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**INVARIANT V α 14 NKT CELLS ARE REQUIRED FOR ALLERGEN-INDUCED
AIRWAY INFLAMMATION AND HYPERREACTIVITY IN AN EXPERIMENTAL
ASTHMA MODEL.**

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ABSTRACT

Airway hyperreactivity (AHR), eosinophilic inflammation, with a Th2-type cytokine profile, and specific Th2 mediated IgE production characterize allergic asthma. Here, we show that OVA-immunized $J\alpha 18^{-/-}$ mice, which are exclusively deficient in the invariant $V\alpha 14^{+}$ (iV $\alpha 14$), CD1d restricted NKT cells, exhibit impaired AHR and airway eosinophilia, decreased IL-4 and IL-5 production in bronchoalveolar lavage fluid (BALF) and reduced OVA-specific IgE compared to wild-type (WT) littermates. Adoptive transfer of wild type iV $\alpha 14$ NKT cells fully reconstitutes the capacity of $J\alpha 18^{-/-}$ mice to develop allergic asthma. Also, specific tetramer staining shows that OVA immunized WT mice have activated (CD69⁺) iV $\alpha 14$ NKT cell. Importantly, anti-CD1d mAb treatment blocked the ability of iV $\alpha 14$ T cells to amplify eosinophil recruitment to airways, and both Th2 cytokine and IgE production following OVA challenge. In conclusion, these findings clearly demonstrate that iV $\alpha 14$ NKT cells are required to participate in allergen-induced Th2 airway inflammation through a CD1d-dependent mechanism.

Introduction

Allergic asthma is a chronic inflammatory disease which is increasing throughout the Western World. This airway inflammation is associated with a predominant Th2 response generating eosinophilia increased mucus production in the lung and airway hyperresponsiveness, and specific IgE antibody synthesis (1,2). The production of Th2 cytokines, such as IL-4 and IL-5, is considered pivotal for the recruitment of eosinophils to the airways and for AHR which are characteristic of asthma. Adoptive transfer of effector Th2 cells into naive mice followed by exposure to inhaled antigens triggers eosinophilic inflammation, mucus hypersecretion and airway hyperresponsiveness, definitively demonstrating the capacity of immune Th2 cells to induce the asthma phenotype (3). It is generally held that rapid generation of IL-4 supports the crucial antigen-specific Th2 cell differentiation. The exact source of this required IL-4 is not known, but besides Th2 cells themselves, cells like mast cells, basophils, γ/δ T cells and Natural Killer (NK) T lymphocytes are thought to be contributors to Th2 immune responses.

NKT cells, are a distinct subset of lymphocytes with NK markers and $\alpha\beta$ TCR, that regulate immune responses in several models of autoimmunity, infection and cancer (4). They are selected and are restricted by the nonpolymorphic MHC class-I-like molecule CD1d and express an invariant V α 14-J α 18 TCR chain (4, 5). Since the stimulation of their TCR by glycolipid ligands, such as exogenous α -galactosylceramide (α -GalCer) presented in the context of CD1d molecules, promptly induces secretion of high concentrations of IL-4 (6), it has been proposed that they may participate in the differentiation of Th2 cells. Indeed, α -GalCer-stimulated NKT lymphocytes, in some cases, promote a protective Th2 immune response (7, 8). However, even though Th2 differentiation can be accomplished normally in NKT cell-deficient mice (9, 10), it cannot be excluded that NKT cells influence the development of some immune responses involving Th2 cells.

In the present study, we demonstrate that the invariant V α 14 (iV α 14) NKT cell subset is required for airway eosinophilia, hyperresponsiveness, Th2 cytokine production, and elevated levels of IgE antibodies, in an experimental model of allergic asthma.

Materials and Methods

Mice.

Six- to eight-week-old male C57BL/6 wild type and $J\alpha 18^{-/-}$ (backcrossed at least 10 times in C57BL/6) (11) mice were bred in our own facilities. All mice were kept in well-controlled animal housing facilities and had free access to tap water and pellet food throughout the experimental period.

Antigen Immunization and Airway Challenge.

