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Cutting Edge: Intravenous Ig Inhibits Invariant NKT Cell-Mediated Allergic Airway Inflammation through FcγRIIA-Dependent Mechanisms

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Despite their increasing use in autoimmunity, inflammatory, and allergic conditions, the mechanism of action of i.v. Iggs (IVIg) is poorly understood. On the basis of the critical role of invariant NKT (iNKT) cells in allergic airway inflammation (AAI) and their constitutive expression of the low-affinity IgG receptor FcγRIIA, we surmised that IVIg targets iNKT cells to exert their anti-inflammatory effect. We found that IVIg treatment significantly inhibited AAI in OVA-sensitized C57BL/6 mice and downregulated α-galactosylceramide–induced iNKT cell activation and cytokine production. Allergic responses were restored in iNKT cell-deficient mice by transferring iNKT cells from PBS- but not from IVIg-treated mice, suggesting that IVIg acts directly on activated iNKT cells that have a critical role in AAI. The inhibitory effects of IVIg on both iNKT cell activation/function and OVA-driven AAI were lost in FcγRIIA−/− mice. Our data unravel an FcγRIIA-dependent inhibitory effect of IVIg on activated iNKT cells that confers protection in AAI.

Intravenous Ig (IVIg) is a therapeutic molecule prepared from pools of plasma obtained from several thousand healthy blood donors. In addition to its use in the treatment of primary and secondary immune deficiencies, IVIg has been used as a therapeutic option in a large number of autoimmune, allergic, and inflammatory conditions including idiopathic thrombocytopenic purpura, Guillain–Barré syndrome, myasthenia gravis, and Kawasaki disease (1–6). Considering studies conducted in mouse models, several mutually non-exclusive mechanisms may account for the therapeutic effects of IVIg (1–3, 7–11). It is believed that the beneficial effect of IVIg may be related to its content of natural polyreactive Abs reactive with a large number of immunologically relevant molecules (12–14). Notably, anti-inflammatory activities of IVIg are mediated through both FcγRIIB- and/or FcγRIIA-dependent and FcγR-independent mechanisms. Thus, IVIg has been shown to exert a regulatory role on both the humoral and cellular compartment of the immune system.

Invariant NKT (iNKT) cells are innate lymphocytes that recognize glycolipids presented by the nonclassical MHC class I b family member CD1d (15–17). iNKT cells release massive amounts of IFN-γ following activation through their semi-invariant TCR, but they are also unique in their ability to secrete high amounts of IL-4 in addition to other Th2 cytokines such as IL-13 (16, 18–21). iNKT cells are therefore implicated in the physiopathology of several autoimmune and inflammatory diseases (19, 22, 23), including allergic airway inflammation (AAI), in asthma (18, 24, 25).

Allergic asthma is a chronic inflammatory disease of lung, the incidence and morbidity of which has increased dramatically in the past few decades, especially in industrialized countries (26, 27). Asthma is a heterogeneous and multifactorial disease and is characterized by an airway hyperreactivity in response to...
a variety of specific and nonspecific stimuli, mucus hypersecretion, pulmonary eosinophilia, Th2 cytokine production, hyperplasia and hypertrophy of smooth muscle, and elevated concentrations of serum IgE (28). IVlg has been considered as one of the therapeutic options in the treatment of asthma, although the underlying mechanisms have not been explored (29, 30). Given the critical role of iNKT cells in AAI, we surmised that IVlg targets this cellular population to exert its anti-inflammatory effect.

**Materials and Methods**

**Mice**

Six- to 8-wk-old male C57BL/6 wild-type (WT) and Jα18−/− mice were bred and maintained in our animal facility under specific pathogen-free conditions. FcyRIIIA−/− mice (N7 C57BL/6J) were purchased from The Jackson Laboratory. Jα18−/− mice were backcrossed for 13 generations onto a C57BL/6 background. Animal experiments were performed according to the French Institutional Committee.

**Induction of AAI and IVlg treatment**

Mice were sensitized with OVA (100 μg, i.p., per mouse; Sigma-Aldrich) absorbed to 1.6 mg aluminum hydroxide gel (Merck). After 7 d, mice were exposed to intranasal Ag (50 mg/d for OVA) or PBS on 3 consecutive d. Inhibition of AAI by IVlg in OVA-sensitized mice was assessed by injecting IVlg (10 mg, i.p., per mouse) 1 h prior to the first of the three consecutive challenges with OVA. In some experiments, OVA-sensitized WT or Jα18−/− mice were adoptively transferred with total splenocytes (2.5 × 10^6 INKT cell equivalents) or sorted iNKT cells (2.5 × 10^4) from WT mice that were pretreated with PBS or IVlg 1 h prior to sacrifice and sacrifice. Bronchoalveolar lavage fluid (BALF) and lungs were recovered 24 h after the last challenge. Total and differential cell counts were determined in the BALF, and histological analysis of lung sections was evaluated in a blinded fashion by a histopathologist (M.H.) as previously described (25). Peribronchial and perivascular inflammation was graded on a subjective scale of 0, 1, 2, and 3 corresponding to no, mild, moderate, or severe inflammation, respectively, with an increment of 0.5 if the inflammation fell between two integers.

