

IL-10- and IL-12-Independent Down-Regulation of Allergic Sensitization by Stimulation of CD40 Signaling¹

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Interaction between CD154 (CD40 ligand) on activated T lymphocytes and its receptor CD40 has been shown to be critically involved in the generation of cell-mediated as well as humoral immunity. CD40 triggering activates dendritic cells (DC), enhances their cytokine production, up-regulates the expression of costimulatory molecules, and induces their maturation. It is unknown how stimulation of CD40 during sensitization to an airborne allergen may affect the outcome of allergic airway inflammation. We took advantage of a mouse model of allergic asthma and a stimulatory mAb to CD40 (FGK45) to study the effects of CD40-mediated DC activation on sensitization to OVA and subsequent development of OVA-induced airway inflammation. Agonistic anti-CD40 mAb (FGK45) injected during sensitization with OVA abrogated the development of allergic airway inflammation upon repeated airway challenges with OVA. Inhibition of bronchial eosinophilia corresponded with reduced Th2 cytokine production and was independent of IL-12, as evidenced by a similar down-regulatory effect of anti-CD40 mAb in IL-12 p40-deficient mice. In addition, FGK45 equally down-regulated allergic airway inflammation in IL-10-deficient mice, indicating an IL-10-independent mechanism of action of FGK45. In conclusion, our results show that CD40 signaling during sensitization shifts the immune response away from Th2 cytokine production and suppresses allergic airway inflammation in an IL-12- and IL-10-independent way, presumably resulting from enhanced DC activation during sensitization. *The Journal of Immunology*, 2006, 177: 5138–5144.

The CD40 ligand (CD40L³ or CD154) is a type II transmembrane protein of the TNF family that is expressed mainly on activated CD4⁺ cells, but also on activated CD8⁺ cells, mast cells, basophils, NK cells, B cells, eosinophils, dendritic cells (DC), and platelets (1–3). CD40 is the receptor for CD40L, a type I membrane glycoprotein of the TNFR/nerve growth factor receptor superfamily. CD40 was first identified as a receptor protein on B cells that activates B cell growth and differentiation in support of Ab production (4). Interaction between CD40L on activated CD4⁺ cells with CD40 on B lymphocytes plays a key role in humoral immune responses, as illustrated by the effects of CD40L deficiencies in patients with the X-linked hyper-IgM syndrome (5). Recently, it became clear that CD40/CD40L

interaction orchestrates many other immune functions, including Th cell differentiation (6) and activation of DC (7). CD40 signaling induces IL-12 production, which biases CD4⁺ T cell responses toward a Th1 profile and stimulates IFN- γ production in primed Th1 cells (8). In addition, ligation of CD40 up-regulates the expression of costimulatory and MHC class II molecules, thereby enhancing Ag-presenting capability (7, 9, 10) and preventing transplantation tolerance (11). Experimental evidence suggests a major role for CD40/CD40L interaction in several pathologic conditions. For example, a blocking mAb against CD40L prevented the induction of rheumatoid arthritis (12), diabetes (13), lupus nephritis (14), autoimmune encephalitis (15), oophoritis (16), and experimental colitis (17). In addition, impaired Th1 responses have been reported in mice lacking CD40L (18–20), and anti-CD40L mAb profoundly skews the ratio of IL-4- vs IFN- γ -producing Th cells toward the Th2 phenotype in graft-vs-host disease (21). In contrast, stimulation of CD40 signaling with an agonistic mAb induces IL-12 production (11, 22–24) and skews the immune response toward Th1 differentiation concomitant with down-regulation of Th2 cytokine production (8). The mechanism behind the beneficial effects of CD40 triggering is believed to result from down-regulation of IL-12 production (17). Indeed, agonistic anti-CD40 mAb induce protective Th1 responses to parasitic infections (22, 23), down-regulate pathogenic Th2 inflammation in response to a Th2-eliciting stimulus (23), and prevent Th2-dependent induction of neonatal transplantation tolerance (11). Consistently, vaccination with CD40L/trimer also induces protective immunity to *Leishmania major* infection and provides resistance to metastatic tumor growth (25). Taken together, the immunomodulatory effects of CD40/CD40L interaction have been linked primarily to Th1 cytokine-mediated diseases through modulation of IL-12 production and with consequences for the Th1/Th2 cytokine balance.

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Received for publication August 25, 2005. Accepted for publication July 14, 2006.

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¹ This work was supported by grants (G.0227.01) from the Fund for Scientific Research-Vlaanderen and Grant OT from the Research Council of the Catholic University of Leuven. P.W.H. is the recipient of a research fellowship of the Fund for Scientific Research-Vlaanderen.

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³ Abbreviations used in this paper: CD40L, CD40 ligand; BAL, bronchoalveolar lavage; DC, dendritic cell; EU, experimental unit; KO, knockout; MGG, May-Grünwald-Giemsa; PBLN, peribronchial lymph node; SN, supernatant; WT, wild type.

