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Regulation of Th2 Responses and Allergic Inflammation through Bystander Activation of CD8$^+$ T Lymphocytes in Early Life

Aurore Dubois,* Nathalie Deruytter,[†,‡,§,1] Brigitte Adams,*[,1] Akira Kanda,[†,‡,§] Sandrine Delbauve,* Sebastien Fleury,[†,‡,§] David Torres,* Angélique François,[¶] Michel Pétein,[¶] Michel Goldman,* David Dombrowicz,[†,‡,§] and Véronique Flamand*

Th2-biased immune responses characterizing neonates may influence the later onset of allergic disease. The contribution of regulatory T cell populations in the prevention of Th2-driven pathologies in early life is poorly documented. We investigated the potential of CD8$^+$ T cells stimulated at birth with alloantigens to modulate the development of allergic airway inflammation. Newborn mice were immunized with semiallogeneic splenocytes or dendritic cells (DCs) and exposed at the adult stage to OVA aeroallergens. DC-immunized animals displayed a strong Th1 and Tc1/Tc2 alloantigen-specific response and were protected against the development of the allergic reaction with reduced airway hyperresponsiveness, mucus production, eosinophilia, allergen-specific IgE and IgG_1, and reduction of lung IL-4, IL-5, IL-10, and IL-13 mRNA levels. By contrast, splenocyte-immunized mice displayed a Th2 and a weak Tc2 alloantigen-specific response and were more sensitive to the development of the allergen-specific inflammation compared with mice unexposed at birth to alloantigens. DC-immunized animals displayed an important increase in the percentage of IFN-γ–producing CD8$^+$CD44^{high}, CD8$^+$CD62L^{high}, and CD8$^+$CD25$^+$ subsets. Adoptive transfers of CD8$^+$ T cells from semiallogeneic DC-immunized animals to adult β_2m-deficient animals prevented the development of allergic response, in particular IgE, IL-4, and IL-13 mRNA production in an IFN-γ–dependent manner, whereas transfers of CD8$^+$ T cells from semiallogeneic splenocyte-immunized mice intensified the lung IL-4 and IL-10 mRNA level and the allergen-specific IgE. These findings demonstrated that neonatal induction of regulatory CD8$^+$ T cells was able to modulate key parameters of later allergic sensitization in a bystander manner, without recognition of MHC class I molecules. ***The Journal of Immunology*****, 2010, 185: 000–000.**

Neonatal life is a unique developmental period characterized by a more pronounced susceptibility to microbial infections and allergic reactions (1). T cell responses in neonatal mice were described as Th2 biased, especially for memory responses (2–4). Therefore, Ag exposure in early life is likely to have an impact on the later development of Th2-associated pathologies, such as allergic disorders. It was established that asthma susceptibility might be enhanced by factors present in early life. Increased production of Th2 cytokines by fetal or neonatal T cells supports this view (5). Thus, strategies aimed at balancing neonatal Th1 and Th2 responses would facilitate the treatments for allergies. Allergic asthma is a chronic airway inflammation that leads to airway obstruction and airway hyperresponsiveness (AHR). The dominant immune disorder is driven by an unregulated Th2 response to aeroallergens that leads to Th2-type cytokine production in the lung. Local increased IL-4 contributes to the recruitment of Th2 cells in the lungs. Overproduction of IL-13 in the lungs is associated with AHR and mucus hypersecretion and, together with IL-5, increases eotaxin production and eosinophilia in the lungs. The induction of allergen-specific IgE is dependent on IL-4.

Extensive studies highlighted the potential of regulatory CD4$^+$ T cells (i.e., natural or induced CD4$^+$CD25$^+$, Tr1, IL-10 regulatory T, or Th1) to prevent atopic sensitization; however, the role of CD8$^+$ T cells in the control of allergic airways inflammation is poorly defined, especially in early life. Adult CD8$^+$ T cells are known to regulate Th2 immune responses against pathogens and alloantigens. Indeed, CD8$^+$ T cells were reported to suppress IgE production, Th2-type cytokine secretion (i.e., IL-4 and IL-5), and airway eosinophilia (6–9). The regulatory role of CD8$^+$ T cells on Th2 pathologies was related to several mechanisms, including cytotoxicity against CD4$^+$ T cells or dendritic cells (DCs) (10, 11), production of IFN-γ or IL-10 (12–14), and induction of IL-18 or IL-12 synthesis by APCs (15, 16).

We and other investigators demonstrated the ability of adult CD8$^+$ T cells to mediate Th2-type pathology suppression in a bystander manner (17–19). In a model of experimental Th2-type graft-versus-host disease induced across a single MHC class II Ag disparity, we demonstrated the role of CD8$^+$ T cells in the control of the pathology, independently of the recognition of any foreign MHC molecules (19).

Considering the neonates, we and other investigators reported that regulatory CD8$^+$ T cells controlling Th2 pathology are effective

*Institut d'Immunologie Médicale, Université Libre de Bruxelles; [¶]Institut de Pathologie et de Génétique, Gosselies, Belgium; [†]Institut National de la Santé et de la Recherche Médicale U547; [‡]Université Lille Nord de France; and [§]Institut Pasteur de Lille, Lille, France

[1]N.D. and B.A. contributed equally to this work.

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Address correspondence and reprint requests to Dr. Véronique Flamand, Institut d'Immunologie Médicale, Université Libre de Bruxelles, Rue Adrienne Bolland, 8, 6041 Gosselies, Belgium. E-mail address: vflamand@ulb.ac.be

Abbreviations used in this paper: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; DC, dendritic cell; LN, lymph node; NIMA, noninherited maternal Ag; UN, untreated.

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in early life. In a model of Th2-mediated autoimmunity, as well as in a model of neonatal transplantation tolerance, regulatory $CD8^+$ T cells were shown to downregulate the Th2-biased response (20, 21). In both cases, the induction of IFN-γ–producing $CD8^+$ T cells was suggested to be part of the mechanism. Moreover, several immunization protocols in neonates documented the induction of adult-like $CD8^+$ T cell responses to attenuated viral vector or to DNA vaccines (22, 23).

