

Protective role of nuclear factor of activated T cells 2 in CD8⁺ long-lived memory T cells in an allergy model

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Background: The transcriptional regulation of cytokines released and controlled by memory T cells is not well understood. Defective IFN- γ production in allergic asthma correlates in human beings with the risk of wheezing in childhood.

Objective: To understand the role of the transcription factor nuclear factor of activated T cells 2 (NFATc2) in memory and effector T cells in the airways in experimental allergic asthma. **Methods:** We used murine models of allergic asthma and adoptive cell transfer of fluorescence-activated sorted cells in a disease model.

Results: Mice lacking NFATc2 developed an increase in airway hyperresponsiveness (AHR), remodeling, and serum IgE levels on ovalbumin sensitization. This phenotype was associated with CD8⁺CD122⁻ T cells deficient in IFN- γ production in the airways. The origin of this phenotype in NFATc2^(-/-) mice was related to an expanded population of lung CD8⁺CD122⁺ (IL-2R β chain) CD127^{hi} (IL-7 receptor [R] α chain⁺) long-lived memory cells. Adoptive transfer of ovalbumin-specific CD8⁺ NFATc2^(-/-) T cells enhanced the AHR generated by NFATc2^(-/-) CD4⁺ T cells in immunodeficient mice, increased IL-17, and reduced IFN- γ production in the reconstituted mice. Depletion of the memory CD8⁺CD122⁺IL-7R^{high} T-cell population corrected the defect in IFN- γ production by lung NFATc2^(-/-) CD8⁺CD122⁻ cells and abrogated the increased AHR observed in NFATc2^(-/-) CD8⁺ T-cell-reconstituted mice with a severe combined immunodeficiency disorder.

Conclusion: Taken together, our results suggest that NFATc2 expression in long-lived memory CD8⁺ T cells controls IL-2 and

IFN- γ production in lung CD8⁺ T cells, which then limits T_H17 and T_H2 development in the airways during allergen challenge. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: NFATc2, CD8⁺ T cells, IL-2, IL-10, IFN- γ , IL-17, long-lived memory cells, CD8⁺CD122⁺(IL-2R β)⁺CD127(IL-7R α)⁺ T cells, allergy, model of human allergic asthma

The generation of T memory cells plays an essential protective role on re-exposure to allergen. On antigen encounter, the initial expansion of naive T cells into T_H effector cells is tightly controlled by transcription factors that activate early growth response genes.^{1,2} In this context, the family of nuclear factor of activated T cells (NFAT) of transcription factors is critical in regulating early gene transcription in response to T-cell receptor-mediated signals in lymphocytes.³

We have focused our current studies on exploring NFATc2 function in T cells in a murine model of experimental allergen sensitization.⁴ We report that NFATc2 deficiency leads to increased airway hyperresponsiveness (AHR) both after sensitization and in the absence of exogenous allergen challenge. Allergen sensitization led not only to the pathological development of T_H2 cells in the lung but also to the expansion of an IL-10-producing CD8⁺CD122⁺IL-7R α ^{high} long-lived memory T-cell population that was accompanied by reduced IFN- γ production by CD8⁺CD122⁻ effector cells and by the expansion of T_H17 cells in the airways after allergen exposure. These results suggest that NFATc2 is a key transcription factor in the suppression of memory CD8⁺CD122⁺IL-7R α ⁺ T cells that contribute to the pathogenesis of allergic responses.

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METHODS

Mice

NFATc2^(-/-) and wild-type mice were on a BALB/c genetic background. IFN- γ ^(-/-) mice (kindly provided by E. Schmitt, University of Mainz, Germany) and control littermates were on a C57BL/6 background. All mice entered the experimental protocol at the age of 6 to 8 weeks.

Allergen sensitization and challenge

Female or male BALB/c, NFATc2^(-/-) mice (6-8 weeks of age) were maintained under specific pathogen-free conditions and received intraperitoneal injections of 100 μ g ovalbumin (Calbiochem, Gibbstown, NJ) complexed with alum (Sigma-Aldrich, Steinheim, Germany) or only saline complex with alum on days 0 and 7 as previously described.⁵ On days 14, 15, and 16, the animals received ovalbumin in PBS per aerosol (10 mg ovalbumin/mL PBS in aerosol solution) or were aerosolized only with PBS in a chamber connected to an aerosol generator (Fohr Medical Instruments, Seeheim,

Abbreviations used

AHR: Airway hyperresponsiveness
BALF: Bronchoalveolar lavage fluid
FACS: Fluorescence-activated cell sorting
NFATc2: Nuclear factor of activated T cells
RI: Airway resistance
SCID: Severe combined immunodeficiency disorder

Germany) for 30 minutes. Twenty-four hours after the last aerosol, invasive body plethysmography was performed and mice killed to isolate spleen/lung cells as described. Purified CD8⁺ T cells from the spleen of ovalbumin-sensitized and challenged NFATc2^(-/-) or wild-type mice were used to reconstitute immunodeficient severe combined immunodeficiency (SCID) mice aerosolized with ovalbumin (1% in PBS) on days 15, 16, 18, and 19. At day 21, invasive body plethysmography was used to measure airway resistance in reconstituted SCID mice.⁶

Assessment of AHR and collection and analysis of bronchoalveolar lavage

To assess AHR, we used a whole-body plethysmograph as previously described.⁷ Data are expressed as mean values of (airway resistance [RI]/baseline RI) ± SEMs. After lung plethysmography, bronchoalveolar lavage of the right lung was performed with 1 mL saline 2 times. BALF supernatants were frozen and subsequently analyzed by ELISA and cell pellet resuspended in PBS and used for fluorescence-activated cell sorting (FACS) analysis as previously described.⁸

Histology

Lungs were removed, fixed in 10% formalin-PBS solution, and embedded in paraffin. Sections 5 μm thick were stained either with hematoxylin and eosin for quantification of inflammation or with Goldner stain for the quantification of peribronchial collagen deposition. A semiquantitative system was used to grade the collagen deposition as previously described.⁵

Isolation and analysis of lung and spleen CD4⁺ and CD8⁺ T cells

Lung CD4⁺ and CD8⁺ T cells and subsets of those were isolated as described in this article's Online Repository at www.jacionline.org.

ELISA

Mouse IL-10, IL-2, eotaxin, IgE, and IFN-γ were detected by using a specific sandwich ELISA (OptEIA; BD PharMingen, Heidelberg, Germany).

Statistical analysis

Differences were evaluated for significance ($P < .05$) by the Student 2-tailed t test for independent events (Excel, PC, Microsoft, Redmond, Wash) by using the statistical analysis of the Excel program. Data are given as mean values ± SEMs.