**Analysis of iNKT cells**

The proportion of iNKT cells as well as their surface expression of CD69 was determined in splenocytes by flow cytometry using fluorochrome-conjugated anti–TCR-α (clone H57-597), anti-CD5 (clone 55-7.3), and anti-CD69 (clone H1.2F3) from BD Biosciences (Le Pont de Claix, France) and an allophycocyanin-conjugated α-galactosylceramide (α-GC)-loaded CD1d tetramer (CD1dα-GC TT) provided by the National Institutes of Health Tetramer Core Facility. To assess iNKT cell activation in vivo, mice received an injection of α-GC (Kinin Brewin; 1 μg/dose, i.v.). The in vivo modulatory effect of IVlg on iNKT cells was examined by injecting IVlg 1 h before α-GC challenge. Sera were recovered 2 h later. To compare iNKT cell activity between WT and FcyRIIIA−/− mice, FACS-sorted iNKT cells as CD1dα-GC TT−CD5− cells (2.5 × 10^4/well) were stimulated with α-GC–pulsed dendritic cells (DC) for 48 h (20).

Levels of cytokines in the BALF, homogenate lung, serum, and cell-free culture supernatants were assessed using ELISA Duoset kits (R&D Systems).

**Statistical analysis**

Data are expressed as the mean values ± SEM for each group of mice. We analyzed statistical comparisons with two-tailed unpaired Student t test and Mann–Whitney U test. We considered a p value <0.05 significant.

**Results and Discussion**

**IVlg inhibits airway inflammation in OVA-sensitized and challenged mice**

We first examined the protective effect of IVlg on the evolution of AAI in OVA-sensitized C57BL/6 mice. As shown in Fig. 1, IVlg treatment inhibited the recruitment of inflammatory cells in the airway passage. Thus, the total number of infiltrating cells (Fig. 1A) and, in particular, the percentage (Fig. 1B) and number (Fig. 1C) of eosinophils, one of the major mediators of allergic responses, was significantly downregulated following IVlg treatment. Despite the notion that C57BL/6 strain develops poor airway hyperreactivity compared with other strains (24, 25), IVlg treatment of OVA-sensitized C57BL/6 mice led to a significant decrease of this cardinal feature of asthma (Supplemental Fig. 1A). However, the production of pro-Th2 cytokines was unaffected by IVlg (Supplemental Fig. 1B). These results thus document the therapeutic benefit of IVlg in severe childhood asthma (4, 29, 30).

**IVlg downregulates the activation and function of iNKT cells**

Activated iNKT cells have been shown to play a crucial role in development of allergic asthma following OVA sensitization and challenge (18, 24, 25). Absence of iNKT cells indeed abolishes experimental AAI. In view of the observed protective effect of IVlg in AAI, we asked whether IVlg treatment has repercussion on the activation and cytokine production by iNKT cells. Following injection of naive or α-GC–pretreated mice with IVlg, we assessed the expression level of the activation marker CD69 on iNKT cells. As shown in Fig. 1D, IVlg treatment significantly downregulated the mean fluorescence intensity (MFI) of CD69 on iNKT cells both in naive or α-GC–pretreated mice. Furthermore, IVlg treatment also reduced significantly the serum levels of IL-4 (Fig. 1E) and IFN-γ (Fig. 1F) following in vivo activation of iNKT cells by α-GC. Moreover, in vitro addition of IVlg alleviates...
IVIg treatment blocks the capacity of iNKT cells to restore AAI

Jα18<sup>−/−</sup> mice lack iNKT cells and are resistant to AAI (18, 24). To evaluate whether IVIg-mediated protection in AAI is due to its direct effect on pathogenic effector iNKT cells, we adoptively transferred iNKT cells from PBS- or IVIg-treated WT mice into iNKT cell-deficient Jα18<sup>−/−</sup> mice. As expected, OVA-presensitized Jα18<sup>−/−</sup> mice that did not receive iNKT cells did not experience AAI upon Ag challenge. OVA-presensitized Jα18<sup>−/−</sup> mice that received total splenocytes or purified iNKT cells from PBS-treated WT mice developed airway inflammation as evidenced by massive lung infiltration by inflammatory cells, including eosinophils (Fig. 2A, 2B). Interestingly, animals that were transferred with iNKT cells from IVIg-treated WT animals showed >50% reduction in total cell infiltration and eosinophilia upon Ag challenge (Fig. 2A, 2B). This result demonstrates that the inhibitory effect of IVIg on AAI can be transferred by iNKT cells and that iNKT cells are therefore less pathogenic upon IVIg treatment.