In the present investigation, we sought to test the potential role of $CD8^+$ T cells stimulated at birth with pro-Tc1 versus pro-Tc2 allogeneic accessory cells to modulate the susceptibility to allergic airway inflammation. For this purpose, we first evaluated the impact of neonatal inoculation of allogeneic spleen cells or DCs on the development of OVA-induced airway allergy during adulthood. Then we addressed the question of the regulatory role of $CD8^+$ T cells in this process by in vivo transfer experiments of adult $CD8^+$ T cells stimulated at birth in both ways. We assessed the ability of neonates to generate opposite regulatory $CD8^+$ T cells and, more particularly, the crucial role of IFN-γ–producing $CD8^+$ T cells to control the later allergic sensitization in a bystander manner, without involvement of MHC class I recognition pathway.

Materials and Methods

Mice

BALB/c (H-2^d) and C57BL/6 (H-2^b) mice were obtained from Harlan (Zeist, The Netherlands). BALB/c β2-microglobulin ($\beta2m^{-/-}$) mice were kindly provided by Dr. J.-C. Guéry (Toulouse, France). A/J (H-2^k) and BALB/c IFN-γ–deficient mice (IFN-$\gamma^{-/-}$) were purchased from The Jackson Laboratory (Bar Harbor, ME) and along with (A/J × BALB/c) F_1 hybrids were housed and bred in our specific pathogen-free animal facility.

Generation of bone marrow-derived DCs

DCs were generated by cultivating bone marrow progenitors in the presence of 20 ng/ml recombinant murine GM-CSF for 10 d, as previously described (24). The DC culture purity was evaluated with an FITC-conjugated anti-CD11c mAb (HL3), and DCs were considered immature based on their low expression of CD80, CD86, and CD40 molecules.

In vivo treatments

BALB/c mice were injected into the retro-orbital vein with 10×10^6 (A/J × BALB/c) F_1 hybrid spleen cells or 10^6 (A/J × BALB/c) F_1 hybrid DCs within the first 24 h of life. For $CD8^+$ T cell-transfer experiments, $CD8^+$ T cells were negatively selected from total lymph nodes (LNs) using a Dynabeads mouse CD8 negative isolation kit, according to the manufacturer's instructions (Invitrogen, Merelbeke, Belgium). Purity >93% was routinely assessed by flow cytometry analysis. A total of 10^6 $CD8^+$ T cells collected from 6–8-week-old wild-type (WT) or IFN-$\gamma^{-/-}$ BALB/c mice were inoculated intratracheally or i.v. in 6–8-week-old $\beta2m^{-/-}$ BALB/c mice.

Mixed lymphocytes culture, cytokine measurement, and CTL assay

Mixed lymphocyte cultures were prepared in complete RPMI 1640 culture medium (Lonza Research Products, Basel, Switzerland) supplemented with 20 mM HEPES, 2 mM glutamine, 1 mM nonessential amino acids, 5% heat-inactivated FCS, and 10^{-5} M 2-ME. $CD4^+$ or $CD8^+$ T cells (2.5×10^6/well) were purified from 6-wk-old BALB/c mice LNs and were stimulated with irradiated (2000 rad) syngeneic BALB/c, allogeneic A/J, or third-party C57BL/6 spleen cells (2.5×10^6/well) in 1 ml culture medium in 48-well flat-bottom plates (Cellstar, Greiner Bio-One, Wemmel, Belgium). $CD4^+$ T cells were purified by positive selection with the anti-CD4 mAb-coupled magnetic beads MACS system (Miltenyi Biotec, Utrecht, The Netherlands) and $CD8^+$ T cells, as described above. Purity of isolated cell suspensions was assessed by immunocytofluorometry (>93%). Cultures were kept at 37°C in a 5% CO_2 atmosphere, and supernatants were harvested after 72 h and analyzed for the presence of cytokines. Quantification of IFN-γ, IL-4, and IL-13 was made using commercially the available ELISA Duoset (R&D Systems, Abingdon, U.K.), and IL-5 was quantified by an Opt EIA set (BD Biosciences, Erembodegem, Belgium). CTL assays were performed as previously described (24).

Abs and CFSE labeling

Pacific blue-conjugated anti-CD8 mAb, biotinylated anti-CD44, anti-CD62L, anti-CD25 mAb, and PE-conjugated streptavidin were purchased from BD Biosciences. A total of 20×10^6/ml $CD8^+$ T cells were labeled with 1 μM CFSE (Invitrogen) for 10 min at 37°C.

Intracellular staining

LNs from 6-wk-old BALB/c mice were stimulated with irradiated (2000 rad) allogeneic A/J spleen cells (1/1 ratio) for 48 h at 37°C and 5% CO_2. Brefeldin A (1 μg/ml; Sigma-Aldrich, Bornem, Belgium) was added for the last night. Then cells were incubated for 10 min with Fc-blocking Abs (2.4G2), stained for surface markers (CD8 Pacific-Blue; BD Biosciences), washed with FACS buffer (PBS 1×, 0.5% BSA ≥96% lyophilized powder), fixed in CytoFix/CytoPerm solution (BD Biosciences), permeabilized with Perm/Wash buffer (BD Biosciences), and labeled with specific cytokine Abs (allophycocyanin-conjugated anti–IFN-γ; BD Biosciences) or isotype controls. Cells were analyzed on a Cyan ADP flow cytometer (DakoCytomation, Everlee, Belgium).

Murine model of asthma

Six- to 8-wk-old mice were sensitized by i.p. injection of 50 μg OVA in 100 μl aluminum hydroxide (alum, Reheis Chemical, Bekerley Heights, NJ) on days 0 and 14. From days 21–23, we exposed mice to OVA (1%) aerosols for 20 min using an ultrasonic nebulizer (Harvard Apparatus, Les Ulis, France).

Measurement of airway responsiveness to methacholine

AHR to increasing concentrations of methacholine was measured by whole-body plethysmography (Emka Technologies, Paris, France). Results are expressed as fold increase enhanced pause values over baseline value (25).

