secrete a burst of cytokines, such as IFN- γ , IL-4, and IL-13 (7). Results obtained in murine models clearly show a relevant role for iNKT cells in the pathogenesis of allergic asthma. In iNKT-knockout mice, OVA-induced allergic inflammation is impaired with decreased AHR, bronchoalveolar lavage fluid (BALF) eosinophilia, and specific IgE- and Th2-related cytokines by mononuclear cells (MNC) from draining lymph nodes (dLNs) (7). When directly activated by intranasal administration of α -GalCer, iNKT cells are the major effectors for the development of AHR in mice lacking conventional CD4⁺ T cells (8). Lastly, it has been shown that a distinct subset of murine NKT cells producing IL-17 upon stimulation with α -GalCer led to lung inflammation (9).

TLR are pattern recognition molecules expressed by many types of cells and represent crucial triggers for adaptive immune response (10). Besides dendritic cells (DC) and B cells, TLR are expressed by T (both T $\alpha\beta$ and T $\gamma\delta$ cells), NKT, and NK cells (10). Natural and synthetic TLR ligands related to viral structures have been shown to trigger endosomal TLR as TLR3, -7/8, and -9 (10). We recently described a synthetic heterocycle chemically related to adenine (9-benzyl-2-butoxy-8-hydroxy adenine), referred to as SA-2, which triggers TLR7 in both human and murine cells (11, 12). TLR-stimulated DCs link innate and adaptive immunity by promoting polarization of effector T cells. The influence of mi-

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Received for publication July 16, 2010. Accepted for publication February 6, 2011.

This work was supported by funds provided by Tuscany Region (Health Research Programme 2009), the Italian Ministry of Education (Programmi di Ricerca di Rilevante Interesse Nazionale projects), the Italian Ministry of Health (Strategic Project 2008), the Italian Association for Cancer Research, and European Union Projects SENS-IT-IV (FP6-LSBH-CT-2006-018861) and INNOCHEM (FP6-LSHB-CT-2005-518167).

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The online version of this article contains supplemental material.

Abbreviations used in this article: AHR, airway hyperresponsiveness; Alum, aluminum hydroxide; BAL, bronchoalveolar lavage; BALF, bronchoalveolar lavage fluid; DC, dendritic cell; dLN, draining lymph node; α -GalCer, α -galactosyl ceramide; iNKT, invariant NKT; i.t., intratracheal; MNC, mononuclear cell; PAS, periodic acid-Schiff; PF, peritoneal fluid; SA-1, 2-butoxy adenine; SA-2, 9-benzyl-2-butoxy-8-hydroxy adenine; TLR7L, TLR7 ligand.

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crobial stimulation on IL-17-mediated responses has been clearly demonstrated for TLR2 or TLR4 ligands, such as dectin and LPS (13, 14), whereas the effect of TLR7 ligand (TLR7L) on IL-17 response has been poorly investigated (15, 16).

In this report, we examined the *in vitro* and *in vivo* activity of SA-2 on IL-17A/F and IL-17-associated molecules. By using murine models of acute and chronic asthma and an α -GalCer-mediated experimental setting, we showed that SA-2 significantly inhibits Th17 response. More importantly, our results provide evidence for a relevant role of the IFN- α /IL-27/IL-10 regulatory axis in SA-2-mediated effects.

Materials and Methods

Reagents

The medium used for *in vitro* cultures was RPMI 1640 (Biocrom), supplemented with 2 mM L-glutamine, 2 mM 2-ME, 100 U/ml penicillin, 100 μ g/ml streptomycin (complete medium) (all from Invitrogen, Milan, Italy), and 5% FCS (Thermo Fisher Scientific, Milan, Italy). Synthetic heterocycle related to adenine (SA-2) and its inactive analog (2-butoxy adenine [SA-1]) were obtained as described (11, 12). Endotoxin levels in modified adenines were >0.003 EU/ml as measured by the *Limulus* ameocyte assay (BioWhittaker, Walkersville, MD). OVA and phorbol 12-myristate 13-acetate/ionomycin was purchased from Sigma-Aldrich (Milan, Italy) and α -GalCer from Alexis Biochemicals (San Diego, CA). Anti-murine CD11c allophycocyanin, IFN- γ allophycocyanin, IL-17A PE, IgG1 PE, as well as anti-I-A/I-E FITC mAbs and Recombinant Soluble Dimeric Mouse CD1d:Ig fusion protein were purchased from BD Biosciences (Mountain View, CA). Anti-mouse IFN- α / β R2, IL-10R α , IL-27p28 (subsequently referred as anti-IFN- α R, anti-IL-10R, and anti-p28), and anti-CCR3 PE Abs were purchased from R&D Systems (Minneapolis, MN), whereas anti-murine F4/80 allophycocyanin, NK1.1 allophycocyanin, IL-13 Alexa Fluor 647, and IL-10 PE Abs were purchased from eBioscience (San Diego, CA). Anti-murine CD4 FITC, CD3 PE and allophycocyanin, CD8 FITC, DX5 FITC, and B220 allophycocyanin mAbs as well as anti-FITC Microbeads were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany).

Murine models

Pathogen-free 7-wk-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 systemically sensitized by *i.p.* injection of OVA (10 μ g) adsorbed in 2.25 μ g aluminum hydroxide (Imject Alum; Pierce, Thermo Fisher Scientific) in 100 μ l on days 0 and 7. Negative controls were sham sensitized with aluminum hydroxide (Alum) following the same protocol. Animals were challenged by two intratracheal (*i.t.*) administrations of OVA (10 μ g in 50 μ l PBS; OVA/OVA mice) or PBS alone (Alum/PBS mice) on days 14 and 18, as described (12). In some experiments, mice from the sensitized group received SA-2 (50 μ g in 100 μ l 20% DMSO in saline; SA-2/OVA mice) or its vehicle (DMSO/OVA mice) via *i.p.* injection 2 d before (day -2) and 4 d after (day +4) the first OVA sensitization. In a second protocol evaluating the effect of SA-2 in a chronic model of asthma, OVA-sensitized mice were repeatedly *i.t.* challenged on days 14, 18, 42, and 45 with OVA (10 μ g in 50 μ l PBS) (S.E.M. Howie, personal communication). Additionally, mice from the sensitized group received SA-2 or DMSO, as described for the acute model. In both protocols, the analysis was performed 3 d after the last OVA challenge. Mice were killed by *i.p.* injection of pentobarbitone (Sigma-Aldrich).

For the α -GalCer-mediated stimulation *in vivo*, C57BL/6 mice received a single *i.p.* administration of α -GalCer (10 μ g/mice) plus SA-2 or SA-1 (50 μ g/mice). In some experiments, mice received the *i.p.* administration of anti-IL-10R, anti-IFN- α R, or anti-p28 Abs 1 h before the coinjection of SA-2 (or SA-1) plus α -GalCer. In some experiments, α -GalCer was *i.v.* administered (10 μ g/mice) in OVA-sensitized mice 48 h after the last OVA *i.t.* challenge, the analysis being performed 1 h later.