RESULTS

Mice deficient in NFATc2 develop AHR associated with peribronchial collagen deposition in the absence of allergen challenge

A direct role of NFATc2 in murine models of asthma has not been previously investigated. We thus asked whether NFATc2 deficiency contributed to the development of an asthmatic disease after ovalbumin sensitization. In this model, NFATc2^(-/-)

mice showed significantly increased AHR after methacholine challenge in the absence of allergen challenge compared with wild-type littermates (Fig 1, A; see this article's Fig E1, upper left panel, in the Online Repository at www.jacionline.org). Moreover, serum IgE levels were significantly increased in NFATc2^(-/-) compared with wild-type mice on immunologic sensitization with ovalbumin (Fig 1, B). Histopathological analysis of NFATc2^(-/-) airways in this model demonstrated increased peribronchial collagen deposition in the smaller airways (Fig 1, C, and quantified in the right panel). These characteristics are consistent with an asthmatic phenotype in NFATc2^(-/-) mice on ovalbumin sensitization.⁹

Impaired IFN-γ production by lung CD8⁺ T cells lacking NFATc2

The risk of wheezing in childhood has been linked to defective IFN-γ production.¹⁰ However, the cellular distribution of this cytokine has not been well defined in this disease. Therefore, we analyzed IFN-γ production by CD8⁺ cells isolated from wild-type or NFATc2^(-/-) lungs in a model of allergen sensitization. Intracellular staining for IFN-γ in lung lymphocytes revealed that ovalbumin-sensitized and unchallenged NFATc2^(-/-) mice had reduced numbers of airway CD8⁺ T cells expressing intracellular IFN-γ (Fig 2, A) compared with wild-type mice. We isolated lung CD8⁺ T cells and confirmed this observation by ELISA. We detected higher levels of IFN-γ in supernatants of wild-type compared with NFATc2^(-/-) lung CD8⁺ T cells (Fig 2, B). In contrast with the reduced production of IFN-γ, NFATc2^(-/-) CD8⁺ T cells produced increased amounts of IL-10 (Fig 2, C, left panel).

Increased memory CD8⁺CD122⁺ IL-7Rα^{high} T cells in the lung of NFATc2^(-/-) and IFN-γ^(-/-) mice

IFN-γ is a well known suppressor of IL-10 production,¹¹ and IL-10 in turn can inhibit IFN-γ production.¹²⁻¹⁴ We therefore postulated that these 2 phenotypes might be related. To test this hypothesis, we assessed IL-10 production by CD8⁺ T cells from mice lacking IFN-γ. Similar to NFATc2-deficient CD8⁺ T cells, lung CD8⁺ T cells from IFN-γ-deficient mice produced increased amounts of IL-10 (Fig 2, C, right panel). However, neither neutralization (Fig 2, D) nor addition of IL-10 influenced IFN-γ release by wild-type or NFATc2^(-/-) CD8⁺ T cells (Fig E1, upper right panel). To investigate further the cellular mechanism responsible for the decreased IFN-γ production by NFATc2^(-/-) CD8⁺ T cells, we examined the status of CD8⁺ T-cell subsets in NFATc2 and IFN-γ knockout mice as well as wild-type control mice (Fig 3, A and B). CD8⁺CD122⁺ cells have recently been described to control the pathological expansion of both CD4⁺ and CD8⁺ T cells and to suppress, in particular, the proliferation of IFN-γ-producing CD8⁺ T cells. However, they do not produce IFN-γ by themselves.^{15,16} Previous literature reported that CD8⁺ T cells expressing the signature marker for long-lived memory cells, the IL-7Rα chain (CD127), so-called *long-lived T memory cells*, are CD122⁺.¹⁷ We thus analyzed IL-7Rα chain (CD127) expression on CD8⁺CD122⁺ cells both in wild-type and NFATc2^(-/-) mice. Interestingly, we found that there was an increased number of CD8⁺CD122⁺ lung and spleen T cells in NFATc2^(-/-) compared with wild-type mice after ovalbumin sensitization, and

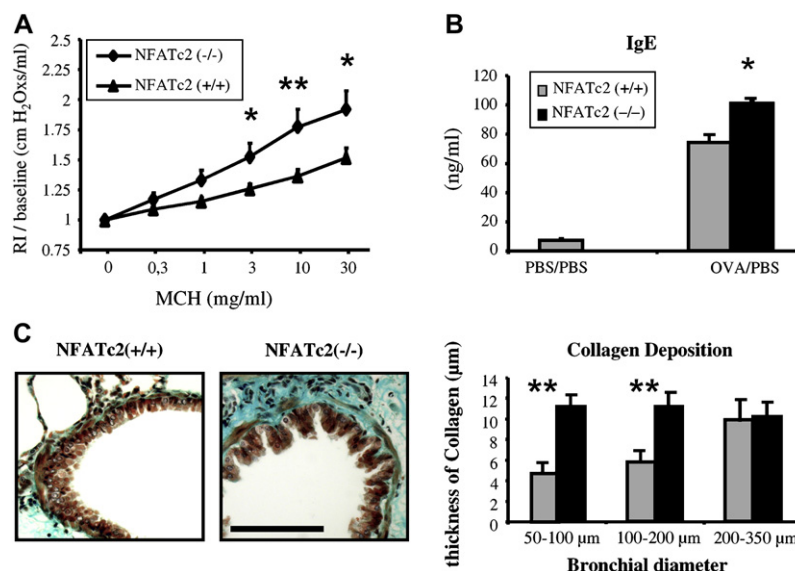


FIG 1. Increased airway lymphocytes, collagen, and AHR in NFATc2^(-/-) mice. AHR was measured in wt and NFATc2^(-/-) BALB/c mice 6 to 8 weeks old and transpulmonary RI measured 24 hours after the last treatment. Dose-response curves to methacholine (MCH) were obtained after administering indicated doses of intratracheally aerosolized MCH. **A**, Eight to 10 BALB/c mice per group were sensitized at days 0 and 7 to ovalbumin (OVA)-alum (OVA-sensitized mice) followed by 3 days local treatment with PBS aerosol on days 14, 15, and 16. NFATc2^(-/-) OVA-sensitized mice displayed increased AHR at higher doses of MCH compared with wt littermates ($n = 11$; $P = .018$, $P = .0065$, and $P = .015$ after 3, 10, and 30 mg/mL of 3 minutes MCH aerosol, respectively). **B**, Increased serum IgE in NFATc2^(-/-) mice after OVA sensitization without OVA challenge ($n = 4-6$; $P = .02826$). **C**, Increased peribronchial collagen deposition in NFATc2^(-/-) lung by Goldner staining (compare right vs left panel and quantified in far right graph). Eight mice and 3 sections/mouse were analyzed at 400 $\times$ magnification on a Zeiss microscope (Carl Zeiss, Göttingen, Germany). The images were all taken at the same magnification. The width of the boxes is exactly 200 μ m, and the space bar represents 100 μ m.

this was true both before (Fig 3, A) and after (see this article's Fig E2 in the Online Repository at www.jacionline.org) ovalbumin challenge. Moreover, we demonstrated that the majority of CD8⁺CD122⁺ cells express the IL-7R α chain and hence can be termed *long-lived T memory cells*.¹⁷ This population of CD8⁺CD122⁺CD127^{hi} T cells composed 20% of the total lung CD8⁺ T cells in NFATc2^(-/-) mice compared with 8% in wild-type littermates and hence was expanded in the absence of NFATc2 (Fig 3, C; Fig E2, lower panels). Concurrent with the expansion of this memory population, we observed a decrease in the number of activated CD8⁺CD25⁺ T cells in NFATc2^(-/-) lung (Fig 3, D; see this article's Fig E3 in the Online Repository at www.jacionline.org). The decrease in CD25 expression on the surface of CD8⁺ T cells in the absence of NFATc2 suggested a downregulation of IL-2 in these cells, because this cytokine regulates expression of its own receptor α chain (IL-2R α chain or CD25). We then analyzed IL-2 production by lung CD8⁺ T cells and found that NFATc2^(-/-) CD8⁺ T cells secrete significantly reduced amounts of IL-2 compared with lung CD8⁺ T cells isolated from the wild-type littermates after ovalbumin sensitization and challenge (Fig 3, E). Thus, NFATc2 has a direct control on IL-2 production, and NFATc deficiency causes a T-cell phenotype reminiscent of anergic CD8⁺ T cells.¹⁸ This T-cell subpopulation leads to an expansion of CD8⁺CD122⁺ T cells that produce more IL-10 and inhibit IFN- γ production in CD8⁺CD122⁻ cells (Fig 3, F and G).