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) fluids were prepared by washing the lungs three times with 1 ml ice-cold PBS. The cells were sedimented by centrifugation at 400 × *g* for 10 min at 4°C. An aliquot of the cell pellets was stained with Turks solution and counted, and 200,000 cells were centrifuged on microscope slides (cytospun at 1000 rpm for 10 min at room temperature). Air-dried preparations were fixed and stained with Diff-Quik (Merz & Dade, Dudingen, Switzerland). Differential counts were made under oil-immersion microscopy. Two fields of 100 cells were counted to determine the number of eosinophils present in the BAL.

Quantification of airway mucus

Lungs were fixed in 10% buffered formalin and embedded in paraffin. Samples were sectioned (4 μm) and stained with periodic acid-Schiff with diastase and hematoxylin. The histologic mucus index was calculated as the percentage of mucus-containing airway epithelia, using three grades of severity: 0–30% (grade 1), 30–60% (grade 2), and 60–100% (grade 3). Three sections per lung were examined in a double-blind fashion.

Real-time quantitative PCR on lung tissue

RNA was extracted from the lungs and mediastinal LNs using a MagnaPure LC RNA Isolation Kit III (Tissue) (Roche Diagnostics, Brussels, Belgium). Reverse-transcription and real-time PCR reactions were carried out using LightCycler-RNA Master Hyprobes (one-step procedure) on a LightCycler apparatus (Roche Diagnostics) to measure IL-4, IL-5, IL-10, IL-13, and IFN-γ mRNA. The sequences of primers and probes are available on request (http://medgen.ugent.be/rtprimerdb/search.php; group name: FLAMAND).

Serum levels of OVA-specific IgE and IgG_1

OVA-specific IgG_1 and IgE were measured by ELISA. For IgG_1 quantification, we incubated Ag-coated Maxisorp plates (Nunc, Langenselbold, Germany) with serial dilutions of sera and biotinylated mAb to IgG_1 (LO-MG1-2 biotin; Université Catholique de Louvain, Brussels, Belgium). For Ag-specific IgE, we first coated the plates with the respective capture mAb (LOME-3; Université Catholique de Louvain, Brussels, Belgium) and incubated them with the serum dilutions. We then added biotinylated OVA (Immunosource).

Statistics

Data are expressed as mean ± SEM. Statistical analysis was performed using the two-tailed nonparametric Mann–Whitney *U* test or, when specified, the Student *t* test. Statistical analysis of enhanced pause values was performed with the repeated-measures ANOVA test.

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Results

Effects of neonatal immunizations with allogeneic DCs or allogeneic splenocytes on the alloreactive CD4$^+$ or CD8$^+$ T cells

Depending on the stimulus, the murine newborn immune system is capable of mounting adult-like Th1-type responses, as well as a partially biased Th2-type response. We first monitored the status of alloreactive T cells by assessing the Th1/Th2-type or Tc1/Tc2-type cytokine production of purified CD4$^+$ or CD8$^+$ T cells, as well as CTL activities. CD4$^+$ T cells that were stimulated at birth with F_1 splenocytes produced large amounts of IL-4 and IL-5 but no IFN-γ in response to A/J stimulators, whereas the ones that were stimulated with F_1 DCs secreted significantly more IFN-γ and produced less IL-4 and IL-5 (Fig. 1*A*).

Compared with CD8$^+$ T cells collected from naive mice or mice immunized at birth with F_1 splenocytes, CD8$^+$ T cells that were sensitized at birth with F_1 DCs produced significantly greater amounts of IFN-γ, IL-13, and IL-5, with a predominance of IFN-γ production. CD8$^+$ T cells that were stimulated with F_1 splenocytes were poorly primed against the alloantigens, with low levels of IFN-γ and IL-5 (Fig. 1*B*). In the presence of IL-2, we were able to induce a low mixed production of IFN-γ, IL-4, IL-5, and IL-13 by CD8$^+$ T cells immunized at birth with F_1 spleen cells (B. Adams, unpublished observations). The alloreactivity of the purified CD4$^+$ or CD8$^+$ T cells isolated from the different groups toward third-party alloantigens was not affected (Fig. 1*A*, 1*B*). We further confirmed, by flow cytometry, the particular ability of allogeneic DCs administered at birth to increase the percentage of IFN-γ$^+$ cells among the total CD8$^+$ T cell population (Fig. 1*C*).

Finally, in agreement with several previous studies (26), when BALB/c mice were injected early in life with F_1 spleen cells, in contrast with F_1 DCs, they were unable to generate anti-donor CTL activities, whereas they developed normal CTL activities against third-party targets (Fig. 1*D*). Overall, we showed the ability of neonatal CD4$^+$ and CD8$^+$ T cells to respond to various allogeneic stimuli. Semiallogeneic splenocytes induced a strong Th2 and a low Tc2 phenotype, with no donor-specific CTL activities, whereas DCs stimulated a strong Th1 and Tc1/Tc2 response with CTL activities.

Mice immunized at birth with semiallogeneic splenocytes display a strong allergic inflammation, whereas mice immunized at birth with semiallogeneic DC are protected

Early life exposure to certain Ags might have a strong impact on the development of asthma. We assessed whether neonatal contact with alloantigens would affect the subsequent development of allergic airway inflammation. BALB/c mice inoculated at birth with semiallogeneic splenocytes or DCs were sensitized and challenged with the allergen OVA at 6 wk of age, and key parameters of allergic reaction were monitored. Compared with mice not treated at birth, AHR, in response to OVA, was diminished in mice that were neonatally exposed to semiallogeneic DCs and was exacerbated in splenocyte-exposed mice (Fig. 2*A*). We observed that the number of eosinophils was elevated following allergen airway