At one weekly intervals, animals were immunized by an intraperitoneal (day 0) and a subcutaneous (day 7) injection with 4 μ g OVA (ICN Biomedicals, Inc, Ohio) adsorbed on 1.6 mg of aluminium hydroxide gel (Merck, Darmstadt, Germany) in 0.4 mL of sterile saline. Beginning on day 14 following the initial immunization, mice were anesthetized with ketamine anesthesia (Imalgene 1000, 35 mg/kg, Merial, Lyon, France) and airway challenged twice (days 14 and 21) intranasally with 10 μ g of OVA in 50 μ L of saline. Control mice received only saline at these times. Twenty-four hours after the last intranasal challenge (day 22), each group consisting of 6 – 20 mice was analyzed for eosinophil infiltration, airway hyperresponsiveness, cytokine levels and antibody production.

In some experiments, at one weekly intervals, animals were immunized by three intraperitoneal injections (days 0, 7 and 14) with 10 μ g OVA adsorbed on 1.6 mg of aluminium hydroxide gel in 0.4 mL of sterile saline. Sera were collected on day 15 and stored for IgE measurement.

Adoptive transfer.

Spleen cells from WT or $J\alpha 18^{-/-}$ mice were depleted of $CD8^+$ T cells, $CD62L^+$, and $CD19^+$ B cells labeled with the corresponding mAbs with anti-rat immunoglobulin-coated magnetic beads (Dyna, Compiègne, France). Among the mAbs used for cell depletion $CD8$ (clone 53.67) and $CD62L$ (clone Mel14) were purified in our laboratory while the anti- $CD19$ (clone 1D3) was purchased from BD-PharMingen(San Diego, CA). The enriched negative selected NKT spleen cell population (containing 10^5 $iV\alpha 14$ NKT cells, as assessed by $CD1d/\alpha$ -GalCer tetramers staining), as

well as a negative control (equivalent numbers of similarly depleted spleen cells from $J\alpha 18^{-/-}$ mice) were injected i.v. into $J\alpha 18^{-/-}$ mice that had been immunized twice with OVA.

Blocking of NKT/CD1d cell interaction in vivo.

In some experiments, in addition to OVA immunization and challenge (as described above), separate groups of mice were also treated i.p. with 0.15 mg of blocking rat anti-CD1d mAb (clone 20H2) or control rat IgG (Sigma-Aldrich, Stenheim, Germany) per mouse on days -1, 0, +2, +6, +7, +9, +13, +14, +16, +20 and +21. No differences in the percentage of $iV\alpha 14$ NKTsplenocytes were observed following anti-CD1d or Ig treatment on day 22 when mice were sacrificed (data not shown).

Determination of Airway Hyperresponsiveness (AHR).

Airway responsiveness was assessed as previously described (12). Briefly, 24 h after the last challenge, conscious mice were placed into a plethysmograph chamber (Buxco Electronics, Sharon, CT), and respiratory parameters were measured after 10 min of aerosol 150mM Methacholine administration (Sigma-Aldrich), delivered for 60 s. Airflow obstruction was expressed as enhanced pause (Penh), calculated as: $Penh = [Te \text{ (expiratory time)} / Tr \text{ (relaxation time)}] - 1 \times [Pef \text{ (peak expiratory flow)} / Pif \text{ (peak inspiratory flow)}]$. The values of Penh expressed per minute were averaged from three determinations recorded every 20 s.

Bronchoalveolar Lavage Fluid (BALF)

Immediately after assessment of AHR, mice were deeply anesthetized by i.p. injection of urethane (15 mg / 10 g body wt) (Sigma-Aldrich), blood collected and resulting serum stored. Airways were washed twice with saline, and the BALF cell differential counts and percentages determined by Diff-Quik (Baxter-Dale AG, Dudingon, Germany) staining of cytopsin slides. Two hundred cells per slide were counted. Aliquots of BALF were stored for cytokine measurement.

Determination of cytokines in BALF and of serial OVA-specific IgE

The levels of IL-4, IL-5, IL-13 and interferon (IFN)- γ in BALF and OVA-specific IgE serum levels were assessed by ELISA as previously described (6, 12) OVA-specific IgE levels of

samples were related to an internal standard from pooled sera of hyperimmunized BALB/c mice. Data were expressed as IgE index calculated as follows: IgE index = [OD sample - OD buffer only]/[OD positive control - OD buffer only].