Allergic asthma is a prototype Th2 cytokine-mediated airway disease characterized by allergen-specific IgE, airway hyperresponsiveness, and eosinophilic inflammation (26). Only limited and rather contradictory data are available on the role of CD40/CD40L interaction in human asthma or experimental airway inflammation. Based on *in vitro* studies, one may speculate that impaired CD40/CD40L interaction, resulting in less IL-12 production by DC (2), would enhance Th2-mediated pathology. However, blockade of CD40L did not affect Th2 cytokine production in bronchial explants of atopic asthmatic patients (27), and mice deficient in the expression of CD40L (28) did not present with aggravated experimental allergic inflammation. However, Tang et al. (29) found a correlation between CD40 expression on airway macrophages and their production of IL-12, and report defective CD40 signaling by airway macrophages in patients with atopic asthma. In contrast, mice deficient in the expression of CD40 (30, 31) did not present with aggravated experimental allergic inflammation.

We hypothesized that the induction of DC activation and maturation through CD40 triggering at the time of allergen priming might affect allergic sensitization, by skewing the Th1/Th2 balance and/or by inducing a protective rather than pathogenic Th2 response. The present work therefore focuses on the effect of stimulation of CD40 signaling on macrophages during sensitization on the development of experimental allergic asthma. We injected agonistic anti-CD40 mAb (32) during systemic sensitization of mice to OVA and studied the effects on sensitization and the subsequent phenotype of airway inflammation. We further investigated whether the effects of anti-CD40 mAb would be mediated via induction of IL-12 or IL-10, as has been reported to occur in mouse models of *Schistosoma mansoni* infection (23) and rheumatoid arthritis (33), respectively.

Materials and Methods

mAbs and reagents

OVA (grade V) and BSA were purchased from Sigma-Aldrich. Agonistic anti-CD40 mAb (rat IgG2a) were purified from the culture supernatants (SN) of the FGK45 hybridoma (32), a gift from A. Rolink (Basel Institute of Immunology (in 1988), Basel, Switzerland). Control rat IgG was purchased from Rockland. Türk's solution was purchased from Merck Diagnostica, and RPMI 1640 was purchased from BioWhittaker. Rat anti-mouse IgE, IgG1, and IgG2a were purchased from BD Pharmingen, and rabbit anti-OVA IgG was obtained from Rockland. Tetramethylbenzidine dihydrochloride was purchased from ACROS. For RT-PCR, TRIzol and Superscript II RT were obtained from Invitrogen Life Technologies.

Mouse model of allergic asthma

Male BALB/c mice (Harlan) were kept under conventional pathogen-free conditions and were actively sensitized by seven *i.p.* injections of 10 μ g of OVA in 0.5 ml of pyrogen-free saline on alternate days from days 1 to 13, as described (34). A total of 250 μ g of agonistic Ab against CD40 (FGK45) or control rat Ig was injected *i.p.* during the sensitization phase, *i.e.*, on days 0, 4, 8, and 12 in mice ($n = 6$ per group).

Mice were then exposed daily for 5 min to nebulized OVA (10 mg/ml; PARI TurboBOY) from days 33 to 40, called the challenge phase. On day 41, eosinophilic airway inflammation was found with typical features reminiscent of human allergic asthma (34). To study the role of IL-12 in FGK45-mediated effects, FGK45 or control rat Ig were given during sensitization, as described above, in age-matched IL-12 p40-deficient (IL-12 knockout (KO)) and wild-type (WT) BALB/c mice (The Jackson Laboratory). The same approach was applied for studying the role of IL-10 in the FGK45-mediated effects. FGK45 or control rat Ig were injected during sensitization in age-matched IL-10 KO and syngeneic WT C57BL/6 mice (The Jackson Laboratory). Each group in this study consisted of 6–12 mice. Experimental procedures were approved by the local Ethical Committee of Animal Experiments.

Measurement of OVA-specific IgE, IgG1, and IgG2a Ab levels

After anesthesia with urethane (*i.p.*, 2.1 g/kg), a retro-orbital bleed was performed on days 28 and 41 and serum frozen until analysis. For ELISA,

96-well plates were coated overnight with rat anti-mouse IgE (10 μ g), rat anti-mouse IgG1 (20 μ g), or rat anti-mouse IgG2a (20 μ g) in 100 μ l of PBS. Remaining binding sites were blocked, and plates were incubated with 100 μ l of diluted serum (1/5 for IgE, 1/1000 for IgG1, and 1/10 for IgG2a). After washing, following substances were sequentially added, incubated, and washed: OVA (1 μ g/100 μ l), peroxidase-labeled rabbit anti-OVA IgG (240 ng/100 μ l), and buffer containing tetramethylbenzidine dihydrochloride (1 μ l/100 μ l) and H₂O₂ (1 μ l/100 μ l). The peroxidase reaction was stopped by adding H₂SO₄, and the OD was measured at 450 nm. Ig levels of a reference pool of serum of highly OVA-sensitized BALB/c mice were assigned a value of 100 experimental units (EU) per ml.