CD8⁺CD122⁺ cells have been previously shown to inhibit IFN- γ production in CD8⁺CD122⁻ cells in an IL-10-dependent manner.^{11,15,16} Analysis of IL-10 production in NFATc2^(-/-) CD8⁺CD122⁺ cells revealed increased levels compared with

wild-type cells (see this article's Fig E4 in the Online Repository at www.jacionline.org). Although IL-10 is a signature cytokine for CD8⁺CD122⁺ cells, it does not contribute to the downregulation of IFN- γ in CD8⁺ T cells. Interestingly, CD8⁺ T cells isolated from lungs of IFN- γ -deficient mice also contained an increased proportion of CD8⁺CD122⁺ T cells (Fig 3, B), and these cells displayed increased IL-10 production as we observed in NFATc2^(-/-) lung (Fig E4, lower panel). We conclude that NFATc2 expression in CD8⁺CD122⁺IL7R α ^{hi} T cells might control both the expansion of these cells and their production of IL-10.

Adoptive transfer of antigen-specific spleen NFATc2^(-/-) CD8⁺ T cells is more potent than wild-type T cells in inducing CD4⁺ T-cell-dependent AHR accompanied by increased IL-17 production in the airways

To test whether CD8⁺ T cells might be responsible for the exacerbated AHR in NFATc2^(-/-) mice (Fig 1, A), we performed adoptive transfer experiments (Fig 4, A-F). SCID mice were reconstituted with CD4⁺ T cells from NFATc2^(-/-) mice alone or together with either wild-type or NFATc2^(-/-) CD8⁺ T cells. As a reference curve, we reconstituted SCID mice with CD4⁺ T cells from NFATc2^(-/-) mice (Fig 4, B, dotted lane) because transfer of CD8⁺ T cells alone did not induce AHR in reconstituted mice (see this article's Fig E5 in the Online Repository at www.jacionline.org). The splenic NFATc2^(-/-) CD8⁺ T cells used to reconstitute these SCID mice released significantly less IFN- γ than wild-type CD8⁺ T cells (Fig 4, A) and homed appropriately to the lung (Fig 4, C). Consistently, the AHR induced by

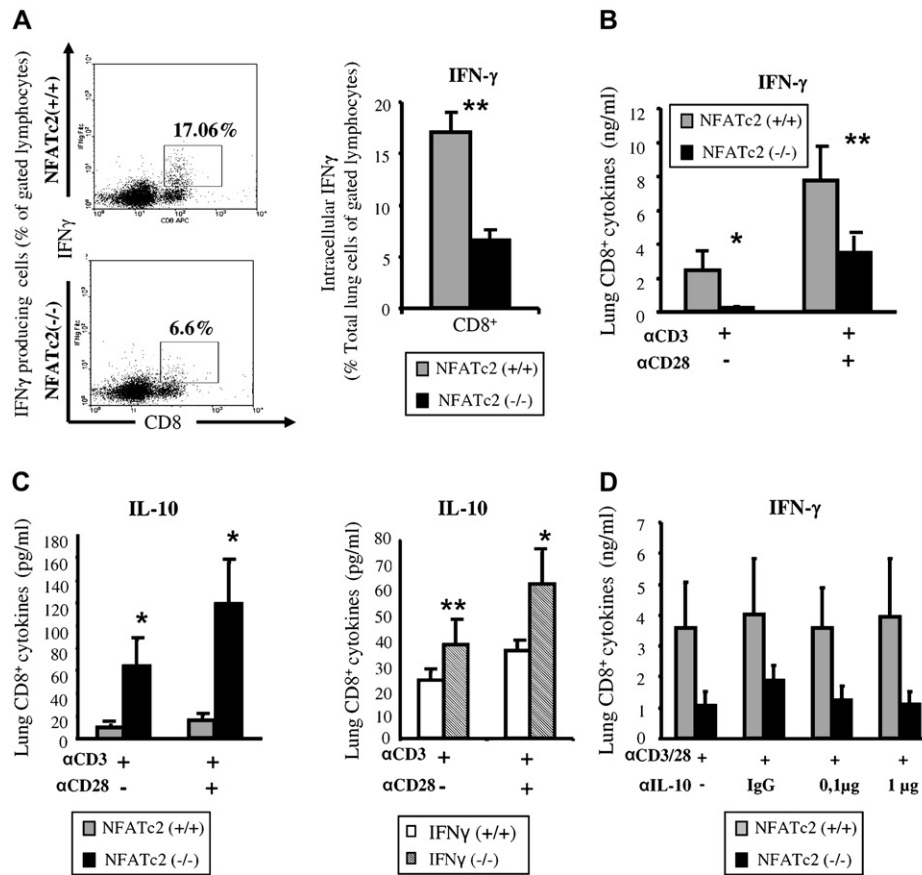


FIG 2. NFATc2 $^{-/-}$ lung CD8 $^{+}$ T cells produce less IFN- γ but more IL-10 than WT lung CD8 $^{+}$ T cells. **A**, Intracellular IFN- γ production by lung CD8 $^{+}$ T cells from ovalbumin-sensitized and challenged mice. *Left panel*, Dot plot analysis of IFN- γ -producing CD8 $^{+}$ T cells; *right panel*, quantification of IFN- γ $^{+}$ cells ($n = 4$; $P = .00103$ for CD8/IFN- γ). **B**, ELISA of IFN- γ released by lung and spleen CD8 $^{+}$ T cells ($n = 4$; $P = .028$, $P = .0065$). **C**, *Left panel*, ELISA for IL-10 production by lung NFATc2 $^{-/-}$ CD8 $^{+}$ T cells from ovalbumin-sensitized and unchallenged mice ($n = 4$; $P = .03$, $P = .04$); *right panel*, IL-10 production is also increased in IFN- γ $^{-/-}$ lung CD8 $^{+}$ T cells ($n = 4$; $P = .02$, $P = .006$) as measured by ELISA. * $P < .05$; ** $P < .01$. **D**, Neutralization of IL-10 by the indicated concentration of anti-IL-10 antibodies did not influence IFN- γ release by wild-type or NFATc2 $^{-/-}$ CD8 $^{+}$ T cells. Values of anti-IL-10 antibodies (α IL-10; R & D Systems) are given as μ g/mL of medium (IgG was present in the cell culture at the final concentration of 1 μ g/mL).

adoptive transfer of NFATc2 $^{-/-}$ CD4 $^{+}$ T cells was not affected by the cotransfer of wt CD8 $^{+}$ ovalbumin-specific spleen T cells. However, cotransfer of NFATc2 $^{-/-}$ CD8 $^{+}$ T cells led to worsening of AHR (Fig 4, B). These findings were associated with increased numbers of eosinophils (Fig 4, D), decreased IFN- γ (Fig 4, E), and increased IL-17 production (Fig 4, F) in the bronchoalveolar lavage fluid (BALF) of mice reconstituted with NFATc2 $^{-/-}$ CD4 $^{+}$ plus NFATc2 $^{-/-}$ CD8 $^{+}$ T cells.