BAL and peritoneal lavage

BAL was performed and BALF analyzed 72 h after the last OVA exposure as described (12). Cells from the peritoneal cavity were collected after the *i.p.* injection of sterile RPMI 1640 (2 ml), followed by an abdomen massage and residual fluid recovery. The cell-free supernatants were used for the detection of cytokines by ELISA kits.

Evaluation of AHR

AHR was measured in unrestrained conscious mice using a whole-body plethysmography device (Buxco Research Systems, Winchester, U.K.) by recording respiratory pressure curves, in response to four increasing doses of inhaled methacholine (Sigma-Aldrich). Enhanced pause (Penh) was used to measure AHR as described (17).

Lung histological analysis

Lungs were dissected after perfusion with saline via the heart to clear the blood. H&E and periodic acid-Schiff (PAS) stainings were performed as described (5, 12). Lung sections were examined at original magnification $\times 200$. For measuring hyperplasia of goblet cells, a score was used according to the percentages of these cells in the epithelial lining: score 0, $<5\%$; score 1, 5–25%; score 2, 25–50%; score 3, 50–75%; and score 4, $>75\%$ goblet cells.

Lung MNC preparation

Lung MNC were prepared as previously described (18), modified by the use of GentleMACS from Miltenyi Biotec. In some experiments, lung MNC were depleted of DX5⁺ cells by immunomagnetic procedure and undepleted, and DX5-depleted lung MNC were stimulated with α -GalCer (100 ng/ml) or PMA/ionomycin for 12 h and then assayed for cytokine mRNA expression.

Cell isolation and cocultures

DC, B, and T cells were purified from spleens of wild-type C57BL/6 mice by positive selection with anti-CD11c, CD19, and CD3 mAbs bound to MACS Microbeads (Miltenyi Biotec) according to the manufacturer's instructions. The enrichment of isolated cells examined by cytometry was consistently $>95\%$. Purified CD11c⁺ or CD19⁺ spleen cells were cocultured with purified 10⁵ CD3⁺ T cells (CD11c⁺/CD3⁺ cell ratio 1:3; CD19⁺/CD3⁺ cell ratio 1:1) in complete medium in the presence of α -GalCer (100 ng/ml) plus SA-2 or SA-1 (2.5 μ g/ml) in 96-well round-bottom plates as described (12). In the coculture system of B cells with DC and T cells, the same ratios were maintained (3:1:3). In some experiments, neutralizing anti-IL-10R, anti-p28 chain, anti-IFN- α R, or isotype control Abs were added to the culture at 10 μ g/ml. Three-day culture supernatants were collected and analyzed for cytokine content.

Serum cytokines (IL-17A, IFN- γ , IL-13, IL-12, and IFN- α) were evaluated by commercial ELISA kits (R&D Systems) at baseline and 4 and 10 h after α -GalCer and SA-2 administration. Detection limits in serum were 5 pg/ml for IL-17A, 2 pg/ml for IFN- γ , 15 pg/ml for IL-13, 8 pg/ml for IL-12, and 12.5 pg/ml for IFN- α .

Ag-specific T cell proliferation and cytokine production

Ag (OVA)-specific proliferation of memory T cells was performed by using MNC derived from mediastinal lymph nodes or from spleen, as described (12).

To evaluate cytokine at the mRNA and protein levels, cells from different sources were cultured at 2×10^6 cells/ml in complete medium with α -GalCer (100 ng/ml) in the presence of SA-2 or SA-1 (2.5 μ g/ml). For mRNA detection, cells were recovered 4 h after stimulation. Three-day culture supernatants were used for evaluating IFN- γ , IL-17A, and IL-13 and measured by ELISA kits (R&D Systems) according to the manufacturer's instructions.

Quantitative mRNA analysis

Total RNA from snap-frozen mouse lungs were extracted using TRIzol reagent (RNAwiz; Invitrogen), whereas total RNA from MNC or purified cell subsets were extracted using the RNeasy mini kit (Qiagen, Milan, Italy). Real-time quantitative PCR was performed as described (12) on an ABI PRISM 7700 Sequence Detector (Applied Biosystems, Warrington, U.K.) with Applied Biosystems predesigned TaqMan Gene Expression assays and reagents (Applied Biosystems), according to the manufacturer's instructions.

ELISPOT assay

IL-17A production by spleen MNC was performed with mouse IL-17 Development Module (R&D Systems) according to the manufacturer's instructions. Image analysis of ELISPOT assays was performed with AID ELISpot reader (AID Autoimmun Diagnostika, Strassberg, Germany).

Statistical analysis

Results are presented as mean values $\pm$ SEM. Statistical analysis was performed using the Student *t* test. The *p* values < 0.05 were considered significant.