Evaluation of eosinophilia in peripheral blood and bronchoalveolar lavage (BAL) fluid

Mice were anesthetized, as described above, at 24 h after the eighth inhalatory challenge with OVA (day 41), and lungs were lavaged five times with 1 ml of PBS at 37°C supplemented with 5% BSA through a tracheal polyethylene catheter (diameter, 0.85 mm). The first lavage was centrifuged at 1400 $\times$ g for 5 min, and the SN was stored at –20°C until measurement of cytokines. The pellet was added to the subsequent four lavages. After centrifugation (1400 $\times$ g, 5 min), cells were washed and resuspended in 200 μ l of PBS. For cell counting, 10 μ l of cell suspension was added to 90 μ l of Türk's solution, and the number of cells was calculated in a Bürker chamber. Differential cell counts were performed on May-Grünwald-Giemsa (MGG)-stained smears of peripheral blood and cytospin preparations of BAL fluid cells. Based on standard hematological criteria, cells were differentiated into eosinophils, neutrophils, lymphocytes, and macrophages.

Histologic analysis

After performing BAL, the right lung was removed and fixed overnight in buffered formalin (5%). After dehydration and embedding in paraffin, 5- μ m sections were stained with the classic H&E. On H&E-stained sections, eosinophils could be easily recognized by their polyglobular nucleus and bright cytoplasmic granules. The maximal thickness of eosinophilic inflammatory infiltrates around two bronchioli and two arterioli in each lung were measured using an eyepiece graticule at a magnification of 25. The average of these four values was calculated for each mouse and expressed in 10^{–2}/mm.

Measurement of IFN- γ , IL-12, IL-5, and IL-4 levels

IL-12 p40, IFN- γ , IL-4, and IL-5 levels in BAL fluid (1/2 diluted) and serum (undiluted) were measured by sandwich ELISA using paired matched Ab, according to the manufacturer's instructions, as described previously (34). ELISA reagents for mouse IL-5 and IFN- γ were purchased from BD Pharmingen. IL-12 p40 was quantified, as described before (35). An ELISA kit from BioSource International was used for measurement of IL-4 levels. The sensitivity of these assays was 300, 2, 2, and 5 pg/ml for IL-12 p40, IFN- γ , IL-4, and IL-5, respectively.

RT-PCR for quantification of cytokines on lung tissue

At 24 h after the eighth challenge, part of the right lung was dissected, immediately frozen in liquid nitrogen, and stored at –80°C. Total RNA was extracted using TRIzol. A constant amount of 1 μ g of target RNA was reverse transcribed using 100 U of Superscript II RT at 42°C for 80 min in the presence of 5 μ M oligo(dT)₁₆. Real-time quantitative RT-PCR was performed for IL-4, IL-5, IL-10, IL-13, IFN- γ , and IL-12 p40 mRNA in the ABI Prism 7700 Sequence detector (Applied Biosystems), as described before (36). The primer and probe sequences used for IL-4, IL-5, IL-10, IL-13, IFN- γ , and IL-12 p40 were as previously published (36). Each PCR amplification was performed in triplicate wells, using the following conditions: 10 min at 94°C, followed by a total of 40 or 45 two-temperature cycles (15 s at 94°C and 1 min at 60°C).

Evaluation of cutaneous hypersensitivity reaction

On days 28 and 41, mice were injected in the right aural concha with OVA (1 μ g in 10 μ l of saline) and with an equal volume of saline in the contralateral concha. After 1 h, the thickness of both conchae was measured using a Mitutoyo Multimeter. The increase in thickness of the right aural concha was expressed in percentage of the thickness of the contralateral concha.

In vitro study on cytokine production by T lymphocytes in vitro

For evaluation of cytokine production in vitro, peribronchial lymph nodes (PBLN) and spleens were dissected and homogenized using a cell strainer (Falcon; BD Biosciences) in RPMI 1640 supplemented with FCS (5%). Spleen homogenates were incubated for 1 min with 5 ml of lysis buffer containing NH_4Cl , KHCO_3 and Na_2EDTA , and then washed with PBS/FCS. Cells were counted using a Coulter counter (Analys).

PBLN cells and splenocytes ($1 \times 10^6/\text{ml}$ each) were cocultured in 1 ml of RPMI 1640 with 10% FCS supplemented with penicillin, streptomycin, L-glutamine, and 0.1% 2-ME, with and without OVA ($10 \mu\text{g}/\text{ml}$) for 5 days at 37°C . The SN were collected and stored at -20°C until cytokine measurement.

Data analyses

Data are expressed as means $\pm$ SEM. Statistical analyses were conducted using the Mann-Whitney *U* test. A difference was considered to be significant when $p < 0.05$.