Once pharmacologically activated by their ligand, iNKT cells are protective rather than pathogenic during AAI (31, 32). For this reason, we also examined whether IVIg-preconditioned iNKT cells were able to transfer protection to normal naive mice upon challenge with OVA. To address this issue, we transferred IVIg-preconditioned iNKT cells from naive mice to OVA-presensitized recipient animals. Neither PBS- nor IVIg-preconditioned iNKT cells from naive mice were able to confer protection to recipient mice upon

production of IL-4 by sorted iNKT cells that were activated by α-GC–loaded DC (Supplemental Fig. 2). These data indicate that IVIg modulates the activation and function of iNKT cells.

**FIGURE 2.** IVIg treatment blocks the capacity of iNKT cells to restore AAI in Jα18<sup>−/−</sup> mice. Groups of OVA-sensitized Jα18<sup>−/−</sup> mice were adoptively transferred with sorted iNKT cells from PBS or IVIg-treated WT mice 1 h prior to the first challenge with OVA. Total infiltrating cells (A) and eosinophils (B) were determined in the BALF in recipient Jα18<sup>−/−</sup> mice 24 h after the last OVA challenge. Results represent means ± SEM from a representative experiment out of two. n = 3–5 mice per group. Mice that did not receive iNKT cells (none) did not respond to OVA challenge.

**FIGURE 3.** Inhibitory effect of IVIg on iNKT cells is FcyRIIIA dependent. WT and FcyRIIIA<sup>−/−</sup> mice were injected with PBS or IVIg before administration of α-GC. After 2 h, the animals were bled and sacrificed. A. MFI of the early activation marker CD69 on splenic iNKT cells. IL-4 (B) and IFN-γ (C) levels were determined in the serum. Data are pooled results from two independent experiments. n = 4 mice per group per experiment. Data represent means ± SEM.

**FIGURE 4.** Inhibitory effect of IVIg on airway inflammation is mediated by FcyRIIIA. WT and FcyRIIIA<sup>−/−</sup> mice were sensitized with OVA and treated with IVIg or PBS 1 h prior to the first challenge with OVA or PBS. Infiltrating total cells (A) or eosinophils (B) in the BALF and histological analysis (C–K) were determined 24 h after the last challenge. Peribronchial scores for PBS- or IVIg-treated OVA-sensitized/challenged WT and FcyRIIIA<sup>−/−</sup> groups of mice are represented in C. Results represent means ± SEM from a representative experiment out of two. WT mice: n = 3 or 5; FcyRIIIA<sup>−/−</sup> mice: n = 5 or 6. Periodic acid-Schiff staining of lung sections did not display robust presence of mucus in both WT and FcyRIIIA<sup>−/−</sup> (not shown). Representative H&E-stained tissue sections of lungs from each group of mice are shown: WT mice treated with PBS (D, E) or IVIg (F, G), and FcyRIIIA<sup>−/−</sup> mice treated with PBS (H, I) or IVIg (J, K). D, F, H, J, original magnification ×20. E, G, I, K, original magnification ×200.
The inhibitory effect of IVIg on activated iNKT cells and AAI is FcγRIIIA dependent

The blockade of cell-surface FcγR is a possible mechanism for the anti-inflammatory properties of IVIg. In view of the fact that iNKT cells constitutively express surface FcγRIIIA but not FcγRI, -IIb, or -IV (33), we examined the anti-inflammatory effect of IVIg on iNKT cells in FcγRIIIA−/− mice. iNKT cell counts were normal in FcγRIIIA−/− mice compared with WT mice (Supplemental Fig. 4A, 4B). In addition, iNKT cells from both strains produced similar levels of IL-4 after in vitro stimulation with α-GC–pulsed DC, suggesting normal functioning of iNKT cells in FcγRIIIA−/− mice (Supplemental Fig. 4C).

To determine whether FcγRIIIA are involved in the inhibitory effect of IVIg on iNKT cells, FcγRIIIA−/− and WT mice were injected with IVIg 1 h before α-GC treatment. Interestingly, the inhibitory effect of IVIg on iNKT cell activation as measured by the expression of CD69 (Fig. 3A) and by the production of IL-4 (Fig. 3B and IFN-γ (Fig. 3C) was lost in FcγRIIIA−/− mice as compared with PBS-treated mice. These findings indicate that the inhibitory effect of IVIg on iNKT cells requires FcγRIIIA.

