

A Novel Low Molecular Weight Inhibitor of Dendritic Cells and B Cells Blocks Allergic Inflammation

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Rationale and Objective: During allergic lung inflammation dendritic cells (DCs) direct the generation and function of effector T-helper type 2 cells. T-helper type 2 cells not only orchestrate the inflammatory processes in the tissue by inducing the accumulation and activation of proinflammatory cells but also induce IgE production by B cells. Thus, inhibitors of DC function should have therapeutic benefits in patients with allergies.

Methods and Measurements: VAF347, a novel low molecular weight immunomodulator, is described and acts as an antiinflammatory compound by a dual mode of action.

Results: VAF347 inhibited the function of human monocyte-derived DCs to induce T-cell proliferation and cytokine production. Mechanistically, this effect may be due to reduced expression of CD86, HLA-DR, and interleukin 6 by DCs. In addition, the compound inhibited IgE synthesis in an isotype-specific fashion by human B lymphocytes. In a mouse model of antigen-induced eosinophilic inflammation, VAF347 blocked lung eosinophilia, mucus hyperplasia, and serum IgE levels, representing the hallmarks of allergic lung inflammation. The biological effects *in vivo* are most likely mediated by the immunoregulatory role of VAF347 on DCs because allergic lung inflammation was also inhibited in B-cell-deficient mice.

Conclusion: VAF347 represents a novel type of immunomodulator by affecting two major pathways in allergic airway pathogenesis: dendritic cell-mediated T-helper-cell activation and induction of IgE production by human B lymphocytes.

Keywords: asthma; immunobiology; pharmacology

Atopic individuals suffering from allergic asthma, atopic dermatitis, or allergic rhinitis demonstrate a high correlation between increased serum IgE levels and hyperreactivity to allergen. IgE is considered a pathologic factor for these diseases (1–3). B lymphocytes produce IgE in response to activated T-helper type 2 (Th2) effector cells and interleukin 4 (IL-4) or IL-13. Besides IgE, Th2 cells orchestrate the inflammatory reactions in the airways of patients with allergic asthma in multiple ways (4, 5). The development of Th2 cells occurs in the draining lymph nodes through the interaction of naive T-cell precursors with antigen-loaded dendritic cells (DCs). At least two contact-dependent signals are necessary to fully activate naive T cells. The primary signal originates from binding of the T-cell receptor to antigenic peptides presented in the context of major histocompatibility molecules on the DCs, whereas the second costimulatory signal is provided by B7 molecules, such as CD80 or CD86, on the DCs with counterreceptors such as CD28 on T cells (6). The additional presence of cytokines, such as IL-4, IL-12, or

IL-6 (7), strongly determines the type and function of T-effector cells being produced. The importance of DCs for the initiation of inflammatory immune responses in allergic diseases has been studied in humans (8, 9) and in experimental animals (10–13). It has been shown that DCs are also involved in the maintenance of these responses by activating memory T cells in sensitized animals (11). Similar data have been reported for patients with allergic asthma in which a rapid influx of DCs into lung tissue was measured after allergen challenge (14). Because of the fast kinetics of this effect it is speculated that the DCs were directly recruited from the blood (15). A similar phenomenon has been observed in patients with allergen-challenged atopic dermatitis and in individuals suffering from allergic rhinitis (16, 17). Given the crucial role of DCs in immune responses, modulation of DC function may have therapeutic benefits in a number of diseases. Here we describe the biological profile of VAF347, a novel low molecular weight compound that was identified in a screening campaign designed for isotype-specific inhibitors of IgE production in B lymphocytes. Further biological profiling of VAF347 in a number of disease-relevant cell types revealed a striking cell-type specificity of VAF347, with DCs being the only other cell type besides B cells that was responsive to the compound. *In vitro*, VAF347 blocks the function of human monocyte-derived DCs to provide T-cell help, possibly by inhibiting the expression of CD86, HLA-DR, and IL-6 by DCs. *In vivo*, VAF347 blocked serum IgE levels in a mouse model of antigen-induced eosinophilic inflammation, thus validating the *in vitro* results. In addition, lung eosinophilia and goblet-cell hyperplasia were inhibited, indicating the antiinflammatory properties of VAF347. The antiinflammatory phenotype is not a consequence of IgE inhibition but is likely due to the effect of VAF347 on DCs.

METHODS

Cell Culture and Reagents

Human spleen biopsies were obtained through the West Hungarian Regional Tissue Bank (Győr, Hungary) from healthy individuals who suffered traumatic lesions. Tissues were obtained with informed consent and in compliance with regulations issued by the Novartis Ethics Committee and state government. Human spleen cells were cultured in Iscove's modified Dulbecco's medium (GIBCO; Invitrogen, Carlsbad, CA), supplemented with 10% fetal calf serum, penicillin (100 U/ml) and streptomycin (100 µg/ml), transferrin (10 µg/ml), and 1.25% soybean lipids. B cells were purified with a negative enrichment kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the B cells was more than 99% as determined by fluorescence-activated cell-sorting (FACS) staining with anti-CD19 monoclonal antibodies (mAbs).