Flow cytometric analysis.

Kinetic analysis of NKT cell activation after exposure to OVA was performed. In brief, 24 h after immunizations and/ or intranasal challenges, mice were sacrificed, splenocytes recovered and stained for 30 min in PBS containing 2% FCS and 0.01 M sodium azide and mCD1d- α -GalCer tetramer-APC. Tetramers were prepared in our laboratory from mCD1d/m β 2m expression vector (13). Cells were then incubated with appropriate dilutions of anti-CD4 PerCP-Cy-5.5 (clone RM4-5), anti-TCR $\alpha\beta$ -FITC (clone H57-597) and anti-CD69-PE (H1.2F3), all purchased from PharMingen. Dead cells were excluded on the basis of forward and side scatter. At least 5×10^5 live lymphoid cells were acquired in each run. Samples were analyzed on a FACScalibur (Becton Dickinson) using CellQuest software.

Statistical Analysis.

Nonparametric Mann-Whitney test was used to calculate significance levels for all measurements. $p < 0.05$ was considered statistically significant.

Results

Airway hyperreactivity and eosinophilic inflammation is reduced in $J\alpha 18^{-/-}$ mice.

A marked feature in this model of allergen-induced asthma is airway hyperreactivity (AHR) and massive airway inflammation observed following OVA-challenge. Our findings clearly demonstrate that OVA-sensitized and -challenged $J\alpha 18^{-/-}$ mice developed decreased AHR after methacholine inhalation as compared to OVA-sensitized and -challenged control mice (Fig. 1), implicating $iV\alpha 14$ NKT cells in AHR. The total cell number in BALF of $J\alpha 18^{-/-}$ mice following two intranasal OVA challenges was decreased by 42% compared to WT mice (Fig. 2A). The percentage of eosinophils, which account for the majority of cells in BALF from OVA-challenged mice (up to 65 %), was decreased by 58% in $iV\alpha 14$ NKT cell-deficient animals (Fig. 2B). Both macrophages and neutrophils also were reduced accounting for the overall decrease ($2.6 \pm 0.2 \times 10^5 \pm 0.2$ vs. $1.7 \pm 0.2 \times 10^5$ macrophages/ml and $2.2 \pm 0.6 \times 10^5$ vs. $1.1 \pm 0.1 \times 10^5$ neutrophils/ml in OVA-challenged WT vs. $J\alpha 18^{-/-}$ mice, respectively). Conversely, lymphocyte recruitment in BALF was not modified $7 \pm 2 \times 10^3$ vs. $4.7 \pm 2 \times 10^3$ lymphocytes/ml in OVA-challenged WT and $J\alpha 18^{-/-}$ mice, respectively). Among these lymphocytes, $iV\alpha 14$ NKT cells were also recruited to the lung of OVA-treated WT mice as assessed by RT-PCR analysis (data not shown).

Both IL-4 and IL-5 production are impaired in bronchoalveolar lavage fluid (BALF) of immunized $J\alpha 18^{-/-}$ mice.

IL-4 and IL-5 levels were markedly reduced in bronchoalveolar lavage fluid (BALF) collected 24 h after challenge from NKT cell-deficient mice as compared to WT littermates (Fig. 2C and D). Impaired IL-5 production in response to OVA challenge was also observed in the serum of $J\alpha 18^{-/-}$ mice (Fig. 2E), showing a systemic defect in the production of this cytokine in the absence of NKT cells. IL-4 levels in the serum were below detection limits (< 10 pg/ml).

OVA-specific IgE secretion is markedly decreased in $J\alpha 18^{-/-}$ mice.