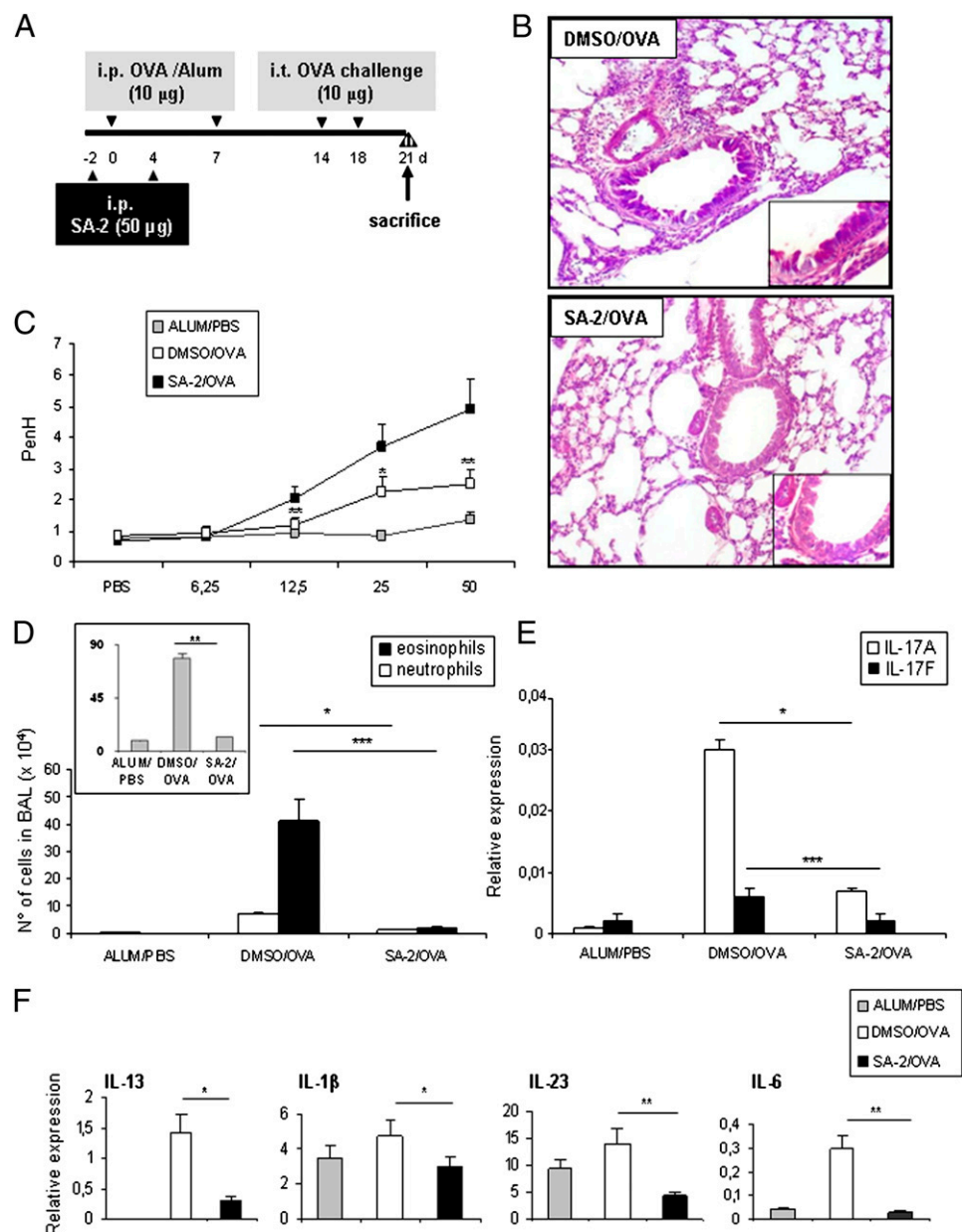
Results

SA-2 inhibits IL-17A production in acute and chronic lung inflammation

To investigate the *in vivo* activity of SA-2 on Ag-induced Th17 response, SA-2 was assessed in the preventive protocol of the acute asthma model as previously described (12) (Fig. 1A). The histochemical analysis showed a reduction of perivascular and peribronchial infiltrates (12) and a decreased proportion of PAS-positive epithelial cells (PAS scores 2.2 ± 0.15 versus 1.18 ± 0.23 ; $p < 0.01$) in the lung of SA-2/OVA mice compared with DMSO/OVA mice (Fig. 1B). SA-2/OVA mice showed decreased AHR and reduced neutrophils (and eosinophils) influx in BALF compared with controls (Fig. 1C, 1D). The lung mRNA expression of IL-17A, IL-17F, and retinoic acid-related orphan receptor

γ t as well as cytokines involved in the development/amplification of Th17 cells (IL-1 β , IL-6, IL-23, and TGF- β) were reduced in SA-2/OVA compared with DMSO/OVA mice (Fig. 1E, 1F and data not shown). Besides the Th17 response inhibition, the decreased AHR could be due to the parallel impairment of type 2-related cytokines (IL-13), as previously described (Fig. 1F) (12). Importantly, the IL-17A mRNA expression was decreased in OVA-stimulated MNC derived from mediastinal dLNs of SA-2/OVA compared with that of DMSO/OVA mice (0.35 ± 0.1 versus 0.04 ± 0.03 gene/ubiquitin ratio; $p < 0.05$). Then, we also analyzed the *in vivo* effects of SA-2 in a chronic model of asthma. SA-2 treatment decreased the total cell recruitment ($83 \pm 11 \times 10^3$ versus $53 \pm 3 \times 10^3$; $p < 0.05$) and the percentage of eosinophils (9.4 ± 4 versus 2.8 ± 1.7 ; $p < 0.05$), and neutrophils (2.5 ± 0.8 versus 0.65 ± 0.5 ; $p < 0.1$) in BALF as well as of the goblet cells in lung tissue (PAS score 1.4 ± 0.5 versus 0.4 ± 0.2 ; $p < 0.05$). In addition, the lung mRNA expression of IL-17A (gene/ubiquitin ratio 0.18 ± 0.11 versus 0.02 ± 0.007 ; $p < 0.05$) and IL-17F (0.16 ± 0.13 versus 0.03 ± 0.002 ; $p < 0.1$) were impaired in SA-2/OVA mice compared with DMSO/OVA mice.

FIGURE 1. SA-2 treatment inhibits the development of Th17 response in an acute asthma model. **A**, C57BL/6 mice were i.p. sensitized and i.t. challenged with OVA, as described in *Materials and Methods*. OVA-sensitized mice were i.p. injected with 50 μ g SA-2 in 20% DMSO (SA-2/OVA mice) or 100 μ l 20% DMSO in saline (DMSO/OVA mice) at -2 d and $+4$ d. **B**, Representative PAS-stained lung tissue sections (original magnification $\times 40$) from DMSO/OVA (upper panel) versus SA-2/OVA (lower panel) mice are reported. **C**, AHR was measured 24 h after the final OVA challenge by plethysmography as described in *Materials and Methods*. Values are expressed as mean ($\pm$ SEM) of PenH (enhanced pause; six animals/group). $*p < 0.05$, $**p < 0.01$. **D**, Absolute values ($\pm$ SEM) of total cells (inset panel), eosinophils, and neutrophils in BAL of SA-2/OVA, DMSO/OVA, and Alum/PBS mice are reported from three experiments (six animals/experiment). $*p < 0.02$, $**p < 0.0025$, $***p < 0.001$. mRNA expression of IL-17A and IL-17F (**E**), IL-13, IL-1 β , IL-6, and IL-23 (**F**) in lung tissue of mice treated with SA-2 was detected by real-time quantitative PCR, as described in *Materials and Methods*. Data are expressed as gene/ubiquitin ratio (mean values $\pm$ SEM, six animals/group). $*p < 0.05$, $**p < 0.025$, $***p < 0.01$.



To define the nature of IL-17A-producing cells responsive to SA-2 activity in lung inflammation, T cell subsets were characterized in BALF, lung, and spleen MNC from OVA/OVA mice versus Alum/PBS control mice. Besides similar proportions of CD4⁺ and CD8⁺ T cells, a significant increase of NKT cells, detected by α -GalCer-loaded CD1d-dimer, was found in BALF ($p < 0.001$) and lung ($p < 0.01$) MNC from OVA/OVA mice compared with control Alum/PBS mice (Supplemental Fig. 1A). Furthermore, MNC obtained from dLNs of OVA/OVA mice produced significant amount of IL-17A upon in vitro stimulation with α -GalCer (NKT-specific stimulus), whereas MNC from control mice (Alum/PBS) did not (Supplemental Fig. 1B). In addition, 1 h after i.v. α -GalCer administration, in OVA-immunized/challenged mice, higher proportions of NKT cells in BALF and lung MNC were found than in α -GalCer-treated control mice. These results paralleled the significant increase of IL-17A mRNA expression ($p < 0.05$) and of the proportion of IL-17A-producing T cells in the same tissue ($p < 0.01$) (Supplemental Fig. 1C). Finally, IL-17A mRNA expression was significantly ($p < 0.05$) reduced in α -GalCer- or PMA/ionomycin-stimulated lung, but not spleen MNC depleted of DX5⁺ cells (Supplemental Fig. 1D).

SA-2 inhibits IL-17A production by activated T cells

To confirm the in vivo results, we then evaluated the in vitro effect of SA-2 on IL-17A (as well as on IFN- γ and IL-13) production by α -GalCer-activated T cells. Spleen cells were stimulated in vitro with α -GalCer (100 ng/ml) in the presence of SA-2 (2.5 μ g/ml) or its inactive analog SA-1 (2.5 μ g/ml). SA-2, but not SA-1, decreased the IL-17A mRNA expression and the number of IL-17-producing cells detected by ELISPOT assay in α -GalCer-stimulated spleen cells (Fig. 2A, 2B). When analyzed at different time points, the IL-17A, IL-13, and IFN- γ production by α -GalCer-stimulated T cells was significantly downregulated by SA-2 at 48 and 72 h of culture (Fig. 2C).