All antibodies used for flow cytometry were obtained from BD Biosciences Pharmingen (San Diego, CA). The design and synthesis of VAF347 ([4-(3-chloro-phenyl)-pyrimidin-2-yl]-[4-trifluoromethyl-phenyl]-amine) are described elsewhere.

Human Immunoglobulin Induction, and Cell Proliferation Assays

Human spleen cells or purified B cells were stimulated with IL-4 (50 ng/ml; Novartis AG, Basel, Switzerland), anti-CD40 mAbs

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(500 ng/ml; provided by S. M. Fu, University of Virginia, Charlottesville, VA), and increasing concentrations of VAF347 to induce polyclonal IgE synthesis. To induce IgG1 production, IL-4 was replaced with IL-10 (100 ng/ml; R&D Systems, Wiesbaden, Germany). On Day 9, supernatants were collected and IgE and IgG1 were measured by ELISA.

Generation of Monocyte-derived DCs

Human peripheral blood monocytes were prepared by elutriation or by negative selection of peripheral blood mononuclear cells, using a monocyte isolation kit (Miltenyi Biotec). Monocytes (typically more than 97% positive for CD14) were differentiated into immature DCs by adding IL-4 (40 ng/ml) and granulocyte-macrophage colony-stimulating factor (GM-CSF; 15 or 50 ng/ml) for 6 to 8 d in the absence or presence of increasing concentrations of VAF347. Cell surface expression of CD14, CD80, HLA-DR, and CD86 was measured by FACS staining. Maturation of DCs was induced by activation with GM-CSF (20 ng/ml; Novartis Pharma, Basel, Switzerland), IFN- γ (100 U/ml; Bender MedSystems, Vienna, Austria), tumor necrosis factor- α (TNF- α ; 20 U/ml; Bender MedSystems), anti-CD40 mAb (4 μ g/ml) and goat anti-mouse IgG (1 μ g/ml; Dianova, Hamburg, Germany) for 48 h. The levels of IL-6, macrophage-derived chemokine (MDC), and thymus- and activation-regulated chemokine (TARC) were measured by ELISA (R&D Systems).

DC-mediated Autologous T-Cell Proliferation Assays and Cytokine Production

Immature DCs were pulsed overnight with keyhole limpet hemocyanin (KLH; 100 μ g/ml; Pierce Biotechnology, Rockford, IL) in AIM V medium (GIBCO) supplemented with 2 mM L-glutamine, human transferrin (1 μ g/ml; Pierce Biotechnology), and insulin (500 ng/ml; Sigma, St. Louis, MO). Afterward, cells were washed and resuspended together with autologous CD4-positive T cells purified by negative immunomagnetic selection (Miltenyi Biotec) at a ratio of 1:20. After 9 d, the primed T cells were washed and restimulated with fresh autologous KLH-pulsed DCs at a DC:T ratio of 1:15 for 3 d. T-cell proliferation was measured by adding 1 μ Ci of tritiated thymidine (specific activity, 3,000 mCi/mmol; GE Healthcare, Little Chalfont, UK) per well during the last 6 h. IL-2 was determined in supernatants from restimulated T cells after 48 h by Luminex technology (R&D Systems). To induce DC-independent T-cell proliferation, 1×10^5 purified T cells (pan T-cell isolation kit; Miltenyi Biotec) were activated with immobilized anti-CD3 mAbs and anti-CD28 mAbs (each at 200 ng/well) in the absence or presence of VAF347. After 24 h, cells and medium were transferred into fresh wells without mAb coating and incubated for another 48 h. T-cell proliferation was measured as described above.

DC Antigen Uptake

Immature monocyte-derived DCs generated in the absence or presence of 50 nM VAF347 were cooled in ice for 30 min or were left at 37°C. Fluorescein isothiocyanate-conjugated ovalbumin (FITC-OVA, 1 μ g/ml; Sigma) was then added and incubated for 15 or 60 min. At these time points, cells were washed with phosphate-buffered saline (PBS) containing 0.1% NaN₃ and resuspended in 2% paraformaldehyde to stop uptake of FITC-OVA. Afterward, the fluorescence intensity of the cells was analyzed by FACS.

Induction of Allergic Lung Inflammation *In Vivo*

On Days 0 and 5, B₆D₂F₁ mice or B-cell-deficient (C57/BL6. μ MT) mice (Jackson Laboratory, Bar Harbor, ME) were sensitized by intraperitoneal injection of 10 μ g of OVA (Sigma) adsorbed to Al(OH)₃ (Serva, Heidelberg, Germany). VAF347 was formulated as a microemulsion (ethanol-Labrafil M2125-corn oil, 150:350:500) and was given orally twice per day at 30 mg/kg between Days 0 and 7 or between Days 10 and 13. For the same time periods supalast tosilate was given orally at 100 mg/kg. To induce allergic lung inflammation, immunized mice were challenged twice on Day 12 with a 1% OVA aerosol for 60 min. Forty-eight hours later, the lungs were lavaged three times, each time with 1 ml of PBS. Eosinophils were counted on May-Gruenwald/Giemsa-stained cytocentrifuge preparations. For the enumeration of tissue eosinophils, fixed lung sections were stained with hematoxylin and

eosin. Mucus-producing cells were stained by the periodic acid-Schiff reaction. Serum IgE levels were determined by ELISA.