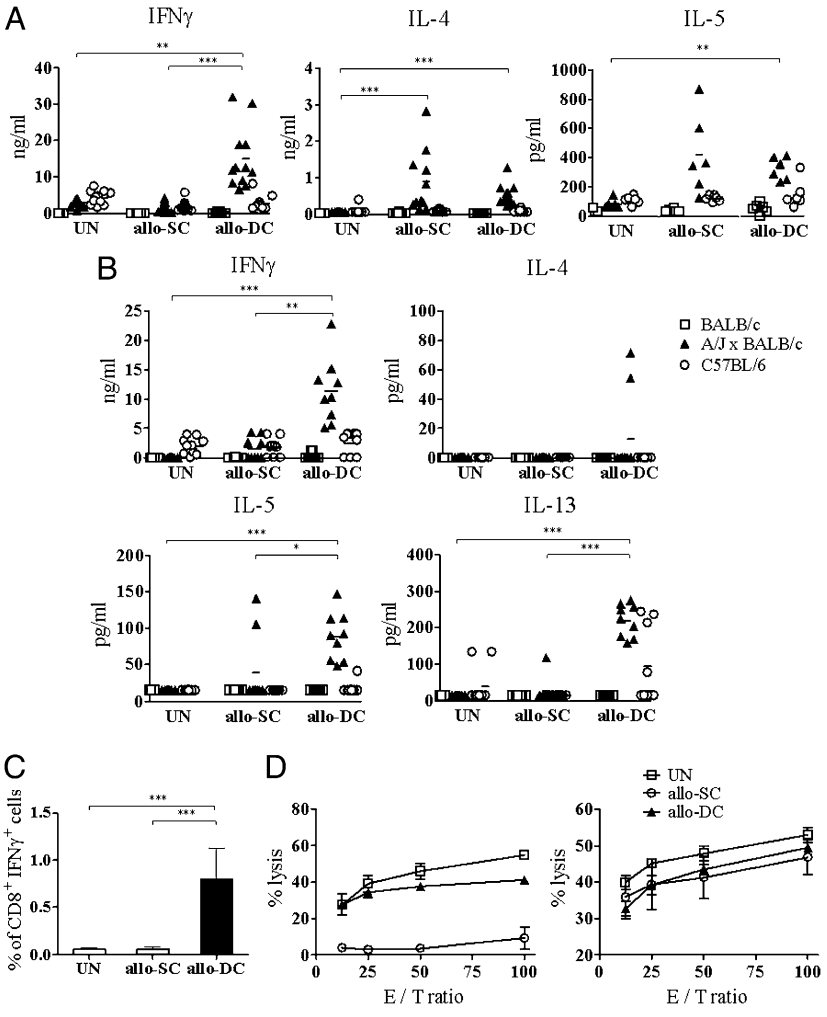


FIGURE 1. Cytokine production and cytotoxic activities of CD4$^+$ and CD8$^+$ T cells. Purified CD4$^+$ T cells (*A*) or CD8$^+$ T cells (*B*) from 6-wk-old naive or neonatally injected BALB/c mice were cultivated with syngeneic (BALB/c), allogeneic (A/J × BALB/c)F_1, or third-party (C57BL/6) spleen cells. Results are individual data from 6–12 mice. *C*, IFN-γ intracytoplasmic staining after (A/J × BALB/c) F_1 alloantigen recall on pooled LN cells collected from six adult mice injected neonatally with F_1 splenocytes or DCs or UN 6-wk-old BALB/c mice collected from three separate experiments. Results are expressed as percentage (mean ± SEM) of IFN-γ$^+$ cells among the CD8$^+$ T cells. *D*, Anti-A/J (*left panel*) or anti-C57BL/6 (*right panel*) CTL activity was generated from 6-wk-old uninjected (□), splenocyte-injected (○), and DC-injected (△) BALB/c mice during a 5-d mixed lymphocyte culture. Results from three to six individual mice are expressed as percentage (mean ± SEM) of specific lysis at different E:T ratios. The results are representative of three independent experiments. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. UN, untreated.

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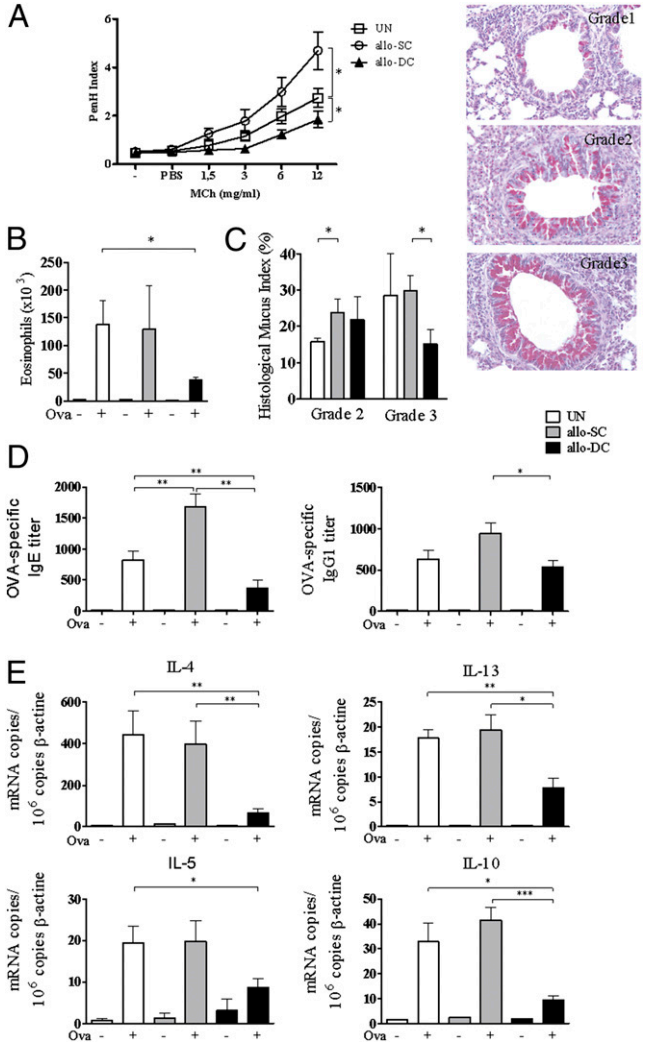