To assess the peripheral response to immunization and subsequent allergen challenge, we measured OVA-specific IgE levels in the sera using ELISA. OVA-immunized and challenged $J\alpha 18^{-/-}$ mice had one eighth ($12.3 \pm 9.7\%$) OVA-specific IgE compared to wild-type (WT) littermates,

indicating that IgE production was severely affected (Fig. 2F). The failure to produce high levels of anti-OVA IgE was not due to the inability of $J\alpha 18^{-/-}$ mice to produce this isotype, because in response to three OVA immunizations but without intranasal challenges they did effectively produce anti-OVA IgE (0.83 ± 0.10 and anti-OVA IgE titer for $J\alpha 18^{-/-}$ and 1.53 ± 0.22 for WT mice).

Adoptive transfer of $iV\alpha 14$ NKT cells restores AHR development, airway eosinophilia as well as IL-4, IL-5 and IgE production in $J\alpha 18^{-/-}$ mice.

To confirm the implication of $iV\alpha 14$ NKT cells in the severity of allergic asthma, we reconstituted OVA-sensitized $J\alpha 18^{-/-}$ mice with $iV\alpha 14$ NKT lymphocytes 24h before the first OVA airway challenge. Adoptive transfer of $iV\alpha 14$ NKT cells, but not of spleen cells from $J\alpha 18^{-/-}$ mice, fully restored the capacity of $J\alpha 18^{-/-}$ mice to develop AHR (Fig. 3A). Moreover, airway eosinophilia (Fig. 3B), both IL-4 (Fig. 3C) and IL-5 (Fig. 3D) production in the BALF as well as specific anti-OVA IgE (Fig. 3E) in the serum of OVA-sensitized and -challenged $J\alpha 18^{-/-}$ mice were restored by the adoptive transfer of $iV\alpha 14$ NKT cells.

$iV\alpha 14$ NKT cells are activated in OVA-treated mice.

We evaluated furthermore whether $iV\alpha 14$ NKT lymphocytes were activated during OVA-induced allergic asthma, as assessed by expression of the early activation marker CD69. Indeed, CD69 expression was up regulated on tetramer $CD1d/\alpha\text{-GalCer}^+ TCR\alpha\beta^+$ splenocytes from OVA-immunized and -challenged mice on day 21 compared with those immunized with OVA but challenged with NaCl (Fig. 4A), demonstrating the activation of these cells following the OVA treatments. Similar results were obtained on day 14 after the first OVA challenge (data not shown).

$iV\alpha 14$ NKT cells mediate Th2 allergen-induced airway inflammation in a CD1d-dependent manner.

We have previously reported that NKT activation of $iV\alpha 14^+$ NKT cells does not necessarily occur in a CD1d-dependent manner, but can also be induced by the pro-inflammatory cytokines IL-12 and IL-18 (14). To evaluate possible CD1d involvement in NKT cell participation in Th2 asthmatic responses, WT mice were treated with blocking anti-CD1d mAb, or with control rat IgG at several time points during OVA immunization and challenge. Figure 4B clearly shows that anti-

CD1d treatment prevents the characteristic eosinophil recruitment to BALF observed after OVA challenge. Similarly, the secretion of both IL-4 (Fig. 4C) and IL-5 (Fig. 4D) levels in the BALF and of specific serum anti-OVA IgE antibodies (Fig. 4E) were diminished when CD1d-dependent interactions were blocked.

Discussion

Our experiments with $J\alpha 18^{-/-}$ mice clearly show that $iV\alpha 14$ NKT lymphocytes are required for the characteristic features of experimental allergic asthma, namely airway inflammation, BALF Th2 cytokine production and the secretion of high levels of specific anti-OVA IgE, that will ultimately induce AHR.

The failure of $J\alpha 18^{-/-}$ mice to develop the typical Th2 inflammation, AHR and IgE responses following OVA airway challenge is not due to a deficiency in Th2 responsiveness since these mice produce high levels of IgE in another protocol using three systemic immunizations with 2.5 higher doses of OVA. These data concur with previous reports showing that $J\alpha 18^{-/-}$ mice are not defective in IgE production (15). Here, we used a protocol where all mice were similarly immunized with OVA and alum, and airway OVA challenge resulting in Th2 inflammation and AHR. Because we found that $iV\alpha 14$ NKT cells are present in the lung of OVA-challenged mice, it is possible that they are involved during challenge rather than necessarily during priming. According to this postulate, we adoptively transferred WT $iV\alpha 14$ NKT cells just before OVA challenge, and then sufficed to restore the capacity of $J\alpha 18^{-/-}$ mice to fully develop airway eosinophilia, AHR, BALF cytokine and anti-OVA IgE production.