Statistical Analysis

Statistical analysis was done by Student *t* test.

RESULTS

VAF347 Inhibits IgE Production by Human B Cells

For the initiation of IgE synthesis by B cells, Th2-cell-derived IL-4 and T/B-cell contact involving CD40 ligand and CD40 on B cells are required. These stimuli induce B-cell activation and differentiation into IgE-secreting plasma cells, a process known as immunoglobulin isotype class switching (18, 19). To identify inhibitors of IgE production, human spleen cells were cultured with IL-4 and agonistic anti-CD40 mAb for 9 d in the presence or absence of test compound. Cell supernatants were quantitated for IgE by ELISA. In these assays, VAF347 inhibited IgE production in a dose-dependent manner with a median inhibitory concentration (IC₅₀) of 1 to 2 nM (Figure 1). The same biological effect was observed when using tonsillar cells as B-cell source (data not shown). Dendritic cells have been described to directly promote isotype switching to IgE in B cells in the absence of T cells (20). To confirm that the effect of VAF347 was due to a direct effect on B cells and not mediated by inhibition of the class switch-promoting function of DCs present in the spleen cell preparation, the experiments were repeated with purified splenic B cells. VAF347 inhibited IgE synthesis with the same potency (Figure 1), demonstrating that the biological activity was due to a direct effect of the compound on B cells. IL-13 is also known to trigger IgE class switching by B cells in conjunction with CD40 signaling (21). VAF347 inhibited IL-13/anti-CD40 mAb-induced IgE secretion in a dose-dependent fashion with a potency (IC₅₀ of 1 nM) indistinguishable from that of IL-4-induced IgE (data not shown). In contrast to IL-4, IL-10 directs B cells to synthesize IgG isotypes rather than IgE (22, 23). To determine the specificity of VAF347 for IgE inhibition, spleen cells were cultured with IL-10 and anti-CD40 antibodies in the presence of the compound. VAF347 was ineffective in reducing the levels of IgG1 in the supernatants even at the highest concentration tested (Figure 1). From these data we concluded that VAF347 inhibited IgE production induced by the prototype Th2 cytokines IL-4 and IL-13 in an isotype-specific fashion. To explore whether VAF347 acted as IL-4 antagonist, the expression level of CD23, a well-characterized IL-4-responsive gene, was measured on cytokine-induced B cells. The data demonstrated that CD23 levels were unchanged in the presence of VAF347 (Table 1), thus indicating that the compound does not act as a general IL-4 antagonist.

VAF347 Modulates the Function of Monocyte-derived DCs

Broader biological profiling of VAF347 in other disease-relevant cell types was performed to determine the cell type specificity of the compound. Whereas no compound effect was observed in T cells, mast cells, fibroblasts, endothelial cells, and keratinocytes, indicating a high degree of cell type specificity, VAF347 affected the function of DCs to induce T-cell activation. In an *in vitro* model of T-cell activation in response to a neoantigen, KLH-pulsed immature monocyte-derived DCs (iDCs) were cocultured with autologous CD4-positive T cells for 9 d followed by restimulation with fresh KLH-pulsed DCs to induce a strong proliferative T-cell response. VAF347 was present throughout the experiment with the exception of the final T-cell challenge. The compound markedly inhibited DC-mediated T-cell proliferation in a dose-dependent fashion (Figure 2A) with a potency comparable to inhibition of IgE in B cells. We also measured