Results

Inhibition of airway inflammation by agonistic anti-CD40 mAb (FGK45) administered during sensitization

In all experiments, BALB/c mice were sensitized by i.p. injection of OVA on alternate days (days 1–13) and then daily challenged with nebulized OVA for 8 days (days 33–40). This protocol results in the induction of allergic airway inflammation, as previously published (37). When FGK45 was injected during sensitization with OVA (days 1–13), this treatment markedly attenuated the severity of allergic inflammation in response to repeated inhalatory OVA challenges. Compared with control IgG-treated mice, FGK45-treated mice had lower total cell counts in BAL fluid ($66.7 \pm 9.3 \times 10^5$ vs $108.2 \pm 12 \times 10^5/\text{ml}$; $p < 0.05$). Differential cell counts of BAL fluid showed that mainly eosinophil and, to a

lesser extent, lymphocyte counts were reduced in the FGK45 group ($p < 0.05$; Fig. 1A), whereas the opposite was found for macrophages (Fig. 1A). Similar cellular shifts were observed in the peripheral blood, where less circulating eosinophils (4.3 ± 0.7 vs $12.2 \pm 1.1\%$ of white cell count; $p < 0.05$) were found in the FGK45-treated group compared with the control, while the opposite was observed for peripheral monocytes (23.4 ± 2.1 vs $7.3 \pm 2.1\%$ of white cell count; $p < 0.05$). In bronchial tissue, the thickness of peribronchial eosinophilic infiltrates was significantly smaller in FGK45-treated than control mice ($35.3 \pm 23.3 \times 10^{-2}$ vs $170.3 \pm 13.2 \times 10^{-2}$ mm, respectively; $p < 0.05$).

FGK45 treatment during sensitization significantly reduced OVA-specific IgG1 production (65.7 ± 11.7 vs 94.1 ± 1.3 EU/ml on day 28; $p < 0.05$), whereas the opposite trend was found for OVA-specific IgG2a (362.3 ± 90.2 vs 257.3 ± 63.2 EU/ml on day 28; $p > 0.05$). Both OVA-specific IgE titers in serum and cutaneous hypersensitivity reactions on day 28 were reduced by FGK45 treatment, without reaching levels of significance (data not shown). Repeated inhalatory OVA challenges of sensitized mice, i.e., 21 days after FGK45 or control IgG treatment, boosted Ig production, resulting in similar serum levels of OVA-specific IgG1, IgE, and IgG2a in both treatment groups on day 41. Data on FGK45-mediated effects on cytokine production will be dealt with in the next paragraph.

FGK45-mediated induction of IL-12 and IL-10 production

On day 41, measurement of IL-12 p40 and IL-10 levels in the serum revealed that FGK45-treated mice presenting with attenuated airway inflammation showed higher levels of circulating IL-12 p40 (3395 ± 269 vs 725 ± 425 ng/ml in controls; $p < 0.05$;