IL-10 produced by DC and B cells are responsible for SA-2-driven IL-17 inhibition in vitro

To better define the mechanism of SA-2-mediated IL-17 inhibition, immunomagnetically purified CD19⁺CD11c⁺ or CD19⁺CD11c⁻ spleen cells (2×10^6 /ml) were cultured with SA-2 or SA-1 (2.5 μ g/ml). IL-10 mRNA expression was significantly upregulated by SA-2, but not SA-1, on both DC and B cells (Table I). When evaluated at the protein level, SA-2 induced higher production of IL-10 in B cells than in DC (1198 ± 61 versus 454 ± 291 pg/ml; $p < 0.05$). In addition, the mRNA expression of IFN- α , IL-27, IL-1 β , IL-23, and IL-12 was increased in SA-2-activated DC but not B cells (Table I and data not shown).

We then compared the effects of SA-2 to inhibit cytokines by α -GalCer-stimulated T cells in two coculture conditions, by using DC or B cells as α -GalCer-presenting cells (DC/T and B/T cell systems). Although a high IL-17A amount was found in the 3-d culture supernatants of DC/T cocultures, it was undetectable in those of the B/T cell system (Fig. 3). Both DC- and B cell-activated T cells were able to induce IFN- γ and IL-13. The addition of SA-2 strongly reduced T cell-derived cytokines in DC/T and B/T cell systems (Fig. 3 and data not shown). Furthermore, the presence of neutralizing anti-IL-10R, anti-p28 chain, and anti-IFN- α R Abs completely restored the IL-17A production in the DC/T system (Fig. 3), whereas they did not induce IL-17A in the B/T system (Fig. 3). IFN- γ and IL-13 inhibition was completely redirected by anti-IL-10R, but not anti-p28 chain or anti-IFN- α R, Abs (Fig. 3 and data not shown).

To better analyze the inhibitory activity of IL-10-producing B cells, we cocultured purified CD19⁺ cells with a DC plus T cell

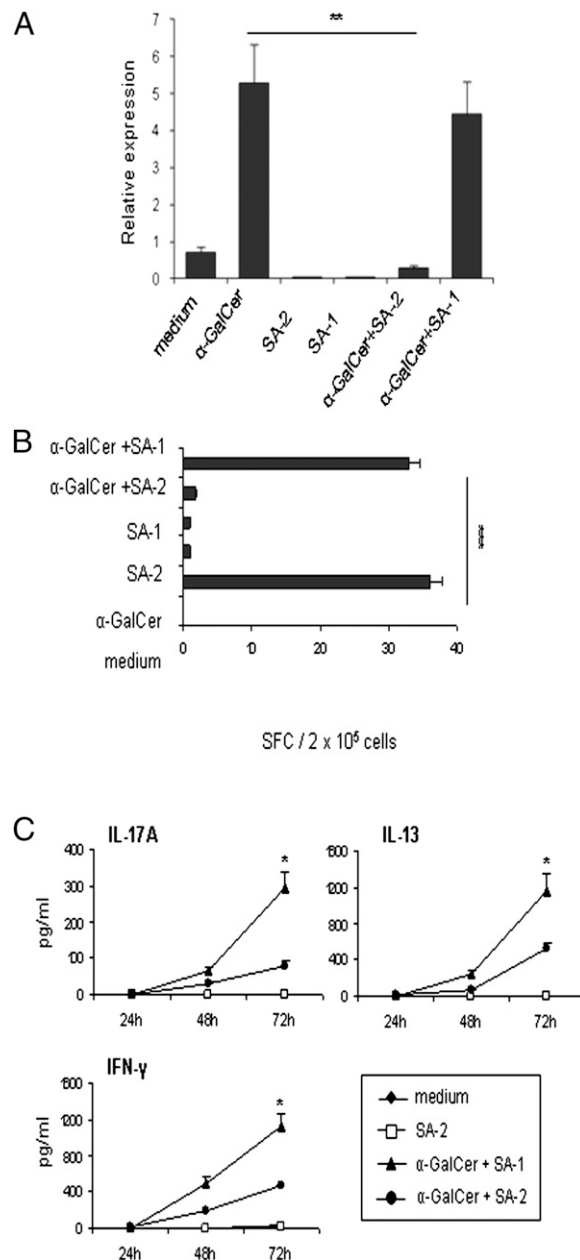


FIGURE 2. SA-2 inhibits IL-17A production by α -GalCer-stimulated cells. **A**, Spleen cells were stimulated with α -GalCer (100 ng/ml) in the presence of SA-2 or SA-1 (2.5 μ g/ml). Four-hour cultured cells were analyzed for IL-17 mRNA expression by using real-time quantitative PCR. Data are expressed as gene/ubiquitin ratio (mean values $\pm$ SEM) of eight separate experiments. **B**, Spleen cells were in vitro stimulated as in **A**, and after 48 h, IL-17-producing cells were analyzed by calibrated ELISPOT assay as described in *Materials and Methods*. Data are expressed as mean values ($\pm$ SEM) of spot-forming cells (SFC)/ 2×10^5 cells from three separate experiments. **C**, Twenty-four-, 48-, and 72-h culture supernatants from spleen cells stimulated as in **A** were assessed for IL-17A, IL-13, and IFN- γ content by ELISA. Data are expressed as the mean values ($\pm$ SEM) of protein in culture supernatants from three separate experiments. * $p < 0.05$, ** $p < 0.02$, *** $p < 0.002$.

system. Unstimulated B cells reduced IL-17A production by α -GalCer-stimulated T cells in the presence of DC (150 ± 12 versus 594 ± 80 pg/ml; $p < 0.01$). The addition of B cells plus SA-2, but not of SA-1, further decreased the cytokine production (67.8 ± 6.1 versus 150 ± 12 pg/ml; $p < 0.05$). The effect of B cells or B cells plus SA-2 on IL-17A production was completely

Table I. SA-2-induced cytokines mRNA expression on DC and B cells

	IL-10	IL-27	IL-23	IL-1 β	IL-12
DC					
SA-1	0.001 $\pm$ 0.001	0.05 $\pm$ 0.03	0.18 $\pm$ 0.16	0.04 $\pm$ 0.008	0.05 $\pm$ 0.02
SA-2	0.97 $\pm$ 0.03*	1.01 $\pm$ 0.24**	2.23 $\pm$ 1.36***	47.00 $\pm$ 7.70****	2.27 $\pm$ 1.74
B cells					
SA-1	0.10 $\pm$ 0.06	0.01 $\pm$ 0.008	0.004 $\pm$ 0.001	0.03 $\pm$ 0.01	0.11 $\pm$ 0.05
SA-2	4.31 $\pm$ 0.93****	0.05 $\pm$ 0.03	0.008 $\pm$ 0.001	0.25 $\pm$ 0.08	0.48 $\pm$ 0.08

DC and B cells were immunomagnetically purified from spleen cell suspension and cultured in vitro in the presence of SA-2 or SA-1 (2.5 μ g/ml) as described in *Materials and Methods*. Four hours later, total RNA was extracted and real-time quantitative PCR was performed. Data are expressed as gene/ubiquitin ratio (mean $\pm$ SEM).