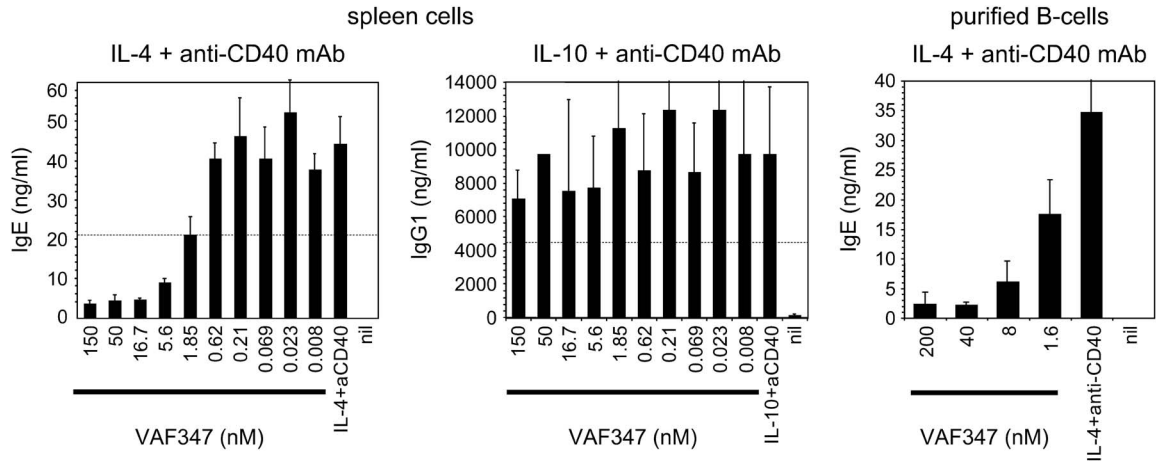


Figure 1. VAF347 inhibits IgE production by human B cells in an isotype-specific fashion. Human splenocytes or purified B cells were incubated with increasing amounts of VAF347 together with interleukin 4 (IL-4) and anti-CD40 monoclonal antibody (mAb) for 9 d to induce IgE production. IgG1 synthesis was induced in parallel cultures by stimulation with IL-10 and anti-CD40 mAb. IgE and IgG1 levels were measured in the cell supernatants by isotype-specific ELISA. One representative experiment of more than 10 is shown.

T-cell cytokine production in the supernatants of DC-rechallenged T cells and found profound inhibition of IL-2 when VAF347 was added during DC generation and T-cell priming (Figure 2B). VAF347 did not directly act on T cells because T-cell proliferation and cytokine production induced in the absence of DCs by antibodies to CD3 and CD28 were not affected (Figure 2C and data not shown).

One mechanistic explanation for the biological effects of VAF347 on DC-mediated T-cell proliferation could be inhibition of antigen uptake by the DCs, a prerequisite for antigen presentation and subsequent T-cell activation. To test for this possibility, iDCs generated in the absence or presence of VAF347 were incubated with FITC-OVA for various time periods. Uptake of antigen was measured as the increase in fluorescence intensity of the cells with time. To control for nonspecific binding of FITC-OVA to the cells, parallel cultures were incubated on ice. Data showed that the fluorescence intensity of cells kept at 37°C strongly increased at 60 min, indicating efficient uptake of the protein, whereas the fluorescence intensity of cells incubated on ice did not increase with time and likely reflects nonspecific binding (Figure 3). VAF347 did not inhibit FITC-OVA uptake at both time points at 37°C, suggesting that a defect in antigen uptake could not account for the immunomodulatory phenotype induced by the compound. Because DCs activate

T cells by a combination of cell contact-dependent signals and secreted products, the expression of selected cell surface receptors was measured on iDCs generated in the presence of increasing concentrations of VAF347. These cells are positive for HLA-DR; express intermediate levels of CD40, CD80, and CD86; and have lost the monocyte marker CD14. Table 2 demonstrates that the compound potentially inhibited the expression of CD86 and HLA-DR in a dose-dependent fashion, whereas CD80 and CD40 expression was much less affected. CD14 was not expressed even in the presence of VAF347 (data not shown). It should be noted that the degree of DC surface marker inhibition was donor cell dependent. In general, in about 75% of the donors the biological effect of the compound on HLA-DR and CD86 could be demonstrated and the degree of inhibition never was higher than 60%. Nevertheless, these data showed that VAF347 does not block monocyte-derived DC differentiation in general but preferentially modulates the surface expression levels of CD86 and HLA-DR in most of the donors tested.

To determine whether VAF347 also affected the synthesis of soluble products by mature DCs, iDCs were cultured with a cocktail containing GM-CSF, TNF- α , IFN- γ , and anti-CD40 antibodies for 48 h to induce DC maturation. Supernatants were analyzed for IL-6 and the chemokines MDC and TARC. All three factors are known to be induced in mature DCs. VAF347 potentially inhibited the production of IL-6 in a dose-dependent fashion with an IC₅₀ of 1 nM (Figure 4), which is close to the concentration needed to block DC surface marker expression and IgE secretion by B cells. In contrast, the secretion of MDC and TARC was not affected by the compound. The specific down-regulation of IL-6 demonstrated that VAF347 did not act as general inhibitor of DC maturation and suggested that a specific signaling pathway(s) induced by the maturation cocktail was targeted by the compound.

Collectively, these data strongly suggested that the inability of DCs to induce T-cell responses may be due to reduced expression of key molecules by DCs involved in T-cell activation, such as CD86, HLA-DR, and IL-6 (6, 24).

TABLE 1. ABSENCE OF EFFECT OF VAF347 ON INTERLEUKIN-4-INDUCED CD23 EXPRESSION ON HUMAN B CELLS

IL-4 and Anti-CD40 mAb	VAF347 (nM)	CD23
-	-	4 (40)
+	-	61 (165)
+	0.1	63 (155)
+	1	63 (161)
+	10	64 (164)
+	100	63 (175)

Definition of abbreviations: IL = interleukin; mAb = monoclonal antibody.
Human splenic B cells were induced with IL-4 (50 ng/ml) and anti-CD40 antibodies (500 ng/ml) for 24 h in the absence or presence of VAF347. Expression levels of CD23 were quantitated by FACS. Data are shown as the percentage of receptor-positive cells relative to the isotype control staining. In parentheses, the mean fluorescence index is shown.