FIGURE 2. Allergic inflammation in mice following neonatal immunization with semiallogeneic splenocytes or DCs. *A*, AHR to methacholine. Twenty untreated 8-wk-old BALB/c mice (□), 11 F_1 spleen cell-injected mice (○), or 11 F_1 DC-injected mice (▲) were sensitized and challenged with OVA. *B*, Numbers of eosinophils in BAL fluid from mice sensitized or not against OVA and immunized or not at birth with semiallogeneic splenocytes or DCs ($n = 6$–14). *C*, Quantitative measurement of airway mucus production. Histological mucus index in OVA-allergic mice immunized or not at birth with semiallogeneic splenocytes or DC ($n = 5$ to 7). $*p < 0.05$; Student *t* test. Photomicrographs of the three grades of mucus-containing airway epithelia (original magnification ×200, periodic acid-Schiff staining). *D*, OVA-specific IgE and IgG_1 levels were monitored in seven individual mice. Values are mean Ig titer. *E*, Total mRNA was extracted from the lung of 7–14 individual mice per experimental group and analyzed by real-time RT-PCR. Cytokine mRNA levels were normalized using β-actin mRNA as a reference. Results are mean ± SEM and were collected from three independent experiments. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

challenge with OVA in mice not treated at birth or injected with F_1 splenocytes, and it was significantly decreased in mice that received F_1 DCs (Fig. 2*B*). Airway mucus accumulation in lungs was quantified. Mice that were inoculated at birth with F_1 splenocytes showed the greatest mucus accumulation, which was characterized by a significant increase in the proportion of grade 2 mucus-containing epithelium, whereas the mice that were inoculated with F_1 DCs showed a significant reduction in the proportion of grade 3 mucus-containing airway epithelium (Fig. 2*C*). We further evaluated allergen-specific IgE and IgG_1 production. As shown in Fig. 2*D*, neonatal F_1 splenocyte inoculation led to an increased production of OVA-specific IgE, as well as a greater production of OVA-specific IgG_1. In contrast, neonatal exposure to F_1 DCs strongly inhibited OVA-specific IgE production, and those treated animals produced significantly less OVA-specific IgG_1 than the animals treated at birth with F_1 splenocytes. As shown in Fig. 2*E*, compared with mice not treated at birth, F_1 splenocytes did not modify the Th2 cytokine environment into the lung following challenge. However, F_1 DCs administered at birth abrogated IL-4, IL-5, IL-10, and IL-13 lung mRNA induction (Fig. 2*E*), as well as the IL-4 mediastinal LN mRNA induction (data not shown) after OVA challenge. These data demonstrated that neonatal administration of Th1/Tc1-type alloantigens (i.e., F_1 DCs) reduced later responses to allergens, whereas administration of Th2/Tc2-type alloantigens (i.e., F_1 splenocytes) increased inflammatory responses to allergens.

$CD8^+$ T cells stimulated at birth with semiallogeneic splenocytes enhance allergen-specific Th2 response, whereas $CD8^+$ T cells stimulated at birth with semiallogeneic DCs prevent Th2 response by an IFN-γ–dependent mechanism

Based on the reported ability of $CD8^+$ T cell to regulate the Th2-type response, we focused our attention on the potential role of $CD8^+$ T cells stimulated at birth with alloantigens to regulate the development of an allergic reaction in adulthood. $CD8^+$ T cells isolated from 6–8-wk-old naive mice or mice injected at birth with semiallogeneic splenocytes or DCs were adoptively transferred to adult β2m-deficient mice ($\beta2m^{-/-}$) that were sensitized and challenged with OVA following the experimental protocol in Fig. 3*A*. $CD8^+$ T cell differentiation in these recipient mice is hindered by the lack of thymic MHC class I expression. As shown in Fig. 3*B* and 3*C*, compared with the transfer of naive $CD8^+$ T cells, transfer of $CD8^+$ T cells isolated from splenocyte-injected mice increased IL-4 and IL-10 lung mRNA production and, more significantly, OVA-specific IgE response following OVA aerosol challenge. In contrast, $CD8^+$ T cells isolated from DC-injected mice downregulated IL-4, IL-10, and IL-13 mRNA production in the lung and the OVA-specific IgE serum levels without modifying local IFN-γ mRNA production. These effects on local cytokine levels and allergen-specific IgE were associated with a partial inhibition of AHR (Fig. 3*D*) and mucus production (Fig. 3*E*) only by allo-DC–immunized $CD8^+$ T cells. OVA-induced IL-5 mRNA production and lung eosinophilia were not significantly inhibited by allo-DC–immunized $CD8^+$ T cells. Our results provide evidence that alloreactive $CD8^+$ T cells stimulated at birth with Th2/Tc2 Ags have the ability to increase allergic reaction, whereas they can downregulate key parameters of such a reaction in adulthood when stimulated with Th1/Tc1 Ags.

We then evaluated the role of $CD8^+$ T cell-derived IFN-γ by transferring DC-immunized $CD8^+$ T cells isolated from WT or IFN-$\gamma^{-/-}$ mice into OVA-sensitized recipient $\beta2m^{-/-}$ mice that were further challenged with OVA following the time schedule provided in Fig. 3*A*. Inhibition of lung IL-4 and IL-13 mRNA and a decrease in serum OVA-specific IgE levels observed after the transfer of F_1 DC-stimulated $CD8^+$ T cells collected from WT mice was abolished when F_1 DC-stimulated IFN-$\gamma^{-/-}$ $CD8^+$ T cells were injected. Interestingly, compared with naive WT and IFN-$\gamma^{-/-}$ $CD8^+$ T cells, F_1 DC-immunized $CD8^+$ T cells collected from WT or IFN-$\gamma^{-/-}$ mice similarly downregulated the lung IL-10 mRNA OVA induction, probably accounting for the relative low production of OVA-specific IgE (Fig. 4). This suggests that the IL-4– and IL-13–dependent IgE production is downregulated by immunized $CD8^+$ T cells in an IFN-γ–dependent manner and that the IL-10–dependent IgE production is regulated by immunized $CD8^+$ T cells in an IFN-γ–independent manner. This phenomenon was induced without any recall of the alloantigen and occurred without MHC class I recognition.