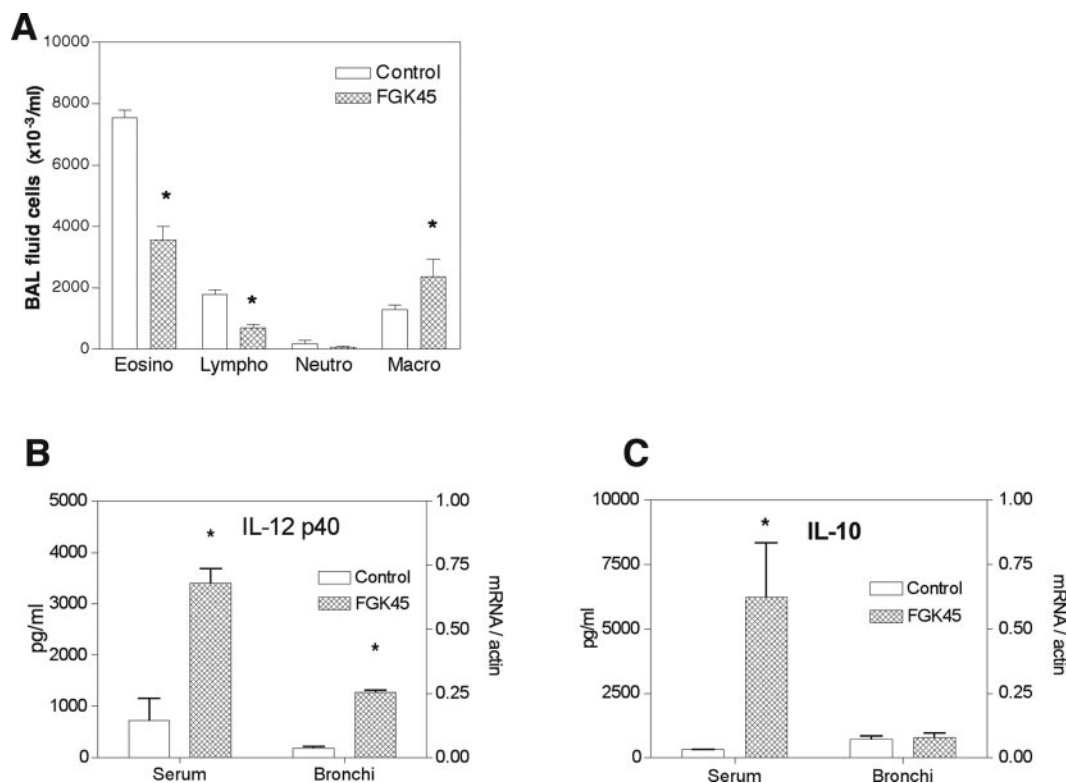


FIGURE 1. Effects of agonistic anti-CD40 mAb (FGK45) administered during sensitization on subsequent development of allergic airway inflammation in response to inhalatory OVA challenge and production of IL-12 p40 and IL-10. Cell counts on cytopins of BAL fluid are presented (A). Titers of IL-12 p40 and IL-10 were measured in serum (B and C) with ELISA and expressed in pg/ml. mRNA levels of IL-12 p40 and IL-10 were determined on lung homogenates using real-time RT-PCR (B and C), and the level of mRNA for IL-12 p40 and IL-10 was expressed relative to mRNA for actin, and multiplied by 10^5 . One representative experiment of three is shown. Each group consisted of six mice. *, $p < 0.05$.

Fig. 1B) and IL-10 (6221 ± 2215 vs 320 ± 10 ; Fig. 1C) in comparison with the controls. In addition, mRNA levels of IL-12 p40 in lung tissue were also higher in the FGK45-treated group (185.2 ± 3.8 vs 127.2 ± 4.6 ; $p < 0.05$; Fig. 1B) as well as on protein level in BAL fluid (data not shown). IL-10 mRNA in the tissue of FGK45-treated mice was not altered by FGK45 (Fig. 1C), nor was there any difference in IL-10 protein level in BAL fluid (data not shown).

IL-12-independent inhibition of airway inflammation by FGK45

As expected from previous studies (38), IL-12 p40-deficient (KO) BALB/c mice developed more severe airway inflammation than WT littermates in response to the protocol for induction of experimental asthma (Fig. 2, A and B). FGK45 treatment down-regulated bronchial inflammation in IL-12 p40 KO or WT mice (Figs. 2 and 3), suggestive of IL-12-independent mecha-

nisms of action of FGK45. Both IL-12 p40 KO and WT mice responded to FGK45 treatment with a potent reduction of bronchial eosinophil and lymphocyte counts (Fig. 2A). Consistent with a reduction of bronchial eosinophilia, lower IL-5 levels were found in BAL fluid of FGK45-treated mice (Fig. 2B). As was observed in WT mice (Fig. 3, A and B), FGK45 treatment reduced the size of peribronchial inflammatory infiltrates in IL-12 KO mice ($45.3 \pm 23.2 \times 10^{-2}/\text{mm}$ vs $210.2 \pm 32.3 \times 10^{-2}/\text{mm}$; $p < 0.05$; Fig. 3, C and D). In both groups of mice, FGK45 reduced bronchial mRNA expression of IL-4, IL-5, and IL-13 (Fig. 2C), without affecting IFN- γ mRNA levels. FGK45 treatment abrogated the production of IL-4 and IL-5 by PBLN, without alteration of IFN- γ production (Fig. 2D). Consistent with aggravated allergic airway inflammation in IL-12 KO mice compared with WT mice, OVA-stimulated PBLN cells of IL-12 KO mice produced more IL-4 and IL-5 and less IFN- γ than WT