VAF347 Inhibits Allergic Lung Inflammation and Serum IgE *In Vivo*

Because DCs and IgE are key players in allergic inflammatory reactions *in vivo*, VAF347 was tested for biological activity in an

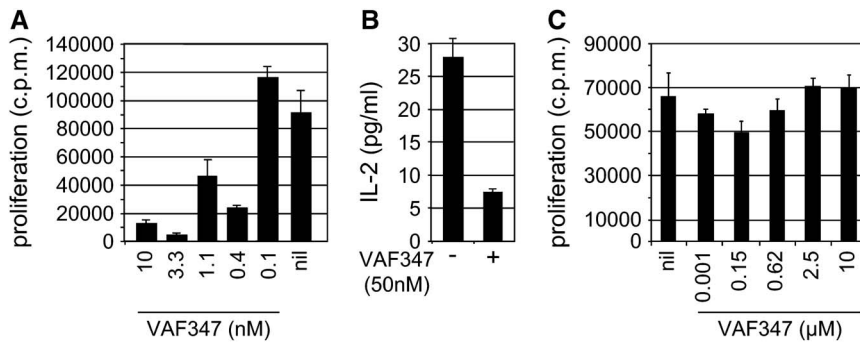


Figure 2. VAF347 inhibits dendritic cell (DC)-mediated autologous T-cell proliferation and cytokine production. T-cell proliferation, induced in a DC-independent fashion, is not affected by the compound. (A) Immature monocyte-derived DCs were pulsed with keyhole limpet hemocyanin (KLH) and subsequently cocultured for 10 d with autologous CD4⁺ T cells. The primed T cells were restimulated with fresh antigen-pulsed DCs and T-cell proliferation was measured on Day 4. VAF347 was present during the first KLH pulse and during T-cell priming. (B) IL-2 was measured in supernatants from restimulated T cells. (C) Purified T cells were stimulated with antibodies against CD3 and CD28 in the presence of increasing VAF347 concentrations and cell proliferation was assessed after 72 h. One representative experiment of three is shown. The error bars indicate the standard deviation of values from six replicates.

antigen-induced mouse model of eosinophilic lung inflammation. Immunization of mice with OVA in Al(OH)₃ followed by challenge with aerosolized OVA induces allergic airway disease, characterized by lung eosinophilia, goblet-cell hyperplasia, and elevated serum IgE levels. Oral treatment of mice with VAF347 (30 mg/kg) twice daily on Days 0–7 led to a strong reduction of total and specific (data not shown) serum IgE levels compared with vehicle-treated animals (Figure 5). For comparison, suplatast tosilate was included in these experiments. This compound, which has been used since 1995 to treat patients with asthma, is considered the most relevant mechanistic comparator based on its proposed mode of action as inhibitor of T-cell-derived IL-4 and IL-6 production (25), resulting in a block of IgE synthesis and pulmonary inflammation *in vivo* (26), and its blocking effect on

CD86 on mouse splenocytes derived from antigen-challenged animals (27). The data demonstrated that VAF347 was superior to suplatast to inhibit serum IgE (Figure 5), with suplatast showing a clear trend of IgE inhibition without reaching statistical significance. Enumeration of the infiltrating cells in the bronchoalveolar fluid 48 h after aerosol challenge revealed a marked reduction of eosinophils in VAF347-treated mice compared with control animals. A similar inhibition of eosinophil influx was observed in lung tissue. Remarkably, also the extent of mucus production by goblet cells was dramatically reduced in VAF347-treated animals (Figure 5). Thus, the major hallmarks of allergic lung inflammation are inhibited by VAF347 in this experimental setting. Interestingly, when VAF347 was applied between Days 10 and 13, no reduction in lung eosinophilia was recorded (Figure 6).