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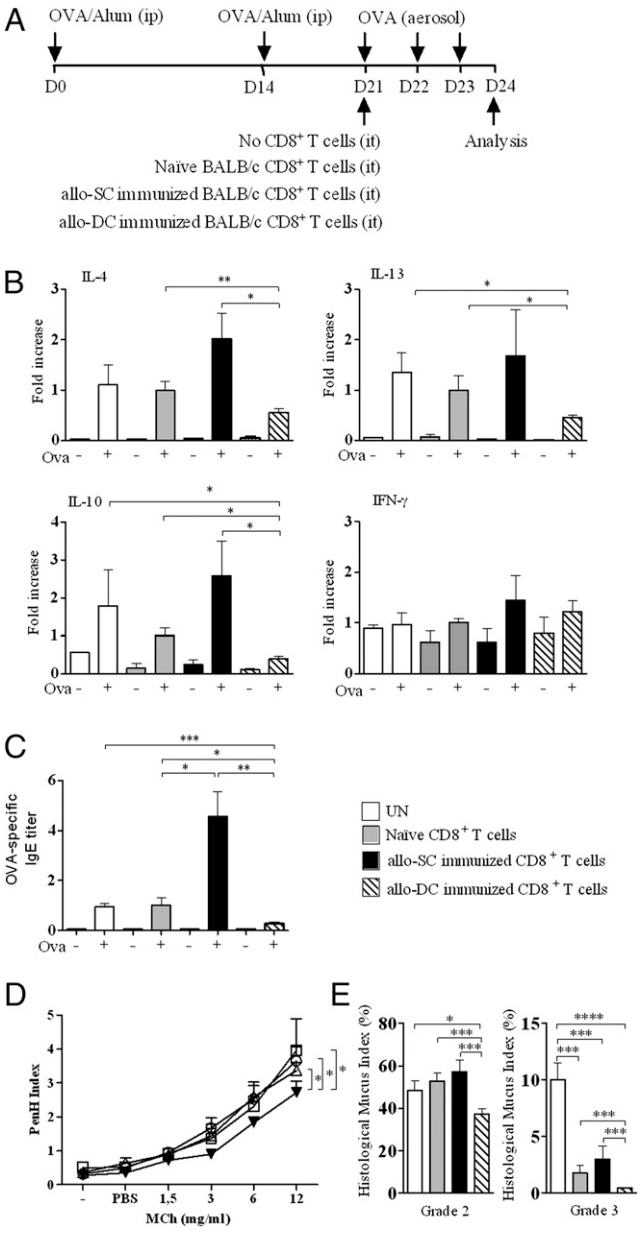


FIGURE 3. $CD8^+$ T cell transfer. *A*, Experimental protocol: six $\beta2m^{-/-}$ mice were injected i.p. with 50 μg of OVA in 100 μl of alum on days 0 and 14. The mice were challenged with OVA aerosols (1%) on days 21–24 and were not treated or were inoculated with $CD8^+$ T cells collected from six naive, six F_1 splenocyte-injected, or six DC-injected BALB/c mice on day 21. *B*, Total RNA was extracted from the lung and analyzed by real-time RT-PCR. Cytokine levels were normalized using β-actin mRNA as a reference and compared with the group of $\beta2m^{-/-}$ mice transferred with naive $CD8^+$ T cells. *C*, OVA-specific IgE was monitored by ELISA and expressed as mean ELISA titer. *D*, AHR to methacholine. Six UN 8-wk-old $\beta2m^{-/-}$mice (□), six $\beta2m^{-/-}$ mice transferred with naive $CD8^+$ T cells (○), six $\beta2m^{-/-}$ mice transferred with allo-SC immunized $CD8^+$ T cells (△), or six $\beta2m^{-/-}$ mice transferred with allo-DC–immunized $CD8^+$ T cells (▼) were sensitized and challenged with OVA before methacholine treatment. *E*, Histological mucus index in OVA-allergic $\beta2m^{-/-}$ mice transferred or not with indicated $CD8^+$ T cells (n = 5–7/group). See the representative grades in Fig. 2*C*. Results are mean ± SEM. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. UN, untreated.

IFN-γ–producing memory and regulatory $CD8^+$ T cells are more present following semiallogeneic DC immunization than upon splenocyte immunization at birth

Memory $CD8^+$ T cells have been described as better providers than $CD4^+$ T cells of an early non-Ag–specific source of IFN-γ. Thus, we evaluated the potential expansion of memory $CD8^+$ T cells following neonatal stimulation with both types of allogeneic stimulation. Because the maintenance of memory $CD8^+$ T cells is regulated by an MHC class I-independent mechanism, we transferred CFSE-labeled $CD8^+$ T cells collected from lymphoid organs of BALB/c mice inoculated at birth with semiallogeneic splenocytes or DCs into adult $\beta2m^{-/-}$ mice. The beginning phenotype of adult $CD8^+$ T cells from untreated mice was 85% ± 3% $CD62L^+CD44^-$ and 2% ± 0.6% $CD25^+$. Adult $CD8^+$ T cells collected from semiallogeneic splenocyte- or DC-immunized mice at birth were 75% ± 5% $CD62L^+CD44^-$ and 4.75% ± 1.5% $CD25^+$. Two days after the i.v. transfer, we visualized $CD8^+$ T cells that produced IFN-γ. We found that $CD8^+CD44^{high}$ and $CD8^+CD62L^{high}$ cells (Fig. 5*A*) producing significant amounts of IFN-γ (Fig. 5*B*) were preferentially expanded after the transfer of semiallogeneic DC-primed $CD8^+$ T cells compared with naive $CD8^+$ T cells and semiallogeneic splenocyte-primed $CD8^+$ T cells. Furthermore, the proportion of the $CD8^+$ $CD25^+$ T cell subset producing IFN-γ was also greater in $CD8^+$ T cells immunized at birth with F_1 DCs (Fig. 5).

Discussion

The present work aimed to examine the effects of neonatal antigenic stimulation on the subsequent allergic response in adult mice. Our data support the hypothesis that sensitivity to airway inflammation, such as asthma, might result, in part, from failure to appropriately activate regulatory pathways through antigenic stimulation involving $CD8^+$ T cells early in life.