Our results differ from those of a previous study using another type of NKT cell-deficient mice, lacking CD1d molecules (16), which demonstrated that allergen-specific IgE levels and eosinophilia were not different from those observed in WT mice. Discrepancies observed may be explained by the different experimental protocols (two versus multiple challenges, timing of mice sacrifice) as well as the genetic background of the mutant mice (129/Sv X C57BL/6 for CD1d^{-/-} and C57BL/6 for $J\alpha 18^{-/-}$ mice). It is unlikely that the genotype (CD1d^{-/-} versus $J\alpha 18^{-/-}$ mice) is responsible for these differences since CD1d^{-/-} mice (backcrossed with C57BL/6) and $J\alpha 18^{-/-}$ mice

were similarly deficient in response to our protocol of immunization and challenge (data not shown). Moreover, in agreement with our results, it has recently been reported that both CD1d^{-/-} and J α 18^{-/-} mice, backcrossed with BALB/c, develop neither AHR nor airway eosinophilia, Th2 cytokine production and high levels of IgE following OVA challenge (17). Taken together, these results clearly demonstrate the requirement of iV α 14 NKT cells in allergen-induced asthma.

Additionally, our findings show that iV α 14NKT cells are present in lungs of OVA-treated mice, that they are systemically activated (up-regulation of CD69), and that their implication in this model is mediated through CD1d interactions. We and others have already demonstrated that NKT cells stimulated with α -GalCer favor Th2 or Th1 protective immune responses in experimental autoimmune diseases or tumors, respectively (4). In contrast, the present study underscores the capacity of activated iV α 14 NKT cells to mediate a pathological Th2 allergy immune response in asthma. It is probable that this activation involves recognition of endogenous antigens associated with CD1d molecules, because OVA itself was unable to stimulate NKT cells neither from naïve nor from OVA-treated mice (day 22) (data not shown). Endogenous antigens capable of stimulating iV α 14 NKT cells remain to be determined, but it has been suggested that the self-antigens, presumably glycolipids, that bind CD1d and together are recognized by V α 14⁺ TCR of these cells.

In conclusion, we have demonstrated that iV α 14 NKT cells mediate Th2 eosinophil airway inflammation and AHR in an allergen-induced asthma model. Thus, NKT cells may mediate or amplify the pathogenic Th2 inflammatory response and crucial airway hyperreactivity in allergic asthma, suggesting that depletion or blockade of NKT cells might be a possible option for the treatment of this disease.

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Footnotes

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Abbreviations

BALF: Bronchoalveolar Lavage Fluid;

OVA: ovalbumin.

Legends to Figures

Figure 1. Decreased AHR development in $J\alpha 18^{-/-}$ mice.

Airway reactivity to methacholine was measured one day after the last challenge with OVA (empty circles and squares) or NaCl (filled circles and squares) in both WT (squares) and $J\alpha 18^{-/-}$ (circles) mice. Data represent the mean \pm SEM enhanced pause (Penh) values from 6 to 20 mice.

Figure 2. Marked decrease in specific airway eosinophilia, IL-4, IL-5 and anti-OVA IgE production in OVA-challenged $J\alpha 18^{-/-}$ mice.

Total (A) and eosinophil cell counts (B) were determined in the BALF of $J\alpha 18^{-/-}$ and wild-type mice 24 h after the second NaCl or OVA challenge on day 22. Levels of IL-4 (C) and IL-5 (D) in BALF as well as IL-5 (E) and specific anti-OVA IgE antibodies (F) in serum were also measured 24 h after the second NaCl or OVA challenge. Data are means \pm SEM from 6 to 20 mice per group. * $p < 0.05$, ** $p < 0.01$.

Figure 3. Decreased AHR development, eosinophilia as well as IL-4, IL-5 and anti-OVA IgE production in $J\alpha 18^{-/-}$ mice are restored by adoptive transfer of NKT cells prior to challenge.