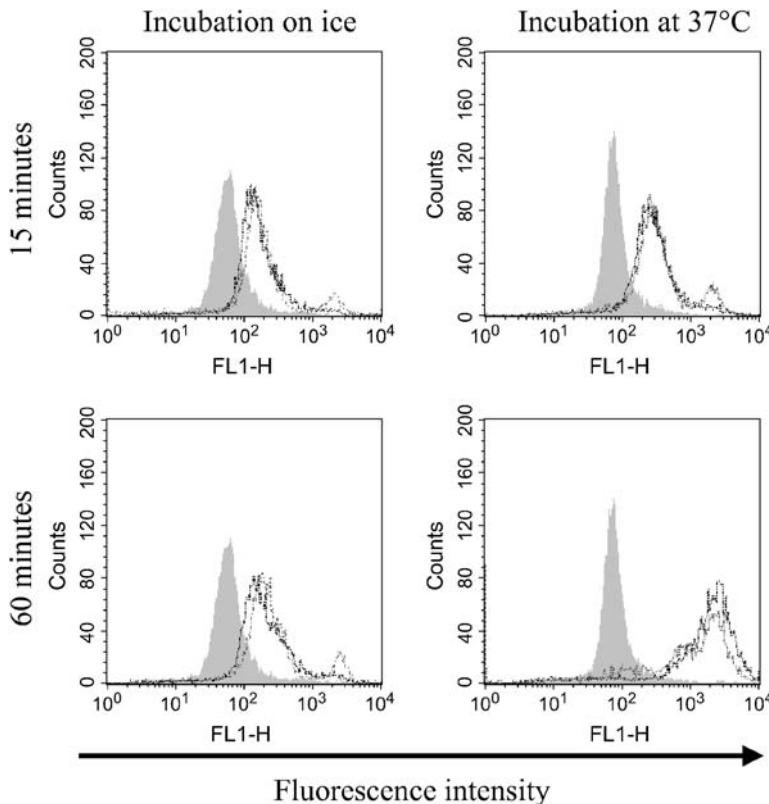


Figure 3. VAF347 does not influence antigen uptake by DCs. Immature DCs were incubated with fluorescein isothiocyanate-conjugated ovalbumin (FITC-OVA) for the indicated times on ice or at 37°C in the absence (gray line) or presence of 50 nM VAF347 (black line). Control (gray shaded area) = fluorescence intensity of cultures measured immediately after addition of FITC-OVA. One representative experiment of three is shown.

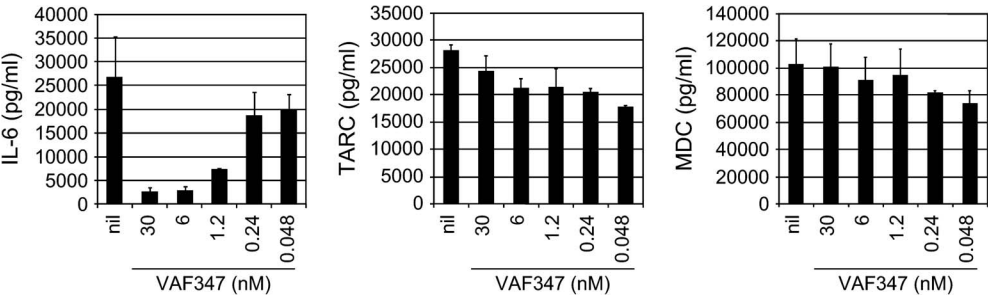


Figure 4. VAF347 inhibits IL-6, but not thymus- and activation-regulated chemokine (TARC) or macrophage-derived chemokine (MDC), expression by maturing DCs. Immature DCs, generated in the presence of increasing concentrations of VAF347, were activated with a cocktail containing granulocyte-macrophage colony-stimulating factor, IFN- γ , tumor necrosis factor- α , anti-CD40 mAb, and goat anti-mouse IgG to cross-link the anti-CD40 mAb. Levels of the soluble factors were measured in the supernatants by ELISA. One representative experiment of four is shown. The error bars indicate the standard deviation of values from four replicates.

Similarly, no effect on serum IgE was measurable. Identical results were obtained when suplatast tosilate was given (Figure 6). These data suggested that VAF347, and also suplatast tosilate, blocked events occurring early during the anti-OVA immune response in this model.

Biological Effects of VAF347 *In Vivo* Are Likely Due to DC Immunomodulation

To explore the relative importance of DCs and B cells for the biological effects of VAF347 in the eosinophilic inflammation model, allergic lung inflammation was induced in B-cell-deficient mice (28) by the same protocol as in wild-type mice. Enumeration of eosinophils in lung tissue 2 d after OVA aerosol challenge demonstrated that VAF347 inhibited eosinophilia in B-cell-deficient animals as potently as in wild-type mice (Figure 7). Similar to the situation in wild-type mice, suplatast tosilate was inferior to VAF347 in blocking eosinophilia. These results demonstrated that B cells and IgE played no major role in eliciting the inflammatory response in this acute inflammation model, in line with previous observations (29). Consequently, the anti-inflammatory activity of VAF347 was likely due to the immunomodulatory effect of the compound on DCs.

DISCUSSION

This study describes the biological profile of a novel immunomodulatory low molecular weight compound with antiinflammatory properties based on a dual mode of action. VAF347 acted as potent inhibitor of IL-4/IL-13 and anti-CD40 antibody-induced IgE class switching in human B cells. The inhibitory effect in

B cells appeared to be isotype specific because IL-10 and CD40-triggered IgG1 synthesis was not affected. The isotype-specific profile is underscored by data demonstrating that IgA class switching, induced by addition of transforming growth factor- β_1 (30), was also not inhibited by the compound (data not shown). The process of IgE isotype class switching can roughly be divided into two consecutive steps (19, 31). Within a few hours, IL-4 induces activation of the IgE germ-line gene promoter and transcription through the IgE constant region gene segments (C ϵ). Coengagement of the CD40 receptor synergizes with IL-4 but has no activation potential on its own. It is believed that transcription through C ϵ makes the DNA accessible for subsequent DNA recombination and targets the recombination machinery to this locus. IL-4/CD40 stimulation also leads to activation of the recombination machinery, in particular the gene encoding activation-induced cytidine deaminase. In a second step, DNA recombination brings the IgE C ϵ gene in close proximity to the preassembled VDJ $_H$ gene segments, thus allowing for transcription and translation of a fully functional IgE molecule. VAF347 may interfere anywhere during this process or may even inhibit IgE protein processing and/or secretion subsequent to class switching. Experiments are underway to pinpoint the mode of action of VAF347 in human B cells.