* p < 0.001, ** p < 0.02, *** p < 0.05, **** p < 0.005.

redirected by the addition of anti-IL-10R, but not isotype control Ab (448 $\pm$ 73 versus 80 $\pm$ 10 pg/ml, p < 0.01 for B cells; and 611 $\pm$ 88 versus 140 $\pm$ 13 pg/ml, p < 0.001 for B cells plus SA-2), thus suggesting that, at least in vitro, IL-10 plays the major role also in IL-17A inhibition.

IL-10 and IL-10-inducing cytokines drive the SA-2-mediated IL-17 inhibition in vivo

To study the in vivo activity of SA-2, mice were i.p. injected with α -GalCer, SA-2, or both, and cytokines were assessed at different time points in the serum. The α -GalCer administration induced IL-17A, IL-13, and IFN- γ , whereas SA-2 did not. When coadministered with α -GalCer, SA-2 decreased IL-17A, IL-13, and IFN- γ in the serum at 4 and 10 h from the treatment (Fig. 4A and data not shown). Next, we studied the in vivo kinetics of IL-10, IL-12, IL-27, and IFN- α in the same setting. Serum IL-10 peaked at 1 h and rapidly declined, being poorly detectable at 4 and 10 h. Serum IFN- α showed two peaks (at 1 and 10 h), whereas IL-27

increased at 4 h and maintained high levels at 10 h (Fig. 4B). In addition, α -GalCer injection induced high levels of serum IL-12 that were significantly inhibited in SA-2-cotreated mice at both 4 h (751 $\pm$ 284 versus 190 $\pm$ 61 pg/ml; p < 0.03) and 10 h (707 $\pm$ 191 versus 72 $\pm$ 30 pg/ml; p < 0.002) posttreatment (Fig. 4B). Accordingly, high IL-10 amounts were detectable 2 h after the treatment in the peritoneal fluid (PF) of the α -GalCer/SA-2 mice, but not in controls (Fig. 4C). In addition, when cultured in vitro for 24 h, PF cells from α -GalCer/SA-2 mice spontaneously produced high IL-10 levels, whereas it was not detectable in the supernatants of splenocytes or cells obtained from mesenteric, mediastinal, and superficial lymph nodes of the same mice. IFN- α and IL-27 were consistently undetectable in these settings (Fig. 4C). Notably, the i.p. administration of anti-IL-10R Ab in α -GalCer/SA-2 mice completely restored serum IL-17A, IFN- γ , and IL-13 levels, whereas the anti-IFN- α R and anti-p28 Abs exerted poor in vivo effect at 4 h posttreatment (Fig. 4D and data not shown).

Because the kinetics of serum cytokines indicated that IL-17A was also inhibited 10 h after the treatment with α -GalCer/SA-2 in the absence of IL-10, we asked whether cytokines other than IL-10 were involved. Thus, we evaluated the activity of anti-IFN- α R Ab in α -GalCer/SA-2 mice 10 h posttreatment. Such Ab increased the IL-17A, IL-13, and IFN- γ mRNA expression, whereas it inhibited the IL-10 transcripts in spleen cells (Fig. 5A), thus suggesting that the delayed IL-17A inhibition was likely due to IFN- α -mediated IL-10 production (Fig. 5A).

Importantly, low but detectable proportions of IL-10- and IL-10/IFN- γ -producing CD4⁺ T cells were found 10 h after α -GalCer/SA-2 administration. The treatment with anti-IFN- α R, but not isotype control Ab, reduced the proportions of IL-10-producing (IFN- γ -positive and IFN- γ -negative) T cells, thus indicating that these regulatory cells were largely dependent on IFN- α production and contribute to the delayed IL-17A inhibition (Fig. 5B).

Discussion

The present study addresses the SA-2 activity on the production of IL-17A and its related molecules. By using a prepriming protocol of treatment in the murine model of acute asthma, we provided evidence that SA-2 prevented the development not only of Th2- but also of Th17-mediated airway inflammation. Indeed, SA-2/OVA mice showed the reduction of both eosinophils and neutrophils in BAL and, as a consequence, of methacholine-driven AHR, which some authors correlated to the decrease of IL-13 or of both IL-13 and IL-17A (19–21). Furthermore, a slight inhibition of SA-2 on the Th1 axis was observed in dLNs and spleen cells of SA-2/OVA mice. Some other reports described the suppressive activity of other synthetic TLR7Ls on AHR and eosinophil infiltration in acute and in long-lasting murine models of asthma (22, 23). The decreased tissue expression of both IL-17A/F and molecules in-

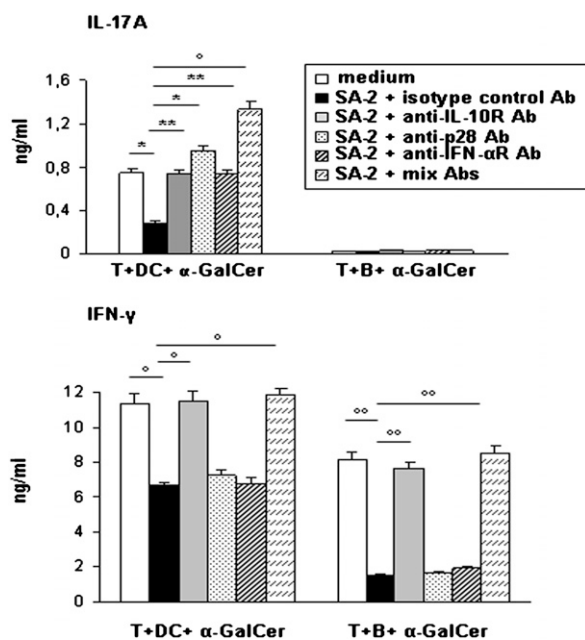
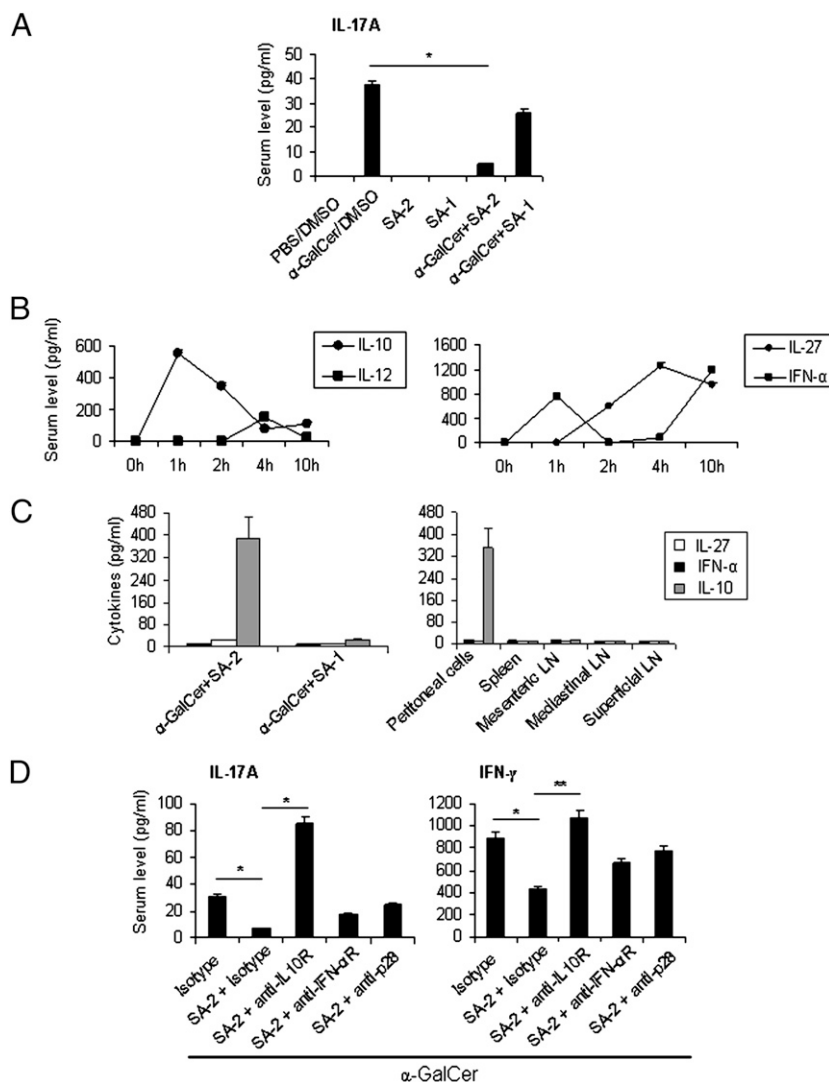