Groups of OVA-immunized and challenged $J\alpha 18^{-/-}$ mice were adoptively transferred with enriched CD1d/ α -GalCer tetramer positive splenocytes. Control $J\alpha 18^{-/-}$ mice were adoptively transferred with splenocytes from $J\alpha 18^{-/-}$ animals. **A.** Airway reactivity to methacholine was measured one day after the last challenge with OVA or NaCl in both WT (squares) and $J\alpha 18^{-/-}$ (triangles) mice. Enriched CD1d/ α -GalCer tetramer positive splenocytes (filled triangles) were adoptively transferred into OVA-immunized and challenged $J\alpha 18^{-/-}$ mice. Control $J\alpha 18^{-/-}$ mice were adoptively transferred with splenocytes obtained from $J\alpha 18^{-/-}$ animals (empty triangles). Data represent the mean \pm SEM enhanced pause (Penh) values from 5 to 10 mice. **B to E.** Twenty-four hours after the last OVA challenge, airway Eosinophil (B) numbers were evaluated and both IL-4 (C) and IL-5 (D) were measured in BALF. Serum levels of OVA-specific IgE (E) were determined by ELISA. Results represent means \pm SEM from 5 to 10 mice. * $p < 0.05$.

Figure 4. OVA-immunization and -challenge activates *iV* α 14 NKT cells, while anti-CD1d mAb treatment diminishes airway eosinophilic recruitment, Th2 cytokine levels and OVA-specific IgE production.

A. Representative FACS profiles showing up regulation of CD69 expression on gated invariant $V\alpha 14$ CD1d/ α -GalCer⁺ TCR $\alpha\beta$ ⁺ splenocytes recovered 24 h after the last OVA or NaCl challenge. **B to E.** Groups of C57BL/6 mice were treated with anti-CD1d mAb or control rat IgG (See Methods) and immunized with OVA and challenged with OVA or NaCl. 24 hours later, serum and BALF were collected. Eosinophil numbers (B) and both IL-4 (C) and IL-5 (D) were measured in BALF while OVA-specific IgE (E) were determined in the serum. Results represent means \pm SEM from 5 to 10 mice. * $p < 0.05$, ** $p < 0.01$.