Importantly, DCs were shown to represent cellular targets for VAF347. The compound potently inhibited DC-mediated T-cell proliferation and cytokine production in the low nanomolar range. The compound effect was not due to impaired antigen uptake by DCs but appeared to be caused by a failure of the DCs to deliver appropriate activation signals to the T cells because the expression of HLA-DR and CD86 on the DC cell surface was at least partially inhibited by VAF347. Both molecules are known to play important roles in productive T-cell activation (7). In addition, DC-derived IL-6 was also strongly inhibited by the compound. An article has described IL-6 as an important cytokine facilitating T-cell activation by rendering T-effector cells refractory to the suppressive activity of T-regulatory cells (24). Thus, inhibition of DC-derived IL-6 by VAF347 during the initiation of T-effector cell activation could have contributed to the observed block in T-cell proliferation. Although the inhibitory effect on CD86 or HLA-DR alone appears unlikely to account for the potent block in DC-mediated T-cell proliferation, it is possible that the combined effects of reduced DC/T-cell contact via HLA-DR and CD86 and lacking resistance toward suppressive T-regulatory cells via decreased IL-6 may account for the overall inhibition of T-cell proliferation and cytokine

TABLE 2. INHIBITION OF DENDRITIC CELL SURFACE MARKERS BY VAF347

VAF347 (nM)	Geometric Mean			
	HLA-DR	CD86	CD80	CD40
0	554.4	21.4	25.9	51.94
1	338.4	10.3	19.4	47.56
10	278.4	10.2	23.1	58.18
100	313.9	10.2	21.6	53.49
1,000	217.7	9.8	21.1	46.19

Immature dendritic cells were generated in the presence of increasing concentrations of VAF347. Expression of the indicated cell surface markers was measured by FACS analysis. Numbers indicate the geometric mean of gated receptor-positive cells from a representative experiment.

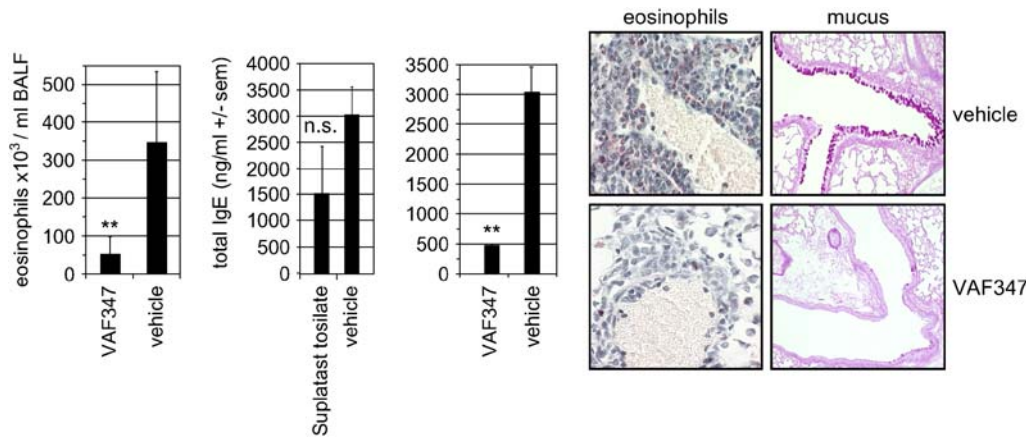


Figure 5. VAF347 inhibits allergic inflammation *in vivo*. OVA-immunized mice were treated orally with VAF347 or suplatast tosilate between Days 0 and 7. Forty-eight hours after challenge with aerosolized OVA, eosinophils were enumerated in the bronchoalveolar fluid (BALF) or in lung tissue. Total IgE was measured in serum by ELISA. Mucus-producing cells were stained in lung sections. ** $p < 0.05$; n.s. = not significant. One representative experiment of three is shown.

production by VAF347. However, at this point other as yet unknown factors cannot be ruled out.

VAF347 inhibited total and antigen-specific IgE synthesis in a mouse model of antigen-induced eosinophilic lung inflammation *in vivo*, thus validating the biological profile of the compound *in vitro*. Induction of IgE in this disease model is initiated by DCs via generation of IL-4-producing Th2 cells. On the basis of the biological profile of VAF347 on B cells and DCs *in vitro*, blockade of serum IgE in this model could be due to the direct inhibitory action of VAF347 on B cells or could be an indirect consequence of diminished Th2 cell generation/function due to the immunomodulatory effect of the compound on DCs or a combined effect on both cell types. None of these possibilities can be ruled out at present.