Depletion of memory NFATc2 $^{-/-}$ CD8 $^{+}$ CD122 $^{+}$ CD127 ^{high} T cells abolished the NFATc2 $^{-/-}$ CD8 $^{+}$ T cell contribution to CD4 $^{+}$ T-cell-induced AHR

To test whether memory CD8 $^{+}$ CD122 $^{+}$ T cells lacking NFATc2 contribute to the asthmatic phenotype in the setting of NFATc2 deficiency in CD4 $^{+}$ T cells, we depleted CD8 $^{+}$ CD122 $^{+}$ cells from total spleen CD8 $^{+}$ NFATc2 $^{-/-}$ T cells by FACS cell sorting and performed adoptive transfer experiments. SCID mice were reconstituted with CD4 $^{+}$ T cells from NFATc2 $^{-/-}$ mice alone or together with either NFATc2 $^{-/-}$ CD8 $^{+}$ CD122 $^{-}$ or NFATc2 $^{-/-}$ CD8 $^{+}$ CD122 $^{+}$ T cells. Three days after the

adoptive transfer, different parameters were measured in these mice. The splenic NFATc2 $^{-/-}$ CD8 $^{+}$ CD122 $^{+}$ T cells used to reconstitute these SCID mice were of high purity (Fig 5, A). Finally, the AHR induced by NFATc2 $^{-/-}$ CD4 $^{+}$ T cells was further augmented when NFATc2 $^{-/-}$ CD8 $^{+}$ CD122 $^{+}$ ovalbumin-specific splenic T cells were cotransferred (Fig 5, B). By contrast, cotransfer of NFATc2 $^{-/-}$ CD8 $^{+}$ CD122 $^{-}$ cells led to amelioration of AHR (Fig 5, B). In addition, cotransfer of CD4 $^{+}$ plus CD8 $^{+}$ CD122 $^{-}$ T cells led to decreased numbers of eosinophils (Fig 5, C), reduced levels of eotaxin (Fig 5, D) and increased levels of IFN- γ (Fig 5, E) in the BALF compared with the cotransfer of CD4 $^{+}$ and NFATc2 $^{-/-}$ CD8 $^{+}$ CD122 $^{+}$ T cells.

Moreover, as shown in Fig 5, F, the NFATc2 $^{-/-}$ CD8 $^{+}$ CD122 $^{-}$ population, when sorted out from the total lung CD8 $^{+}$ T-cell population, produced significantly more IFN- γ than the corresponding cell population isolated from wild-type littermates.

Taken together, these data demonstrate an important inhibitory function exerted by CD8 $^{+}$ CD122 $^{+}$ cells on CD8 $^{+}$ CD122 $^{-}$ cells *in vivo* and *ex vivo* that is controlled by the transcription factor NFATc2.