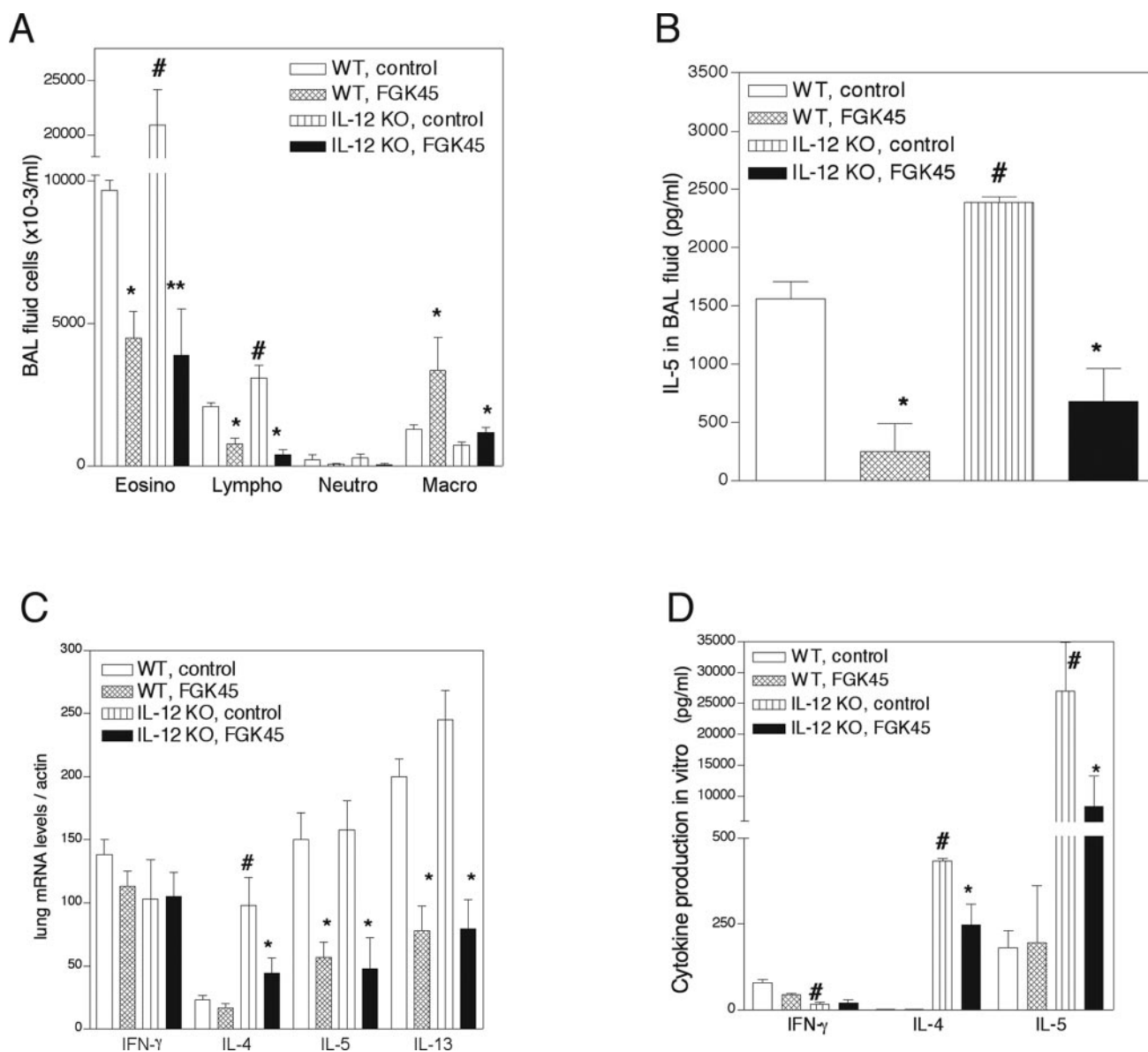


FIGURE 2. Effects of agonistic anti-CD40 mAb (FGK45) administered during sensitization on allergic inflammation in IL-12 KO and WT BALB/c mice. Differential cell counts in BAL fluid on day 41 were performed on MGG-stained cytopspins (A). IL-5 levels in BAL fluid were measured with ELISA (B). mRNA levels of IFN- γ , IL-4, IL-5, and IL-13 were measured on lung homogenates with real-time RT-PCR. The level of mRNA for different cytokines was expressed relative to mRNA for actin, and multiplied by 10^5 for IL-4 and IL-13 and by 10^6 for IFN- γ and IL-5 (C). Production of IFN- γ , IL-4, and IL-5 by OVA-stimulated PBLN cells was measured in culture SNs using ELISA (D). Each group consisted of four to eight mice, and one representative experiment of two is shown. #, $p < 0.05$ vs WT, control mice; *, $p < 0.05$ vs IgG-treated mice with similar background.

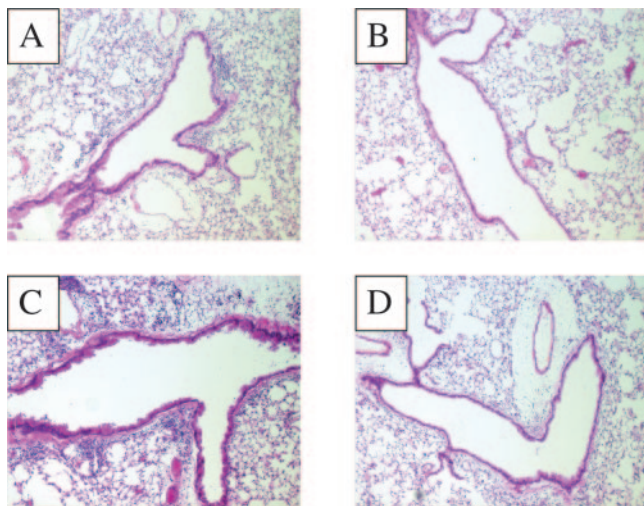


FIGURE 3. Histologic sections of peribronchial tissue of WT (A and B) and IL-12 KO (C and D) mice treated with either control Ig (A and C) or agonistic anti-CD40 mAb (FGK45; B and D) during sensitization. Peribronchial tissue sections were stained with the classic MGG stain, allowing the appreciation of peribronchial inflammatory infiltrates.

littermates (Fig. 2D). No differences in total T cell numbers were found in PBLN of mice in different treatment groups (data not shown).