FIGURE 3. SA-2 activity on cytokine production by α -GalCer-stimulated T cells in the presence of different APC. Immunomagnetically purified CD19⁺CD11c⁺ or CD19⁺CD11c⁺ cells (2×10^6 /ml) were cultured for 72 h with purified T cells in the presence of α -GalCer (100 ng/ml) or α -GalCer plus SA-2 (2.5 μ g/ml) as described in *Materials and Methods*. The effects of anti-IL-10R, anti-p28 chain, anti-IFN- α R Abs (10 μ g/ml), and their mixture were evaluated on the production of IL-17A (upper panel) and IFN- γ (lower panel). The mean values ($\pm$ SEM) of three separate experiments are reported. * p < 0.05, ** p < 0.02, *** p < 0.01, **** p < 0.005, ° p < 0.0005, °° p < 0.002.

FIGURE 4. Effects of SA-2 on α -GalCer-driven cytokine production in vivo. **A**, C57BL/6 mice were i.p. injected with α -GalCer (10 μ g/mice) plus SA-2 or SA-1 (50 μ g/mice) and the serum IL-17A levels measured after 4 h by ELISA. Data are expressed as the mean values ($\pm$ SEM) obtained in three separate experiments (six animals/group). * p < 0.0025. **B**, Kinetics of serum cytokine levels after α -GalCer plus SA-2 i.p. administration. Data are expressed as the mean values ($\pm$ SEM) of three separate experiments. **C**, Cytokine content in PF (left panel) and cytokine production (right panel) by cells obtained from different sources 2 h after α -GalCer plus SA-2 i.p. treatment. Cells were then in vitro cultured in medium for additional 24 h as described in *Materials and Methods*. The spontaneous IL-10, IFN- α , and IL-27 production in culture supernatants were evaluated by ELISA. Data are expressed as the mean values ($\pm$ SEM) obtained from four separate experiments. **D**, Upregulation of cytokines by in vivo pretreatment with neutralizing anti-IL-10R or anti-IFN- α or anti-p28 Abs. Neutralizing and isotype control Abs (100 μ g/mice) were i.p. administered 1 h before α -GalCer plus SA-2 treatment and the serum IL-17A, IL-13, and IFN- γ levels evaluated 4 h later by ELISA. Data are expressed as the mean values ($\pm$ SEM) obtained in two separate experiments (six mice/group). * p < 0.05, ** p < 0.01.



involved in Th17 development/amplification (IL-1 β , IL-6, and IL-23) paralleled the impaired expression of IL-17A by memory T cells of mediastinal dLNs. Similar results were obtained in a chronic model of airway inflammation. In addition, by inhibiting fibrogenic molecules such as IL-1 β , IL-13, IL-17A/F, and TGF- β , SA-2 may also play a role in downregulating tissue fibrosis and remodeling related to chronic inflammation.

To better define the target cells of SA-2 activity, the nature of IL-17A-producing cells in airways of OVA-sensitized mice was investigated. We showed that the proportion of NKT cells was significantly increased in the lung and BALF of these mice. Moreover, if stimulated with α -GalCer in vitro or in vivo, BALF or dLN MNC from OVA/OVA mice produced higher levels of IL-17A compared with those from control mice. Finally, taking into account that IL-17A-producing cells in vitro generated (anti-CD3 Ab plus IL-23) from spleen cells of C57BL/6 mice are TCR β ⁺ NKT cells expressing DX5 (CD49b) (24), when depleted of DX5⁺ NKT cells, lung MNC from OVA/OVA mice expressed lower levels of IL-17A mRNA. These data agree with other reports showing the accumulation of NKT cells in BALF and lung of murine models and asthmatic patients (9, 25, 26). The prevalent IL-17A production in allergen-induced airway inflammation by a CD1d-activated NK1.1-negative NKT cell subset or by human type II CCR6⁺CD161⁺NKT cells producing or not type 2 cytokines have also been described (27–31). Several mechanisms have

been proposed to explain how the allergen-induced airway inflammation allows for the lung accumulation of CD1d-reactive NKT cells releasing type 1-, 2-, and 17-associated cytokines (21, 32). These findings have also allowed us to design in vitro and in vivo systems to easily monitor SA-2 effects on IL-17A production. In fact, spleen cells cultured in vitro with SA-2 plus α -GalCer produced lower amounts of IL-17A than controls. Accordingly, the IL-17A serum levels detectable 4 and 10 h after the i.p. administration of α -GalCer were reduced in mice cotreated with SA-2. Upon these conditions, SA-2 inhibited also the serum levels of NKT-derived IFN- γ and IL-13 and APC-produced IL-12. This indirectly confirms the IL-17A inhibition because this cytokine has been recently described as one of the strongest inducer of IL-12 production by APC (33). On the whole, these data reinforce the concept that SA-2 exerts regulatory, rather than proinflammatory, activity. Indeed, we previously showed that the dampening effects of SA-2 on Th2 cells in vivo was not due to increased regulatory T cells or IDO activity, but to the induction of IFN- α , IL-27, and IL-10 (12). In this study, we confirmed that SA-2 induced IL-10 by DC and B cells. DC, but not B cells, produced IFN- α and IL-27 upon SA-2 stimulation, whereas the regulatory (TGF- β and IL-35) or inflammatory (IL-1 β , IL-12 and IL-23) cytokines were unchanged. This suggests that SA-2 prevalently regulates T cells through the production of IFN- α /IL-27/IL-10 by DC and of IL-10 by B cells.