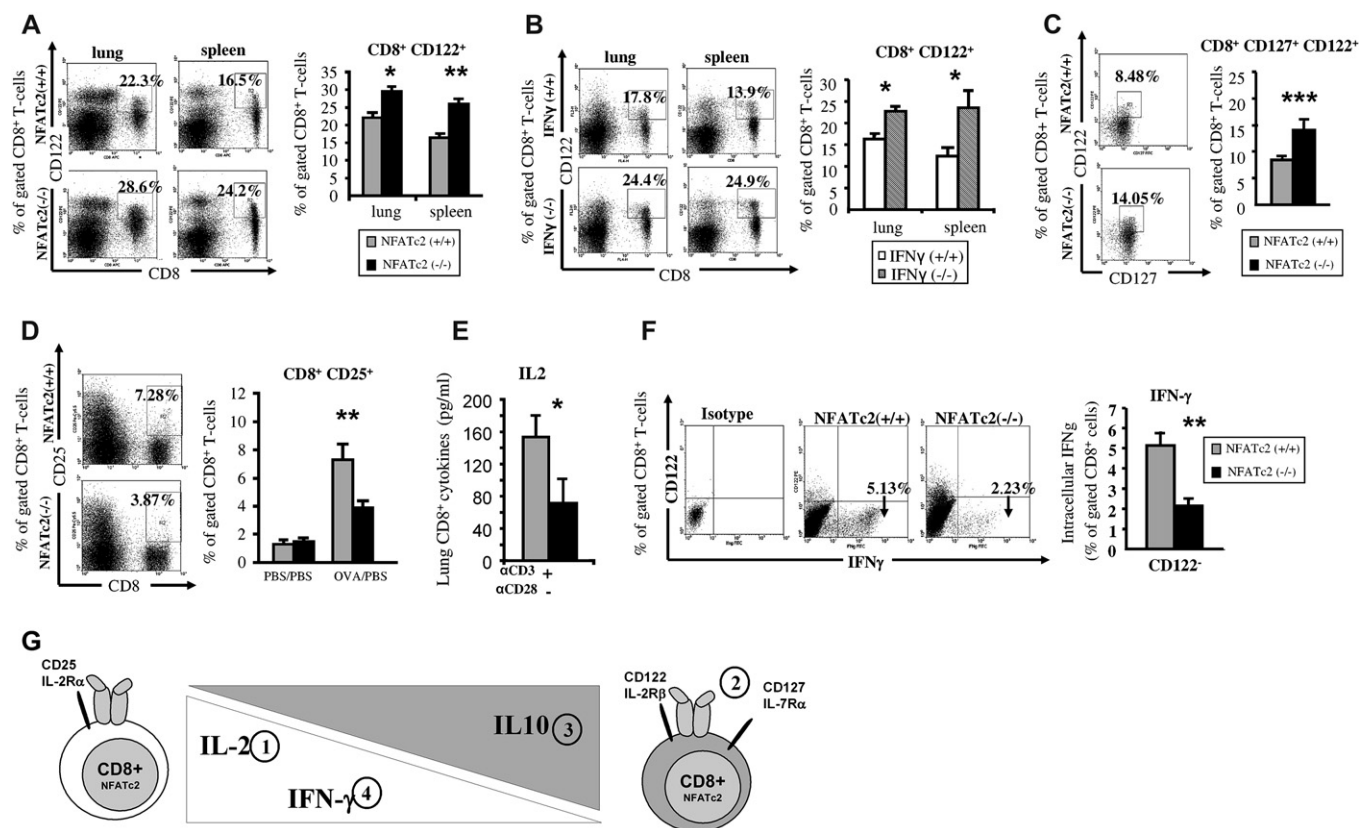


FIG 3. Increased number of CD8⁺CD122⁺CD127^{high} (IL-7Rα) cells in the lung and spleen of NFATc2^(-/-) and IFN-γ^(-/-) mice. **A**, NFATc2^(-/-) mice have increased numbers of CD8⁺CD122⁺ T cells both in the lung and in the spleen before ovalbumin (OVA) challenge (OVA/PBS) as shown by dot plot analysis (left panel) and depicted quantitatively (right panel; $n = 4$; $P = .0012$, $P = .036$). **B**, Increased numbers of CD8⁺CD122⁺ T cells in lungs and spleen of IFN-γ^(-/-) mice (middle panel; $n = 4$; $P = .048$, $P = .016$). **C**, CD8⁺CD122⁺ cells also expressed the IL-7Rα chain (CD127) and were thereafter defined as long-lived memory cells. Long-lived memory cells were increased in the lungs of NFATc2^(-/-) mice. A representative dot plot is shown in the left panel (quantification is depicted on the right; $n = 4$; $P = .0022$, $P = .00015$). **D**, The CD8⁺CD25⁺ T effector population is downregulated in NFATc2^(-/-) lung 24 hours after allergen challenge ($n = 4$; $P = .0078$, respectively). **E**, NFATc2^(-/-) CD8⁺ lung T cells produced less IL-2 compared with those isolated from wild-type mice ($n = 4$; $P = .037$). **F**, NFATc2^(-/-) CD8⁺CD122⁻ lung T cells produced less IFN-γ than wt ($n = 4$; $P = .0068$) as shown by FACS analysis before allergen challenge (OVA/PBS). **G**, Graph depicting the hypothetical sequence of events in the lung NFATc2^(-/-) CD8⁺ T-cell population. In the absence of NFATc2^(-/-), CD8⁺ T cells display changes characteristic of anergic T cells and produce less IL-2 (1). Because IL-2 controls CD25 expression, CD8⁺CD25⁺ T cells are also decreased in the absence of NFATc2. This event leads to an expansion of the CD8⁺CD122⁺ T-cell population (2), which releases IL-10 (3). Finally, CD8⁺CD122⁺ T cells inhibit IFN-γ production by CD8⁺CD122⁻ T cells (4).

DISCUSSION

The current study reveals an unexpected role of the nuclear transcription factor NFATc2 in integrating the function of CD4⁺ and CD8⁺ T cells in a murine model of allergic sensitization. The consequences of NFATc2 deficiency for the lung involved long-lived memory CD8⁺CD122⁺CD127^{high} (IL-7Rα) T cells.

An expansion of the T_H2 cell compartment has been previously described by us and others in mice lacking NFATc2,^{19,20} and we demonstrate here that in the setting of an allergic diathesis, there is a further induction of this response. Furthermore, we found that in the lung, the vast majority of IFN-γ is produced by CD8⁺ T cells in an NFATc2-dependent manner, as evidenced by diminished IFN-γ production in NFATc2^(-/-) lung CD8⁺ T cells. Interestingly, in adoptive CD4⁺ and CD8⁺ cotransfer experiments, we demonstrated increased IL-17 in the airways of CD4⁺NFATc2^(-/-) CD8⁺NFATc2^(-/-) reconstituted SCID mice, confirming the

inhibitory function *in vivo* of IFN-γ on IL-17 production. Although there is scarce literature on the role of IL-17A in asthma, it has been reported to be both a positive and negative regulator of the asthmatic response.²¹ Levels of IL-17 are increased in both lung and blood of patients with allergic asthma and correlate with asthma severity,²² and IL-17R knockout mice fail to undergo antigen sensitization and hence to develop allergic asthma.²¹

IFN-γ was produced by the CD8⁺CD122⁻ but not the CD8⁺CD122⁺ subset of CD8⁺ T cells in both wild-type and NFATc2^(-/-) mice, consistent with previous reports.^{15,16} Interestingly, we found an expansion of the CD8⁺CD122⁺ subset in the NFATc2^(-/-) lung: CD8⁺CD122⁺ cells represented approximately 20% of total lung CD8⁺ T cells in NFATc2^(-/-) versus 8% in wild-type lung. The CD8⁺CD25⁺ subset represented a restricted proportion of the lung CD8⁺ T cells (2% to 4%) and was reduced in number in the NFATc2^(-/-) lung CD8⁺ T-cell compartment. This

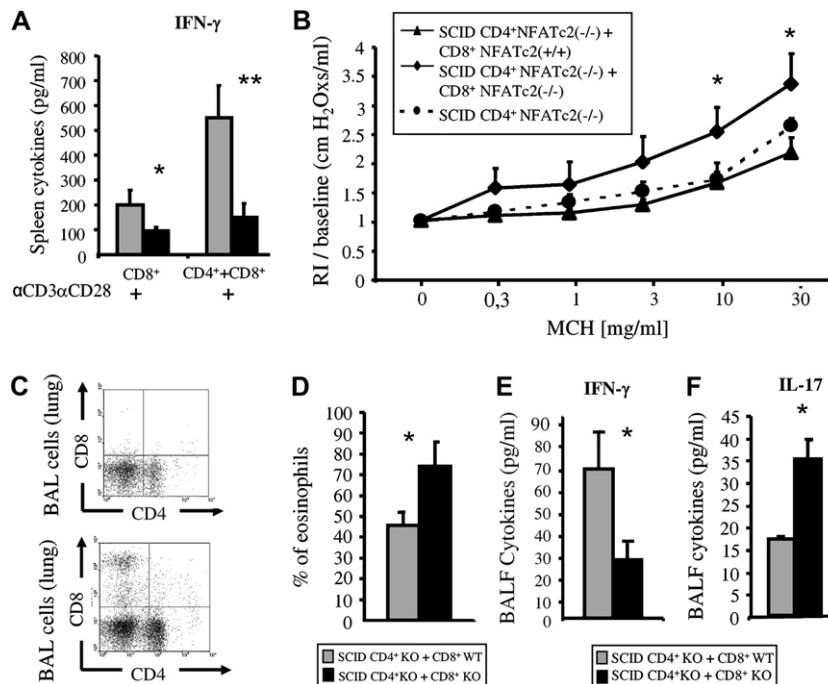


FIG 4. The CD8 $^{+}$ CD122 $^{+}$ subset inhibits IFN- γ production in NFATc2(-/-) CD8 $^{+}$ CD122 $^{-}$ cells and induces AHR on adoptive cotransfer of NFATc2(-/-) CD4 $^{+}$ and CD8 $^{+}$ T cells. **A** and **B**, Adoptive transfer of CD8 $^{+}$ T cells from NFATc2(-/-) compared with WT mice increased the AHR generated by NFATc2(-/-) CD4 $^{+}$ T cells ($n = 4$; $P = .028$, $P = .014$). **A**, The NFATc2(-/-) CD8 $^{+}$ T cells used to reconstitute these SCID mice released significantly less IFN- γ per cell compared with wt CD8 $^{+}$ T cells. The total amount of IFN- γ released by both NFATc2(-/-) CD4 $^{+}$ and NFATc2(-/-) CD8 $^{+}$ T cells (right panel) was significantly lower than wt (left panel). **B**, AHR is ameliorated by cotransfer of wild-type but exacerbated by cotransfer of NFATc2(-/-) CD8 $^{+}$ T cells. SCID mice were reconstituted with NFATc2(-/-) CD4 $^{+}$ T cells alone or together with either wild-type CD8 $^{+}$ T cells or NFATc2(-/-) CD8 $^{+}$ T cells from OVA-sensitized and challenged mice and AHR measured in reconstituted mice. As reference, the AHR of SCID mice reconstituted with NFATc2(-/-) CD4 $^{+}$ T cells alone is shown. **C**, Both CD4 $^{+}$ and CD8 $^{+}$ T cells homed to the lung of reconstituted mice as demonstrated by FACS analysis. **D**, Increased number of eosinophils in SCID mice reconstituted with NFATc2(-/-) CD4 $^{+}$ T cells together with NFATc2(-/-) CD8 $^{+}$ cells ($n = 4$; $P = .036$). **E** and **F**, Decreased IFN- γ ($n = 4$; $P = .03$) and increased IL-17 ($n = 4$; $P = .023$) in lungs of SCID mice reconstituted with NFATc2(-/-) CD4 $^{+}$ and CD8 $^{+}$ T cells. BAL, Bronchoalveolar lavage; MCH, methacholine; KO, knockout.