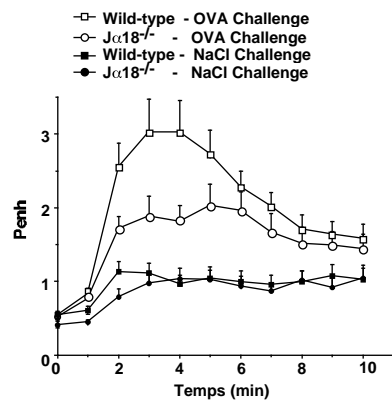


Figure 1

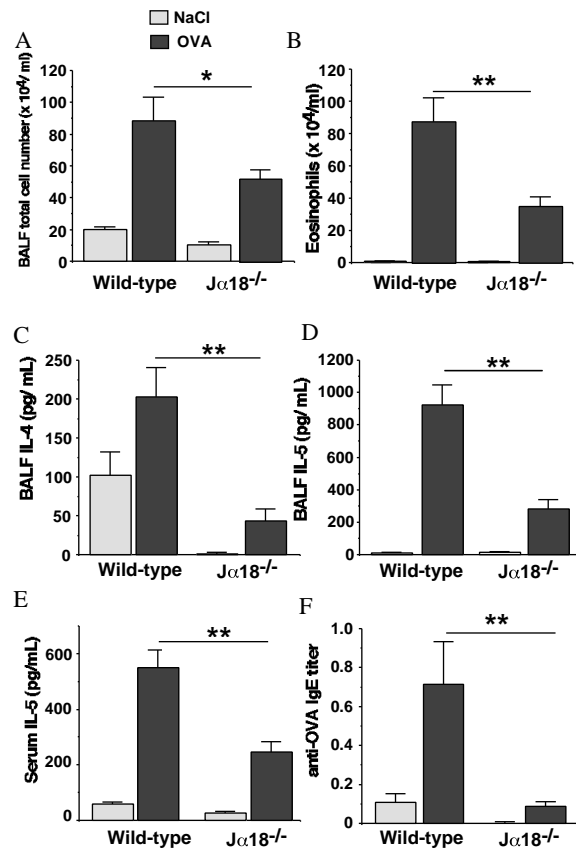


Figure 2

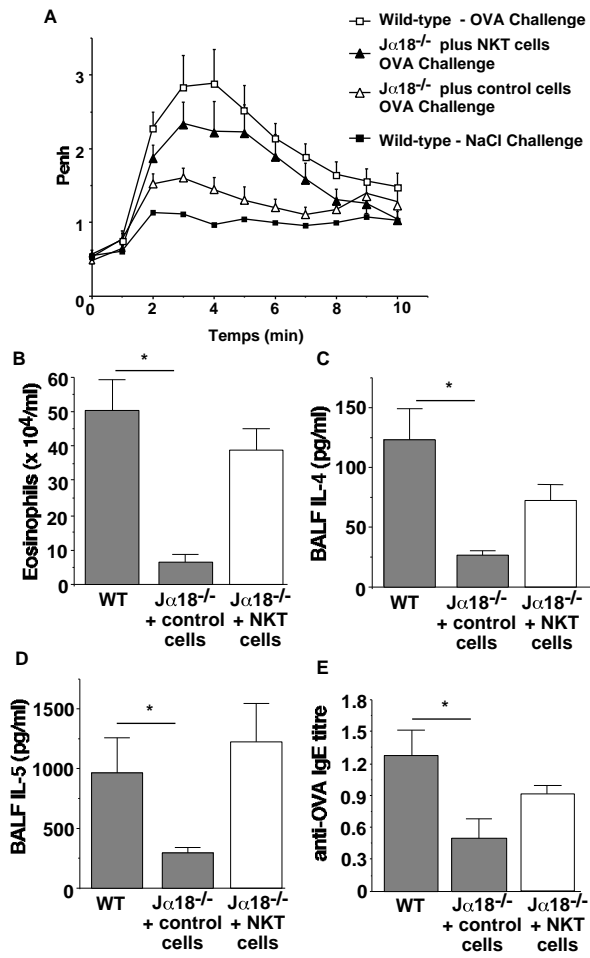


Figure 3

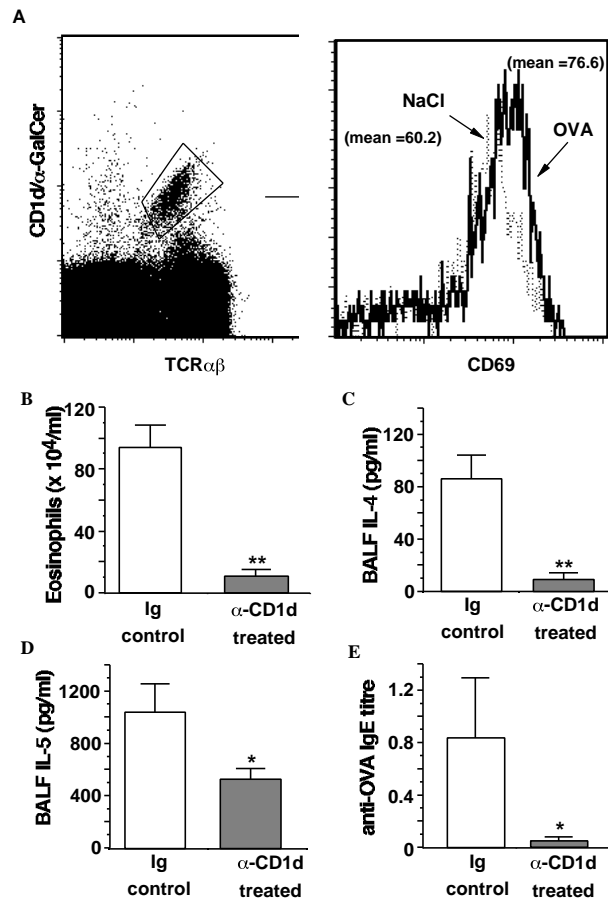


Figure 4