These findings led us to determine whether IVIg inhibits iNKT cell-mediated AAI through FcγRIIIA-dependent mechanisms. Consistent with this view, we found that FcγRIIIA deficiency led to a quasicomplete loss of the anti-inflammatory effect of IVIg treatment in OVA-sensitized and challenged animals (Fig. 4). Indeed, the total number of infiltrating cells (Fig. 4A) and eosinophils (Fig. 4B), as well as peribronchial and perivascular inflammation (Fig. 4C–K), was significantly decreased following IVIg treatment in WT mice but not in FcγRIIIA−/− mice. It is of note that WT and FcγRIIIA−/− mice had a similar capacity to develop AAI in response to OVA immunization and challenge. Together, our data demonstrate an FcγRIIIA-dependent inhibitory effect exerted by IVIg on activated iNKT cells that confers protection in AAI. Together, these data allow us to infer that the beneficial effect of IVIg on iNKT cell-mediated allergic airway inflammation implicates FcγRIIIA, although we do not provide evidence of a direct effect of IVIg on FcγRIIIA expressed by iNKT cells in vivo.

A well-accepted paradigm is that the inhibitory functions of IgG are mediated by FcγRIIB, which contains a cytoplasmic immune tyrosine-based inhibitory motif that recruits phosphatases, leading to suppression of intracellular signal transduction (34). By contrast, FcγRIIIA is established as an activating receptor that is required for multiple Ab–immune complex-dependent inflammatory responses in vitro and in vivo. However, Park-Min et al. (35) have shown that inhibition of IFN-γ responses by immune complexes is independent of FcγRIIB. These authors revealed that immune complexes that are contained in IVIg inhibit IFN-γ signaling in macrophages by mechanisms dependent on FcγRIIIA, although being an activating receptor, and suggest that inhibition of IFN-γ signaling contributes to the therapeutic efficacy of IVIg (35, 36). Consistent with these data, our results demonstrate an inhibitory role for FcγRIIIA in IVIg-mediated suppression of iNKT cell activation. This suppressive effect on iNKT cells contributes to the anti-inflammatory effects of IVIg in airway inflammation. Interestingly, the NK cell-dependent effect of IVIg has also been suggested to be of relevance in the prevention of recurrent pregnancy loss (5). Strategies aimed at functional inhibition of iNKT cells by targeting through IVIg alone or in combination with other appropriate therapeutics should help in paving the way for fighting allergic conditions and in maintaining a critical balance between tumor immunity and self-tolerance.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplementary Fig. 1: IVIg-treatment inhibits bronchopulmonary hyperreactivity but not local Th2-type cytokine production in OVA-sensitized and -challenged mice. WT mice were sensitized with OVA and treated with IVIg or PBS 1h prior to the first challenge with OVA or PBS. (A) Bronchopulmonary hyperreactivity to metacholine was measured 1d after the last challenge. Data represent the mean±s.e.m. Penh values of a representative experiment out of two (n=4-6 mice per group). The difference between OVA-sensitized and -challenged mice treated with IVIg and OVA-sensitized and -challenged mice treated with PBS is significant (P=0.006, ANOVA repeated measure). (B) The levels (means±s.e.m) of IL-4 in BALF and IL-5 and eotaxin in the lung homogenates were determined 24h after the last challenge. Data are pooled results from two independent experiments. n=6 to 11 mice per group. NS: not significant.
**Supplementary Fig.2: In vitro inhibitory effect of IVIg on IL-4 production by iNKT cells.** Freshly sorted iNKT cells as CD1d:α-GC TT⁺ CD5⁺ cells (2.5x10⁴ cells/well) from spleen from WT mice were cultured for 48h with α-GC-pulsed DC (DC α-GC) in presence or absence of IVIg (8mg/ml). IL-4 levels in cell-free culture supernatants were measured. Data are pooled results from three independent experiments. Data represent means±s.e.m.
Supplementary Fig. 3: IVIg-preconditioned iNKT cells from naïve mice do not confer protection to WT recipients. OVA-sensitized WT mice were transferred with iNKT-enriched cells (2x10⁶/mouse) from PBS- or IVIg-treated mice 1h prior to the first challenge with OVA or PBS. Infiltrating total (A) cells and eosinophils (B) in the BALF were determined 24h after the last challenge. Data are pooled results from two independent experiments. n=9 mice per group. Data represent means±s.e.m. NS: not significant.
Supplementary Fig. 4: Analysis of iNKT cells in FcγRIIIA−/− mice. Representative flow cytometry plots defining the iNKT cell population as α-GC:CD1d TT+ CD5+ cells (A). Percentage of iNKT cells in the spleen of naïve WT or FcγRIIIA−/− mice (B). Data are expressed as mean ± s.e.m. of a representative experiment out of two (n=4 mice per group). FACS-sorted splenic iNKT cells from WT or FcγRIIIA mice were stimulated with α-GC-pulsed DC for 48h. IL-4 levels in cell-free culture supernatants were measured by ELISA (C). Data are expressed as mean ± s.e.m. of a representative experiment out of two (n=3 mice per group).