finding suggested that NFAT deficiency causes a T-cell phenotype reminiscent of anergic CD8 $^{+}$ T cells¹⁸ in the lung. Anergy is induced by hyporesponsiveness to inhaled antigen characterized by T lymphocytes with a blockade in IL-2 secretion. Indeed, we found that lung NFATc2(-/-) CD8 $^{+}$ T cells secrete reduced amounts of IL-2 compared with those isolated from the wild-type littermates. It has been described that anergic CD8 $^{+}$ T cells have defective nuclear translocation of NFATc2.¹⁸ We thus believe that the defective IL-2 production is directly controlled by NFATc2, resulting in a downregulation of CD25 expression.

Many NFATc2-deficient CD8 $^{+}$ CD122 $^{+}$ T cells expressed the signature marker of long-lived memory cells, the IL-7R α chain (CD127). Two cytokines, IL-7 and IL-15, are known to be involved in maintaining the numbers of CD8 $^{+}$ T memory cells *in vivo*.²³⁻²⁷ Hence, the increased expression of the IL-7R α (CD127) chain on NFATc2(-/-) compared with wild-type CD8 $^{+}$ CD122 $^{+}$ lung cells is of note. The function exerted by CD8 $^{+}$ CD122 $^{+}$ T cells remains unclear,²³⁻²⁷ although it has been demonstrated that CD8 $^{+}$ CD122 $^{+}$ T cells are regulatory cells important in controlling CD8 $^{+}$ CD122 $^{-}$ T cells. Moreover, we found that specific depletion of the CD8 $^{+}$ CD122 $^{+}$ T-cell subset restored IFN- γ production by NFATc2(-/-) CD8 $^{+}$ CD122 $^{-}$ T cells in adoptive transfer experiments demonstrating effects of

the former cells on IFN- γ production by the latter cells that could be mediated either via cell contact-dependent mechanisms or soluble factors. In any case, our experiments delineate the pathological role of a memory CD8 $^{+}$ CD122 $^{+}$ primary subpopulation on AHR *in vivo* and *ex vivo*. Depletion of CD8 $^{+}$ CD122 $^{+}$ cells abolished the contribution of the CD8 $^{+}$ T-cell population to the AHR generated by CD4 $^{+}$ T cells in immune-deficient mice. Moreover, they control IFN- γ production by CD8 $^{+}$ CD122 $^{-}$ T cells. The exact mechanisms by which NFATc2(-/-) CD8 $^{+}$ CD122 $^{+}$ T cells control IFN- γ production by CD8 $^{+}$ CD122 $^{-}$ T cells as well as the role of CD8 $^{+}$ CD122 $^{+}$ T cells in organs other than lung remain to be elucidated.

On allergen exposure, antigen-specific T cells undergo clonal expansion and differentiate into effector cells. The mechanisms responsible for the generation of T-cell memory are only beginning to be understood. The current data suggest that in the absence of NFATc2, the long-lived memory CD8 $^{+}$ CD122 $^{+}$ CD127 $^{+}$ T cell is pathologically expanded after allergen sensitization in the absence of NFATc2, leading to the downregulation of the protective cytokine IFN- γ in the complementary CD8 $^{+}$ CD122 $^{-}$ cell population. We further identify NFATc2 as a major transcription factor controlling the development of IL-15/IL-7-dependent CD8 $^{+}$ memory function in the lung.

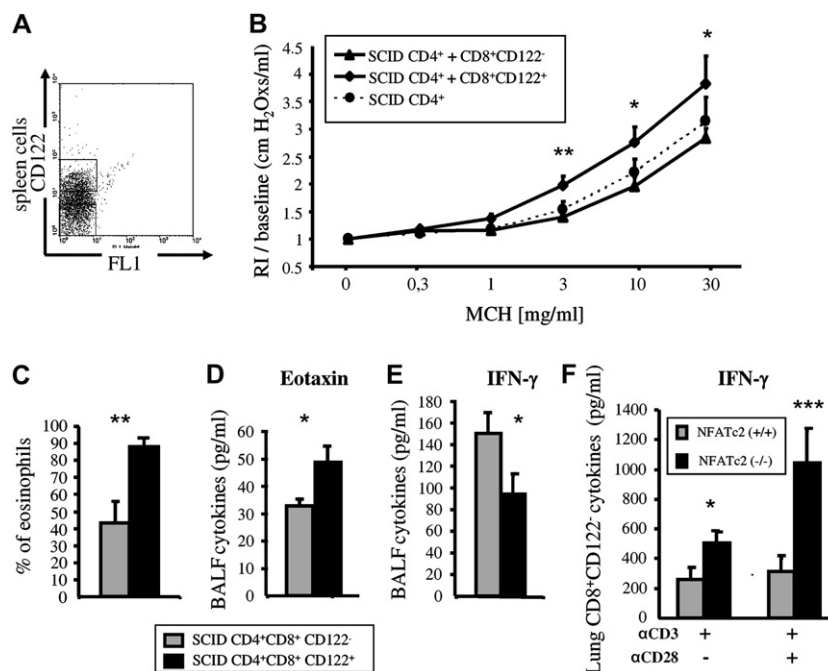


FIG 5. The sorted CD8⁺CD122⁺ subset induces AHR on adoptive cotransfer of NFATc2^(-/-) CD4⁺ and CD8⁺ T cells. **A**, FACS of splenic CD8⁺ NFATc2^(-/-) T cells that had been depleted of CD8⁺CD122⁺ T cells. The 2 resulting cell populations, CD8⁺CD122⁺ and CD8⁺CD122⁻, were used to reconstitute SCID mice along with CD4⁺ NFATc2^(-/-) T cells. **B**, SCID mice that received CD8⁺CD122⁻ NFATc2^(-/-) cells but not CD8⁺CD122⁺ NFATc2^(-/-) cells were protected from AHR. **C** and **D**, Decreased numbers of eosinophils and levels of eotaxin in SCID mice reconstituted with NFATc2^(-/-) CD4⁺ T cells together with NFATc2^(-/-) CD8⁺CD122⁻ cells ($n = 4$; $P = .0057$ and $n = 4-6$; $P = .0344$). **E**, Increased IFN- γ levels in BALF of SCID mice reconstituted as described on depletion of CD8⁺CD122⁺ T cells before transfer ($n = 4$; $P = .0298$). **F**, Lung CD8⁺ T cells from both wt and NFATc2^(-/-) mice were depleted of CD8⁺CD122⁺ T cells, and the 4 resulting cell populations CD8⁺CD122⁺ and CD8⁺CD122⁻, were cultured with anti-CD3 and anti-CD3/CD28 overnight and the supernatant analyzed for IFN- γ by ELISA ($n = 5-9$; $P = .04$, $P = .005$). As shown, the inhibition of IFN- γ production by CD8⁺CD122⁻ T cells was reversed by CD8⁺CD122⁺ T-cell depletion. *MCH*, Methacholine; *FL*, fluorescence.