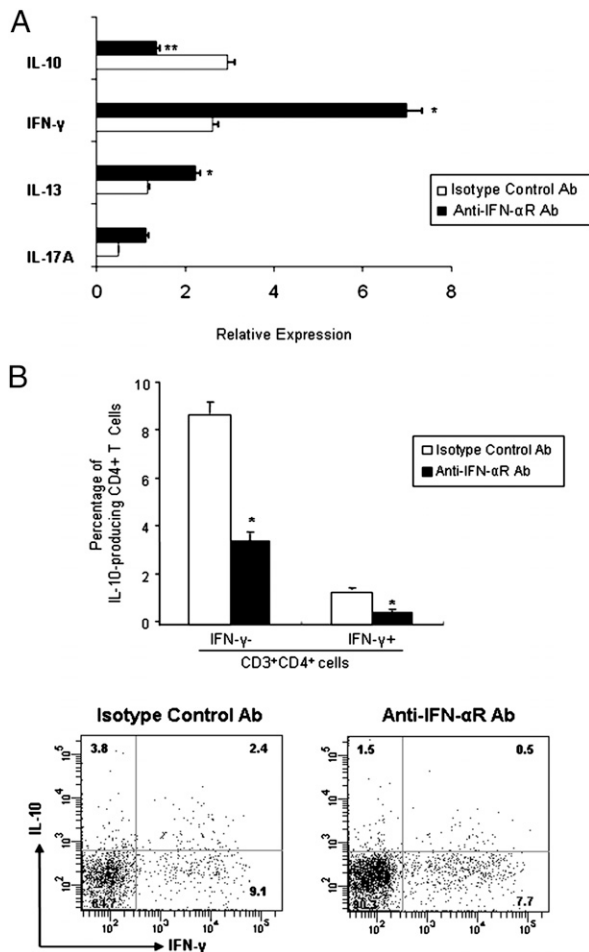


FIGURE 5. In vivo effects of anti-IFN- α R Ab on cytokine mRNA expression and IL-10-producing T cells. **A**, Spleen mRNA expression of IL-10, IFN- γ , IL-13, and IL-17A detected 10 h posttreatment described in Fig. 4D. mRNA were detected by real-time quantitative PCR and data expressed as gene/ubiquitin ratio (mean values $\pm$ SEM, six animals/group). * p < 0.05, ** p < 0.01. **B**, The proportion of IL-10-producing T cells was downregulated by in vivo i.p. administration of anti-IFN- α R Ab. Cytometric analysis in spleen CD4 $^{+}$ T cell-gated cells of IL-10- and IL-10/IFN- γ -producing lymphocytes was evaluated 10 h posttreatment described in Fig. 4D. The mean values ($\pm$ SEM) obtained from two separate experiments are reported (upper panel). A representative experiment on CD4 $^{+}$ T cell-gated spleen cells of IL-10- and IL-10/IFN- γ -producing cells is also shown (lower panel). * p < 0.02.

The induction of regulatory mechanisms by TLR7L has been confirmed by other reports. The TLR7 triggering (by S-28463) has been shown to play an anti-inflammatory role in murine allergic asthma by inhibiting both type 2 and type 1 cytokines (22). Moreover, the intranasal administration of the TLR7L (resiquimod) suppresses experimental asthma through two distinct mechanisms, one mediated by type I IFNs (as SA-2) inducing acute suppression and the second mediated by immunoregulatory CD8 $^{+}$ T cells and IFN- γ , which confers long-term protection (23).

T cells cocultured with α -GalCer-stimulated DC produced high levels of IL-17A, IFN- γ , and IL-13, which were downregulated by SA-2. By contrast, when T cells were cocultured with the α -GalCer plus B cells, IFN- γ , and IL-13, but not IL-17A, were produced, thus confirming that DC but not B cells provide signals essential for Th17 development and amplification, such as IL-1 β and IL-23. In contrast, it cannot be excluded that DC can be activated and modulated through the cooperative action of both

TLR7L and α -GalCer-activated NKT cells, thus driving quantitative and qualitative differences in T cell responses, as it has been recently described (34).

The experiments with neutralizing Abs strongly suggest that IFN- α , IL-27, and IL-10 play a role in inhibiting IL-17A in the DC/T cell system. IL-17A production was inhibited by culturing DC/T cells with unstimulated B cells, and much more, with B cells plus SA-2, this inhibition being completely redirected by the addition of anti-IL-10R Ab. It reinforces the concept that IL-10-producing B cells have a relevant role in the in vitro IL-17A inhibition and that, likely, the SA-2-induced IL-27 and IFN- α by DC can indirectly improve additional IL-10 production by APC or T cells (35, 36).

Moreover, the i.p. administration of SA-2 plus α -GalCer impaired IL-17A (as well as IL-13 and IFN- γ) serum levels. We have shown that the early (4 h) and delayed (10 h) SA-2-mediated inhibition of IL-17A response is related to different mechanisms. The serum IL-17A levels were completely restored by anti-IL-10R and poorly affected by anti-IFN- α R Abs at 4 h after α -GalCer treatment. However, IL-17A mRNA expression was strongly upregulated by anti-IFN- α R and poorly by anti-IL-10R Abs at 10 h posttreatment. This double effect was probably due to the different kinetics of IFN- α and IL-10 in α -GalCer plus SA-2-treated mice. Indeed, 1 to 2 h after the treatment, we observed the peak of serum IL-10 and IFN- α , which rapidly declined, whereas a second peak of serum IFN- α and a concomitant high level of IL-27, with no detectable IL-10, were present at 10 h. The two IFN- α peaks agree with the described two-phase induction of IFN- α by DC (37). The early IFN- α production has been shown to be produced by plasmacytoid DC triggered by pathogen-associated molecules patterns, whereas the second amplifying phase represents a feedback response mostly sustained by conventional DC (37). In our system, the early serum IFN- α peak is due to IFN- α -producing resident peritoneal cells stimulated by SA-2, which can directly enroll and amplify spleen conventional DC to produce IFN- α . It has been shown that IFN- α not only amplifies TLR signaling cascade (by increasing the expression of TLR7R on other cells) (38) but, importantly, also downregulates murine or human Th17 cells by several mechanisms as the reduction of IL-17-favoring molecules (i.e., osteopontin), the increase of IL-17-inhibiting cytokines as IL-27, or the upregulation of IL-10-producing Th cells by IL-27 itself (39, 40). Indeed, in vivo treatment with anti-IFN- α R Ab strongly inhibited IL-10 mRNA expression in the spleen, thus suggesting a role for IFN- α -mediated IL-10 production in our model. The detection of IL-10-producing T (including Th1) cells in the spleen 12 h, but not 4 h, after α -GalCer plus SA-2 treatment confirms the previous data. Accordingly, the proportion of IL-10-producing T cells in SA-2-treated mice was inhibited by the anti-IFN- α R- and, poorly, anti-IL-10R Abs. These data are consistent with the increased IFN- α in the serum and elevated IL-10-producing T cells during the remission of respiratory symptoms in severe asthma and Churg-Strauss syndrome (41). Thus, it is reasonable to hypothesize that IL-10 and IL-10-inducing cytokines are the major players for the early and delayed inhibition of NKT cell-derived cytokines, including IL-17A.