IL-10-independent inhibition of airway inflammation by FGK45

To investigate whether IL-10 plays a role in the FGK45-mediated effects on allergic sensitization, IL-10-deficient and WT mice of C57BL/6 background were used for study. As was found in WT BALB/c mice, FGK45 injection during sensitization of WT C57BL/6 mice potently reduced the development of airway eosinophilia in response to inhalation of nebulized OVA. FGK45 treatment reduced the total cell count in BAL fluid both in WT (46.3 ± 6.2 vs $86.0 \pm 26.1 \times 10^5/\text{ml}$; $p < 0.05$) as well as in IL-10 KO C57BL/6 mice (45.8 ± 7.9 vs $67.2 \pm 10.7 \times 10^5/\text{ml}$; $p < 0.05$). Differential cell counts in BAL fluid are depicted on Fig. 4A and show a manifest reduction of eosinophilic and lymphocyte infiltration in the bronchial lumen by FGK45 both in WT and IL-10

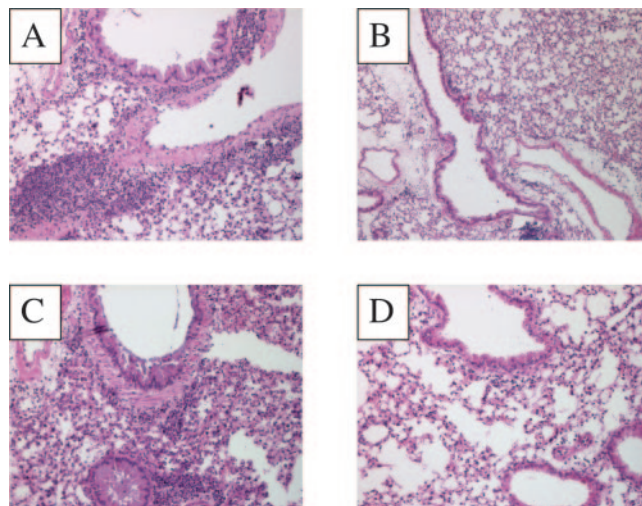


FIGURE 5. Histologic sections of peribronchial tissue of WT (A and B) and IL-10 KO (C and D) mice treated with either control Ig (A and C) or agonistic anti-CD40 mAb (FGK45; B and D) during sensitization. Peribronchial tissue sections were stained with the classic MGG stain, allowing the appreciation of peribronchial inflammatory infiltrates.

KO mice. Similar observations were made when quantifying the degree of bronchial inflammation on histologic sections through the bronchi (Fig. 5). Consistent with reduced bronchial eosinophilic inflammation, less Th2 cytokines, especially IL-4, were produced both in WT as well as in IL-10 KO C57BL/6 mice after administration of FGK45 (Fig. 4B).

Discussion

Our data show that stimulation of CD40 at the time of sensitization markedly attenuates the subsequent manifestation of allergic airway inflammation. The beneficial effects of agonistic anti-CD40 mAb on bronchial eosinophilia inflammation were accompanied by a reduction of Th2 cytokine production and occurred in an IL-12- and IL-10-independent way.

Stimulation of CD40 signaling during Ag priming resulted in a reduction of bronchial eosinophilia following subsequent inhalatory allergen challenges. Down-regulation of eosinophilic inflammation by FGK45 was accompanied by and can potentially be

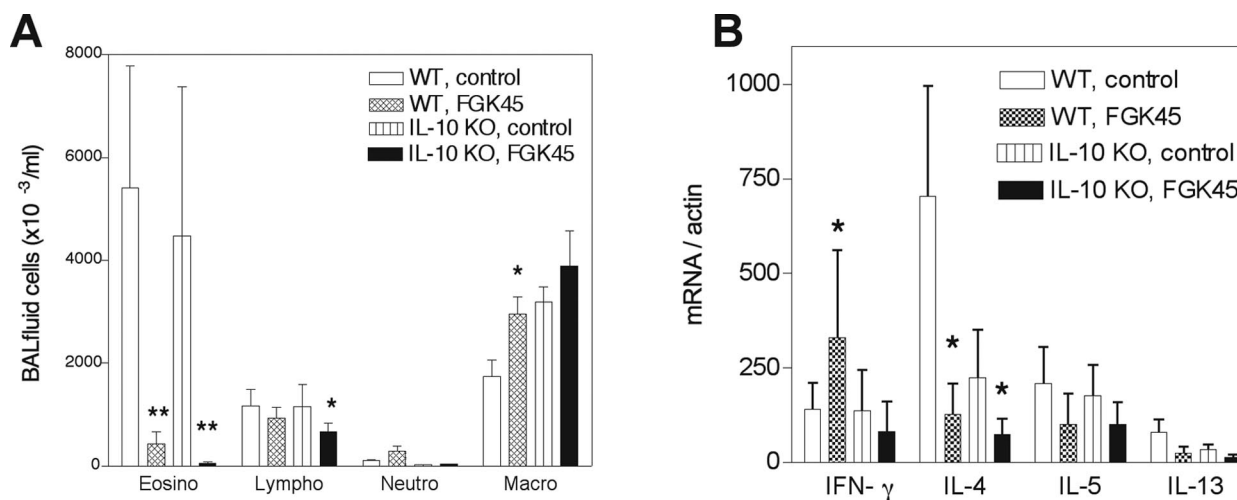


FIGURE 4. Effects of administration of agonistic anti-CD40 mAb (FGK45) during sensitization on allergic inflammation in IL-10 WT and KO C57BL/6 mice. Differential cell counts in BAL fluid were performed on MGG-stained cytospins (A). On lung homogenates, mRNA levels of IFN- γ , IL-4, IL-5, and IL-13 were determined by quantitative real-time RT-PCR, expressed relative to mRNA for actin, and multiplied by 10^5 (B). Each group consisted of six to eight mice. One representative experiment of two is shown. *, $p < 0.05$ and **, $p < 0.001$ vs control Ig-treated mice.