The clinical relevance of these findings for human allergic diseases is further underlined by the observation that defective activation and nuclear translocation of NFATc2 have been associated with decreased IL-2 and IFN- γ production by CD8⁺ T cells in patients with Wiskott-Aldrich syndrome.²⁸ Our data thus suggest that increasing NFATc2 levels in memory T cells might restore the IFN- γ deficiency detected in patients with asthma.

We thank Isabel Ernst and Magali Burdet for their technical assistance.

Key message

- Increasing NFATc2 levels or inducing its nuclear translocation on dephosphorylation in CD8⁺ T cells might reduce the number of lung long-lived CD8⁺CD122⁺ (IL-2/15R β chain)⁺CD127(IL-7R α)⁺ cells, decreasing AHR by restoring IFN- γ deficiency detected in some patients with asthma.
- The results of this study suggest that NFATc2 expression in long-lived memory CD8⁺ T cells controls IL-2 and IFN- γ production in lung CD8⁺ T cells that then limits T_H17 and T_H2 development in the airways during allergen challenge.

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METHODS

Reconstitution of SCID mice with ovalbumin-specific splenic CD4⁺, CD8⁺, CD8⁺CD122⁺, or CD8⁺CD122⁻ T cells

Immunodeficient SCID mice were reconstituted by intraperitoneal injection of 2×10^6 CD4⁺ NFATc2^(-/-) plus 1.5×10^6 CD8⁺ NFATc2^(-/-) or CD8⁺ NFATc2^(+/+) T cells. CD4 and CD8⁺ T cells were isolated from the spleen of ovalbumin-sensitized and challenged NFATc2^(-/-) and NFATc2^(+/+) mice as described by positive magnetic selection according to the manufacturer's instructions. In additional experiments, SCID mice were reconstituted intraperitoneally with CD8⁺ T cells that had been sorted (FACS Vantage; BD PharMingen, Heidelberg, Germany) into 2 cell populations, CD8⁺CD122⁺ and CD8⁺CD122⁻ (2×10^6 CD4⁺ NFATc2^(-/-) and 1.5×10^6 CD8⁺CD122⁺ or 2×10^6 CD4⁺ NFATc2^(-/-) and 1.5×10^6 CD8⁺CD122⁻ NFATc2^(-/-) T cells). One day before and 1 and 3 days after adoptive cell transfer, SCID mice were aerosolized with 1% ovalbumin for 30 minutes. Twenty-four hours later, after the last allergen challenge, lung function was measured by invasive body plethysmography, BALF was retrieved, and lungs were removed and stored frozen until used for further analysis.

Isolation and analysis of lung and spleen CD4⁺ and CD8⁺ T cells

Lung CD4⁺ and CD8⁺ T cells were isolated as previously described.^{E1} After 3 washes in complete medium, the CD4-positive T cells were cultured with plate-bound anti-CD3 or anti-CD3 anti-CD28 antibodies (CD3 clone 145-2C11, 5 µg/mL; CD28 clone 37.51, 2 µg/mL; BD PharMingen) for 24 hours at a density of 10^6 cells/mL. The supernatant was analyzed for cytokine production by ELISA. CD4⁺ T cells from the spleen of ovalbumin-sensitized and challenged NFATc2^(-/-) or wt mice were used to reconstitute immunodeficient C17/SCID mice.

In some experiments, CD8⁺CD122⁺ and CD8⁺CD122⁻ cells were sorted by FACS Vantage (BD PharMingen) by using anti-CD122 (TM-β1, phycoerythrin [PE]) antibody (BD PharMingen). In additional experiments, CD8⁺CD122⁻ cells were depleted of CD8⁺CD122⁺ cells by using a 2-step sequential positive isolation by incubating first with rat antimouse anti-CD122 (TM-β1 PE) (BD PharMingen), followed by magnetic cell sorting using the anti-PE-MultiSortKit (Miltenyi Biotec, Heidelberg, Germany). Finally, the CD122⁺ cells underwent a second positive selection to separate CD8⁺CD122⁺ cells from CD8⁺CD122⁻ cells.

FACS analysis

To ascertain routinely the purity (purity >94%) of the CD4⁺ T-cell isolation, 5×10^5 cells were washed with 1 mL PBS, then incubated for 30 minutes

in 100 µL PBS containing 5 µg/mL anti-CD4 (L3T4-PE; BD PharMingen). Total lung and spleen cells were stained with FACS antibodies for 30 minutes at 4°C, washed in PBS, then analyzed with a FACSCalibur (BD PharMingen). The following antibodies were used: antimouse CD4 (L3T4-PE), antimouse CD8α (53-6.7-allophycocyanin), antimouse CD49b (DX5-fluorescein isothiocyanate [FITC]; BD PharMingen), antimouse CD69 (H1.2F3 FITC; BD PharMingen), antimouse CD69 (H1.2F3 PE; BD PharMingen), CD44 (Pgp-1, Ly-24 PE Cy5), rat antimouse anti-CD122 (TM-β1 PE; BD PharMingen), antimouse CD4 (L3T4-APC; BD PharMingen), and rat antimouse CD127 (A7R34-FITC; eBioscience, San Diego, Calif). Isotype controls were IgG_{2a} (G155-178; BD Biosciences, San Diego, Calif), IgG_{2b} (eB149/10H5), IgG (eBio299Arm; eBioscience), and IgM (RGM01; Caltag, Karlsruhe, Germany).

Intracellular protein analysis

For intracellular staining of IFN-γ, freshly isolated lung cells were stimulated with 1 ng/mL phorbol 12-myristate 13-acetate (Sigma Chemical, St Louis, Minn) and 1 µmol ionomycin and Brefeldin A (BD PharMingen) (5 ng/mL) for 4 hours at 37°C or for intracellular staining with IL-10 with phorbol 12-myristate 13-acetate, ionomycin, LPS (1 µg/mL), and Golgi-Stop (BD PharMingen). Cells were stained with anti-CD4 (L3T4-PE; BD PharMingen) and anti-CD8α (53-6.7-APC; BD PharMingen) or CD8α (53-6.7-2 FITC; BD PharMingen) and CD122 (TM-β1 PE; BD PharMingen) antibodies for 30 minutes at 4°C. Thereafter, cells were fixed with PBS containing 2% (vol/vol) paraformaldehyde for 15 minutes at room temperature and were washed twice with PBS and once with PBS containing 0.1% (wt/vol) saponin, 0.5% BSA, and 0.01% NaN₃ (permeabilization fluid). Cells were then incubated with FITC-labeled antibodies against IFN-γ (XMG 1.2- FITC; BD PharMingen) or with APC-labeled antibodies against IL-10 (JES5-16E3-APC; BD PharMingen) for 30 minutes at 4°C in permeabilization fluid and washed twice.

ELISA

The lower sensitivity threshold for the ELISA kit used was as follows: IgE, 1.3 ng/mL; IL-10, 31.3 pg/mL; IFN-γ, 31.3 pg/mL; IL-17, 15.625 pg/mL; eos-taxin: 7.1825 pg/ml; IL-2: 3 pg/ml.