Although the neonatal immune system is predisposed to Th2 cell activation, maturation of the immune response, driven by contact with Ag in early life, may take different pathways, depending on the stimulus. Indeed, we and other investigators showed in murine models that neonatal exposure to semiallogeneic splenocytes stimulates Th2 and Tc2 responses, whereas inoculation of semiallogeneic DCs may induce Th1 and Tc1 activation (27–30). We showed in this article the immunomodulatory effects induced by (A/J × BALB/c) F_1 alloantigens early in life on the later development of allergic responses to OVA aeroallergens. Exposure to semiallogeneic splenocytes led to a severe allergic airway reaction, with a more pronounced OVA-specific IgE production, mucus accumulation in the lung, and AHR. In contrast, early life exposure to semiallogeneic DCs decreased OVA-specific IgE and IgG_1 production, eosinophilia, mucus production, as well as the Th2-type cytokine mRNA and AHR. We showed that the neonatal antigenic experience may lead to different types of regulatory $CD8^+$ T cells able to enhance or prevent some key parameters of allergic disease in adulthood. Indeed, the neonatal activation of Tc2 $CD8^+$ T cells with semiallogeneic splenocytes may aggravate the subsequent development of allergic responses to irrelevant Ags by increasing the lung levels of IL-4 and IL-10 upon allergen challenge and the allergen-specific IgE levels. In contrast, the neonatal activation of Tc1 $CD8^+$ T cells with semiallogeneic DCs may decrease the severity of allergic disease later in life, mostly by decreasing the lung levels of IL-4, IL-10, and IL-13; the seric OVA-specific IgE levels; and mucus production. IL-17 induction was not observed as a potential regulatory cytokine in this setting (data not shown). As the major APCs present in the semiallogeneic splenocyte inoculum, B lymphocytes are most likely to be responsible for the induction of the Tc2 phenotype, and IL-10 secretion by such activated $CD8^+$

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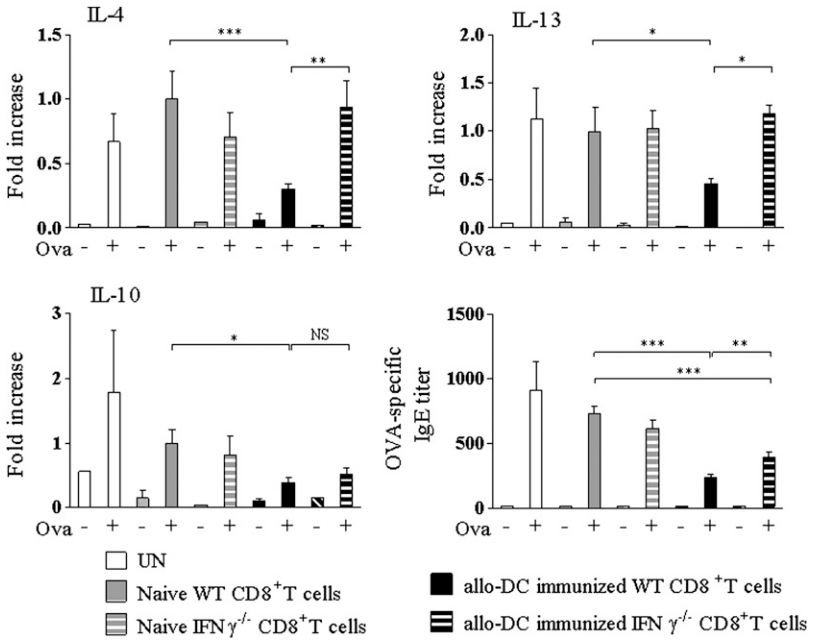


FIGURE 4. Th2-type cytokines and IgE levels after in vivo IFN-$\gamma^{-/-}$ $CD8^+$ T cell transfer. Six individual $\beta 2m^{-/-}$ mice were UN or inoculated with $CD8^+$ T cells collected from four naive, five F_1 DC-injected WT, or five IFN-$\gamma^{-/-}$ BALB/c mice and were sensitized or not and challenged with OVA. Total RNA was extracted from the lung and analyzed by real-time RT-PCR. Cytokine levels were normalized using β-actin mRNA as a reference. OVA-specific IgE was monitored by ELISA and expressed as mean of ELISA titer. Results are mean ± SEM collected from two independent experiments. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. UN, untreated.

T cells is likely to contribute to the enhancement of the allergic reaction (B. Adams, A. Dubois, S. Delbauve, I. Debock, F. Lhommé, M. Goldman, V. Flamand, submitted for publication). We expected the induction of these two types of regulatory $CD8^+$ T cells to be restricted to the neonatal stage, but it would be interesting to evaluate how similar regulatory $CD8^+$ T cells could be induced at the adult stage.

Based on our observations with semiallogeneic spleen cells or DCs, we speculate that the nature of Ag exposure in early life has a dominant role in shaping nonspecific memory (i.e., Th2/Tc2-promoting Ag exposure in early life may interfere with their capacity to mount protecting Th1-mediated responses and may reinforce helminth protection, whereas Th1/Tc1-promoting Ags control the development of atopic disease and may favor autoimmunity and increased susceptibility to helminth infection).

The importance of IFN-γ in the prevention of Th2-type pathologies was highlighted in several studies showing that one of the major factors associated with protection from allergic disorders is the ability of pathogens to induce strong IFN-γ production by T cells (31, 32). The IFN-γ–dependent inhibitory effects of Th1-type $CD4^+$ T cells on allergic airway eosinophilia and mucus production were described (33), and vaccination with Th1-directing adjuvants may activate $CD4^+$ T cells to suppress the development of allergen-induced Th2-type responses (34). Still, memory $CD8^+$ T cells were described as a better provider than memory $CD4^+$ T cells of an early non-Ag–induced source of IFN-γ. The regulation of allergic airway inflammation by MHC class I-restricted allergen-specific $CD8^+$ T cells able to produce IFN-γ in response to IL-12 secretion by lung DCs was reported (35). $CD8^+$ T cells are considered key players in the immune protection against intracellular infections and tumor eradication, but their powerful cytotoxic activities may also contribute to rejection of organ transplants and autoimmunity. After pathogen clearance, memory $CD8^+$ T cells survive, whereas effector $CD8^+$ T cells die. $CD8^+$ T cell memory can be maintained in the absence of Ag and even in the absence of MHC molecules through a mechanism of self-renewing division called homeostatic proliferation that is dependent of IL-7 and IL-15 (36). Because of their low lymphocyte numbers in lymphoid organs, neonates may be particularly efficient for lymphopenia-induced proliferation (37). It was reported that an IL-7–dependent homeostatic proliferation of $CD8^+$ T cells in neonatal mice allows the generation of natural memory T cells that rapidly produce IFN-γ when transferred in neonate recipients (38).