explained by decreased production of IL-4, IL-5, and IL-13 (30, 39, 40). IL-12 has the capacity to inhibit Th2 differentiation at the time of Ag priming (8) and to reduce bronchial eosinophilia in allergic inflammation (38). In view of enhanced bronchial and systemic production of IL-12 after administration of anti-CD40 mAb and the reported IL-12-mediated effects of anti-CD40 mAb in other murine models (23, 33), we hypothesized that the beneficial effects of anti-CD40 mAb would be mediated via induction of IL-12. Unexpectedly, FGK45 also down-regulated the allergic inflammatory response in IL-12-deficient mice, indicative of alternative IL-12-independent pathways. However, our data are in line with the observation by Kuipers et al. (41) that LPS stimulation of airway DC suppresses Th2 development of eosinophilic airway inflammation in an IL-12-independent way. We therefore speculated on CD40-mediated induction of other mediators with inhibitory potential on Th2 differentiation. IL-10 has powerful anti-inflammatory activities (42). Despite the contradictory results that have been reported regarding the immunosuppressive capacity of IL-10 in allergic airway inflammation (43, 44), we explored the effects of IL-10 production as a possible explanation for the beneficial effects of FGK45 treatment. CD40 signaling has been reported to mediate production of IL-10 in vitro (7) as well as in vivo (33). Mauri et al. (33) found up-regulation of IL-10 production by activated Ag-specific T lymphocytes from mice treated with FGK45. Consistently, we found high levels of circulating IL-10 after FGK45 therapy. In our model of repeated exposure of sensitized mice to nebulized allergens, the FGK45-mediated beneficial effect on allergic airway inflammation was independent of the induction of IL-10, as a similar reduction of bronchial eosinophilia was found in both IL-10-producing and -deficient mice. In a *S. mansoni* infection model, Martin et al. (23) reported that IFN- γ is responsible for the inhibition of Th2 cytokine production by anti-CD40 mAb. In this study, IFN- γ is unlikely involved in down-regulating Th2 cytokine production, as both bronchial as well as systemic production of IFN- γ remained low after FGK45 therapy. Similarly, the production of TGF- β , another cytokine with anti-inflammatory capacity in allergic asthma (45), was not altered on mRNA and protein level by anti-CD40 treatment (our unpublished observation). Induction of IL-18 may also represent a mechanism by which allergic airway inflammation is down-regulated after administration of anti-CD40 mAb. IL-18 has been reported to reduce eosinophilic airway inflammation and replace an established Th2-biased immune response with a Th1-biased response (46). Therefore, the IL-18 pathway merits further exploration.

In parallel with down-regulating the eosinophilic and lymphocytic influx, anti-CD40 mAb slightly enhanced bronchial macrophage numbers. This phenomenon may represent a late effect of IL-12-induced stimulation of extramedullary hemopoiesis (47). When mice were sacrificed at several time points after an injection of FGK45, we observed a marked splenomegaly already present at day 1 after injection, which then progressively regressed by day 14, and was not accompanied by any sign of wasting disease (our unpublished observation). The splenomegaly can be related to by CD40-mediated induction of IL-12 (47) and/or GM-CSF production (48).

Of note, the beneficial effects of FGK45 treatment on the allergic airway inflammation were accompanied by a marked shift of cytokine production away from Th2 cytokine production toward a Th1 cytokine predominance in C57BL/6 WT mice (Fig. 4B). After FGK45 treatment, BALB/c mice did not show increased Th1 cytokine expression, but presented with a marked reduction of Th2 cytokine expression. In both mouse strains, high levels of IL-12 p40 were measured in bronchi as well as in serum after FGK45 treatment. It is noteworthy to mention that the beneficial effects of

anti-CD40 mAb on allergic inflammation occur concomitantly with the induction of IL-12. The association between amelioration of allergic airway inflammation and induction of Th1 cytokines is supported by clinical studies showing increased IFN- γ levels after successful immunotherapy of allergic patients (49, 50) and reduction of bronchial eosinophilia by exogenous IL-12 (51).

In conclusion, in this study we demonstrated in a mouse model that stimulation of CD40 signaling during sensitization redirects the allergic response away from the Th2 cytokine phenotype normally induced in allergic airway inflammation and reduces the subsequent development of allergic airway inflammation in response to inhaled allergen. Hence, our data underline the critical role of the activation state of DC at the time of Ag priming in the control of the development of allergic airway inflammation.

Acknowledgments

We thank E. Dewil and the staff of the animal housing department for taking care of the mice.

Disclosures

The authors have no financial conflict of interest.

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