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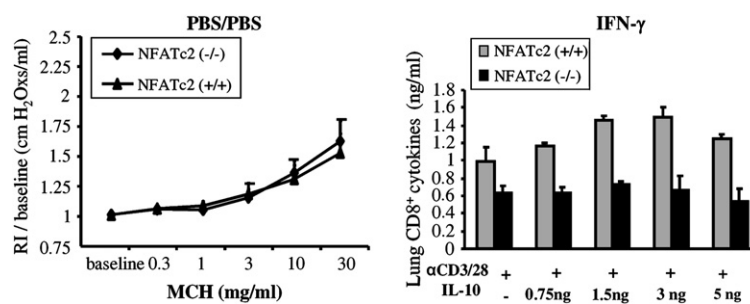


FIG E1. *Left*, Naive mice lacking NFATc2 show comparable AHR to the wild-type littermates ($n = 5$). *Right*, Addition of different amounts of recombinant IL-10 (R&D Systems, Minneapolis, Minn) did not alter IFN- γ levels in lung CD8 $^{+}$ T cells isolated from unchallenged mice ($n = 4$). *MCH*, Methacholine.

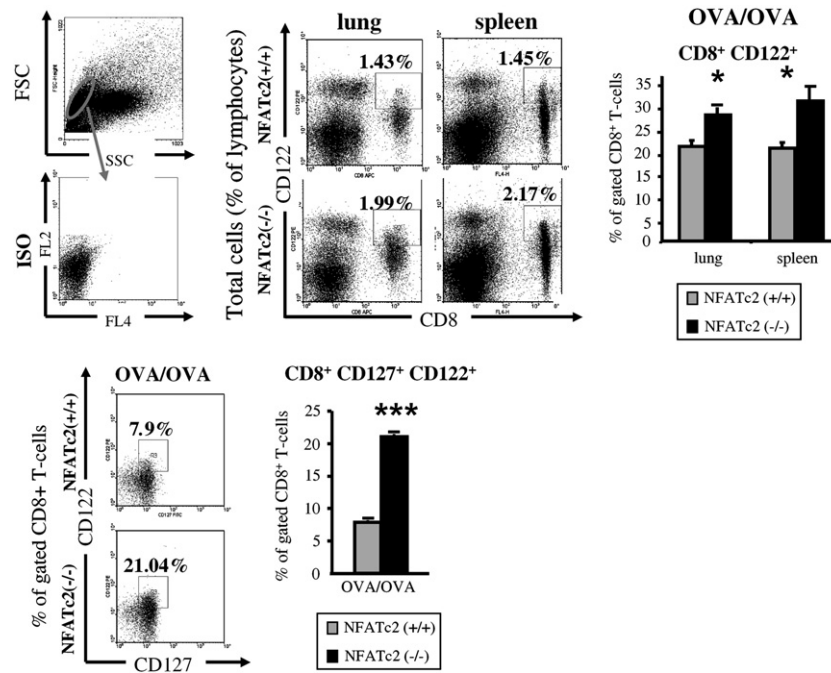


FIG E2. Upper lanes, Ovalbumin (OVA) challenge after OVA sensitization in NFATc2^{-/-} mice led to increased numbers of CD8⁺CD122⁺ T cells compared with wild-type littermates ($n = 4$; $P = .012$, $P = .037$). Left, Isotype control. Lower lanes, OVA challenge after OVA sensitization in NFATc2^{-/-} mice led to increased numbers of CD8⁺CD122⁺CD127⁺ T cells compared with wild-type littermates ($n = 4$; $P = .0022$). Left, Isotype control. FCS, Forward scatter; ISO, isotype control; FL, fluorescence.

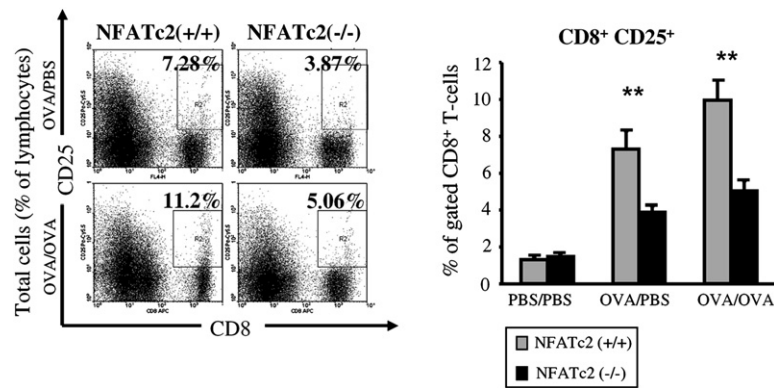


FIG E3. CD8⁺CD25⁺ cells were decreased in NFATc2^(-/-) mice after ovalbumin (OVA) sensitization (n = 4; $P = .0078$) and OVA sensitization and OVA challenge (n = 4; $P = .0027$).

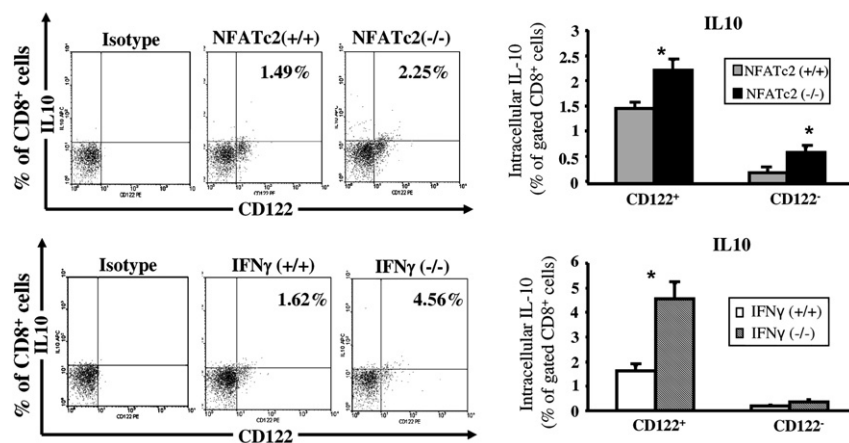


FIG E4. Increased number of CD8⁺CD122⁺ lung T cells expressing intracellular IL-10 in NFATc2^(-/-) and IFN-γ^(-/-) mice. Increased numbers of CD8⁺CD122⁺ lung T cells from NFATc2^(-/-) mice ($n = 4$; $P = .037$, $P = .011$; upper panels) and IFN-γ^(-/-) mice ($n = 4$; $P = .013$; lower panels) after allergen challenge as compared to the number of CD8⁺CD122⁺ T cells isolated from the NFATc2^(+/+) and IFN-γ^(+/+) mice, respectively. These cells express intracellular IL-10 as shown by FACS analysis.

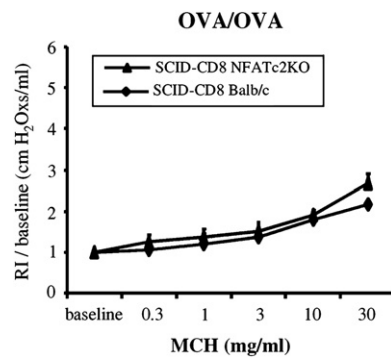


FIG E5. SCID mice reconstituted with either wild-type or NFATc2^(-/-) CD8⁺ T cells (1.5×10^6 CD8⁺ T cells) do not show increased AHR ($n = 5$). *MCH*, Methacholine; *OVA*, ovalbumin; *KO*, knockout.