The suppressive activity of SA-2 on Th17 response is, however, at odds with a recent paper suggesting that TLR7 ligation in humans actually promotes Th17 responses (42). The use of a different TLR7L (imiquimod) and an unusual in vitro model (human naive T cells cultured with allogeneic plasmacytoid DC, which per se can induce IL-17A) as well as the not well-defined interindividual responses to TLR7L, render our and Yu et al.'s data (42) not comparable at all. By contrast, other reports agree with

results of the present paper. Indeed, it has been shown that TLR7 activation by respiratory virus Ags induces the downregulation of the IL-23/IL-17A axis in vivo (43).

On the whole, the results referred to in this study imply two major remarks on the role of T or B cells producing IL-10 in SA-2-treated mice. The induction of IL-10-producing Th1 cells upon SA-2 treatment fits with previous reports, describing these cells in mice undergoing chronic stimulation with Ags (44–48). A regulatory activity of naive and memory B cells producing IL-10 has been recently suggested (49, 50). Indeed, the ultimate effect of B cells producing IL-10 is to constrain pathology (irrespective of whether it is driven by Th1, Th2, or Th17 cell responses) by a direct activity on CD4⁺ T effector cells, a reduction of Ag priming by DC, or, lastly, an enhanced activation of IL-10-producing T cells (50, 51). This is also in agreement with our previous result that SA-2 does not favor the onset of autoantibodies in vivo (12).

In conclusion, whatever the complexity of regulatory mechanism(s) involved, our in vitro and in vivo data provide evidence that SA-2 inhibits both Th17 and Th2 responses with no induction of autoimmunity, thus making this compound a suitable candidate for novel vaccine formulations in allergy.

Disclosures

The authors have no financial conflicts of interest.

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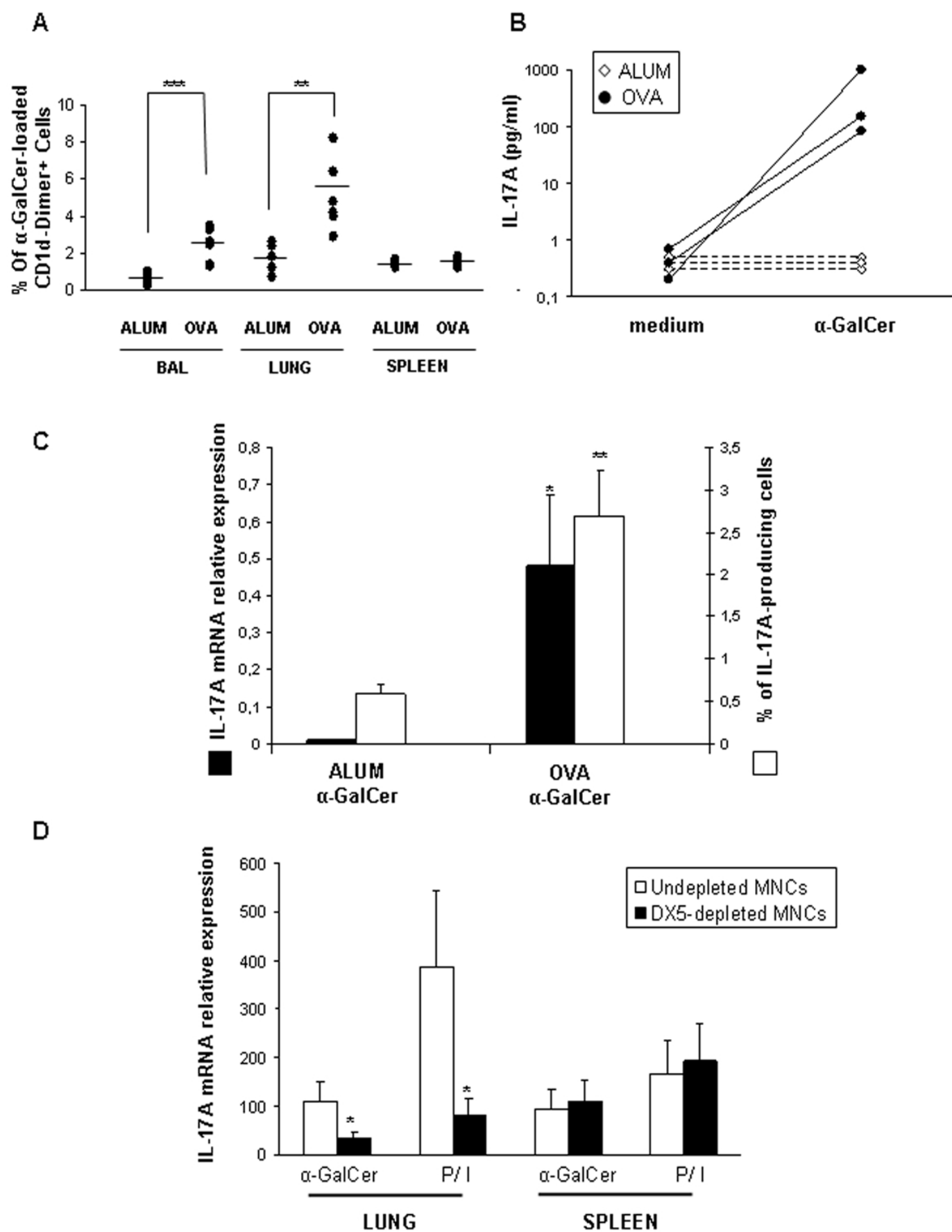


Figure 1S

SUPPLEMENTARY FIGURE 1. IL-17A-producing NKT cells accumulate in OVA-mediated airway inflammation. *A*, Lung, BALF and spleen MNC were obtained from sensitized mice 48 h after OVA i.t. challenge. Alum/PBS mice were used as control. Cells were stained with α -GalCer-loaded (or empty) CD1d-dimer as described in *Materials and Methods*. The proportion of NKT cells among lymphocyte-gated cells is reported (6 animals/group). ** $p < 0.01$, *** $p < 0.001$. *B*, MNC (2×10^6 /ml) obtained from mediastinal dLN of OVA/OVA and Alum/PBS mice (3 animals/group) were *in vitro* stimulated with, or without, α -GalCer (100 ng/ml). IL-17A was measured in 72h-culture supernatants as reported in *Materials and Methods*. *C*, OVA/OVA and Alum/PBS mice received i.v. α -GalCer administration (10 μ g/mice) 48h after the second i.t. challenge. One hour later, lung MNC were analysed for IL-17A mRNA expression by RT-PCR (data expressed as gene/ubiquitin ratio) and for intra-cytoplasmic IL-17A production by cytometry, as reported in *Materials and Methods*. Mean values ($\pm$ SEM) are reported (6 animals/group). * $p < 0.05$, ** $p < 0.01$. *D*, Relative expression of IL-17A mRNA (data expressed as gene/ubiquitin ratio) of lung and spleen MNC depleted, or not, of DX5+ cells and stimulated with α -GalCer or PMA/ionomycin is reported as described in *Materials and Methods*. Results indicate the ratio between the relative expression of mRNA obtained from OVA/OVA and Alum/PBS mice (6 animals/group). * $p < 0.05$.