In the absence of obvious TCR-mediated stimulus for $CD8^+$ T cell activation in the present model of OVA-induced airway allergy, it is likely that memory $CD8^+$ T cells play a dominant role

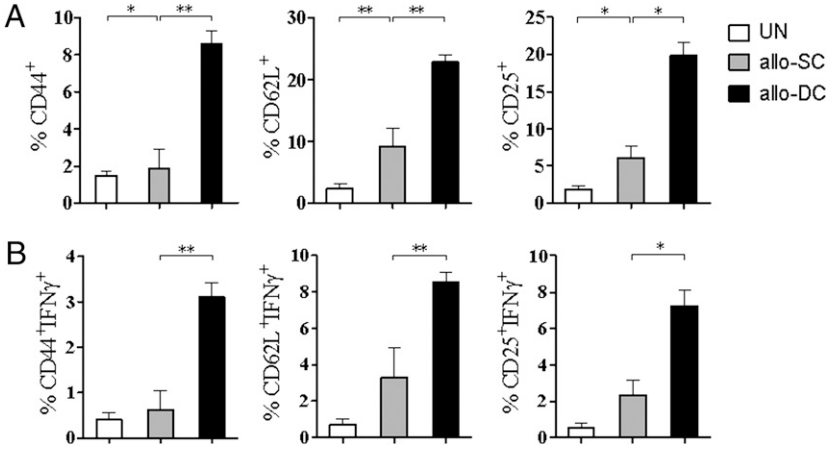


FIGURE 5. Expansion of $CD44^{high}$, $CD62L^{high}$, and $CD25^+$ subsets producing IFN-γ in $CD8^+$ T cells immunized at birth with F_1 DCs. A total of 4×10^6 purified $CD8^+$ T cells collected from 6–8-wk-old naive, F_1 spleen cells-injected BALB/c mice, or F_1 DC-injected BALB/c mice were labeled with CFSE and inoculated i.v. in adult $\beta 2m^{-/-}$ mice. *A*, Two days later, $CD8^+CFSE^+$ cells expressing CD25, $CD44^{high}$, or $CD62L^{high}$ were monitored by flow cytometry. *B*, IFN-γ intracellular staining was performed in the three $CD8^+$ subsets without recall with alloantigens. Data are expressed as mean (± SEM) percentage of positive staining of five $\beta 2m^{-/-}$ mice collected in three independent experiments. $*p < 0.05$; $**p < 0.01$.

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in the induction of IFN-γ synthesis, either directly or indirectly. Long-lived memory $CD8^+$ T cells have been recognized as high producers of IFN-γ able to rapidly respond through cross-reactive Ag-specific TCRs or in a nonspecific manner through cytokines present in the environment (17, 31). We observed the ability of the $CD8^+$ T cells to regulate asthma in a bystander manner (i.e., $CD8^+$ T cells collected from neonatally semiallogeneic DC-inoculated mice) to expand the proportions of $CD44^{high}$, $CD62L^{high}$, and $CD25^+$ subsets that produce IFN-γ. This supports the concept that neonatal exposure to some inflammatory signals provokes a lymphopenia-driven proliferation that drives naive T cells to differentiate directly into memory or regulatory T cells (39). The ability of $CD8^+$ T cells to inhibit lung Th2 responses and allergen-specific IgE responses is consistent with other studies. Memory $CD8^+$ T cells may reside locally for a long period postinfection [e.g., in the lung after influenza infection (18)] and may inhibit, in a bystander manner, allergic airway inflammation through an IFN-γ–dependent pathway. The ability of nonspecific memory $CD8^+$ T cells to rapidly produce IFN-γ and to inhibit the development of a Th2-type response is dependent on their capacity to respond to the local presence of IL-12 and IL-18. Furthermore, $CD8^+$ T cells stimulate DCs to produce IL-12 and, thereby, can promote IFN-γ production by activated $CD4^+$ T cells that control the allergen-specific response, especially IL-4 and IgE production (7, 31). Together with IL-12, CD40 and NKG2D ligation could be involved in the stimulation of memory $CD8^+$ T cells (40–42). In the present model, we ruled out the involvement of MHC class I recognition (classical or nonclassical MHC class I molecules, such as Qa-1, a MHC-Ib product) in the activity of regulatory $CD8^+$ T cells, because semiallogeneic DC-stimulated $CD8^+$ T cells were able to inhibit Th2 cytokine production and OVA-specific IgE production when transferred to OVA-sensitized β2m-deficient mice.

A natural exposure to alloantigens may occur in early life. Indeed, noninherited maternal Ags (NIMAs) can be transferred to the fetus across the placenta or to the neonate via breast milk. Several studies demonstrated that, depending on the nature and the level of NIMA-like alloantigens present in newborns, tolerance or priming responses may take place, leading to long-term heart allograft survival (43) and suppression of graft-versus-host reaction (44) or to the opposite: acute rejection of renal grafts (45) and vigorous T cell responses (46). A recent study demonstrated that the human fetal immune system exposed to maternal microchimerism is able to mount NIMA alloantigen-specific $CD4^+$ and $CD8^+$ T cell responses and that $CD4^+$ $CD25^+Foxp3^+$ T cells able to control the fetal alloreactive T cells were induced by maternal alloantigens (47). Therefore, we speculate that $CD8^+$ T cells stimulated during the fetal life with NIMA-like alloantigens may become regulatory or memory $CD8^+$ T cells able to influence, in a bystander manner, the development of Th-2–driven allergic disease.

Therefore, investigations are needed to further understand how potent regulatory or memory $CD8^+$ T cells may be induced early in life in a way that could affect the outcome of allergic diseases in later life. Early-life vaccination targeting the CD8 population is promising as a novel treatment for allergy and asthma, because it has the potential to antagonize Th2- and IgE-dependent components of the disease.

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Disclosures

The authors have no financial conflicts of interest.

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