More importantly, VAF347 blocked the hallmarks of allergic lung inflammation in this mouse model. This effect was likely due to the activity of VAF347 on DCs because the compound blocked allergic lung inflammation in B-cell-deficient mice. Obviously, B cells and IgE had no major role for initiation of the disease in the model used in this study. Support for this notion comes from data demonstrating that the requirement for IgE in the development of lung eosinophilia on antigen challenge is dependent on the experimental setup of the model (29). The experimental protocol in our experiments did not support a role for IgE in facilitating lung inflammation, thus corroborating the data obtained in B-cell-deficient mice.

VAF347 inhibited serum IgE and eosinophil influx when given during antigen sensitization but not when applied at the time of antigen challenge. This treatment scheme resembles that used in patients with allergies, raising questions concerning whether VAF347 will have a therapeutic effect on patients with established asthma. Human asthma is characterized as a chronic inflammatory disease of the lungs, in which the degree of the inflammatory state varies depending on the exacerbation frequency. The animal model used here cannot be considered a true model of human asthma because (1) the antigen-induced lung inflammation does not reflect the chronic inflammatory condition in human patients with asthma and (2) IgE does not play a role in the inflammatory condition in the model. This is different from the situation in human patients with asthma, in whom IgE obviously plays a major role based on positive clinical data with the nonanaphylactogenic anti-IgE antibody omalizumab (32); and (3) suplatast tosilate presents a discrepancy, in its lack of biological activity in this model when given during antigen challenge and its proven clinical effects in human patients with asthma (33). Taken together, only a carefully planned

proof-of-concept study in human patients with asthma will answer questions concerning the therapeutic potential of VAF347.

It should be noted that CD86 was implicated in skewing T-cell activation toward the generation of Th2 cells, which are known to drive allergic inflammatory reactions (34, 35), and that blockade of CD86 function with neutralizing antibodies resulted in reduced allergic inflammation (36, 37), although these findings are inconsistent (38). It is tempting to speculate that the specificity of VAF347 to inhibit CD86 expression on DCs over other costimulatory molecules (e.g., CD80) may contribute to the observed biological activity *in vivo*. It will be interesting to explore whether the compound will have biological activity in Th1-cell-driven inflammatory responses *in vivo*.

The existence of two VAF347-sensitive cell types raises a question concerning whether the compound targets the same molecule(s) in DCs and B cells to produce its biological effects. Support for the same or similar target(s) comes from the fact that the potency of the compound is virtually identical in both cell types. In addition, IL-4 is an important factor to direct B cells toward IgE secretion and to drive monocyte differentiation into DCs. This raised the possibility that VAF347 acts as general IL-4 antagonist. This appears not to be the case because CD23 surface expression, induced by IL-4 on primary B cells, was not affected by VAF347 even at high concentrations. IL-4 can signal via two different signal transduction cascades, one

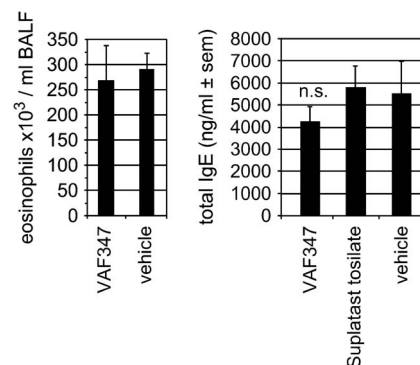


Figure 6. VAF347 acts during antigen sensitization. OVA-immunized mice were treated orally with VAF347 or suplatast tosilate between Days 10 and 13. Forty-eight hours after challenge with aerosolized OVA, eosinophils were enumerated in the BALF and total serum IgE was measured by ELISA. One representative experiment of three is shown.

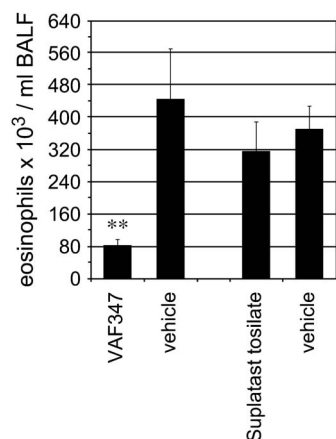


Figure 7. VAF347 inhibits lung eosinophilia in B-cell-deficient mice. OVA-immunized B-cell knockout mice were treated orally with VAF347 or suplatast tosilate between Days 0 and 7. Forty-eight hours after challenge with aerosolized OVA, eosinophils were enumerated in the BALF. **p < 0.05. One representative experiment of two is shown.

driven by STAT6 (signal transduction and activator of transcription-6), the other one by insulin receptor substrate proteins (39). IL-4-induced CD23 expression is known to be dependent on STAT6 (40). Thus, this pathway is considered less likely to be the target of VAF347. One additional common signal transducing molecule shared in the two cell types is nuclear factor- κ B (NF- κ B). It is known that CD40, GM-CSF, and TNF- α all use NF- κ B as signaling intermediate. Therefore, interference with this pathway by VAF347 must be considered a possibility. However, such a scenario cannot explain the fact that IL-10/anti-CD40-induced IgG1 production by human B cells is not affected by the compound. In addition, expression of the chemokines MDC and TARC is under the control of NF- κ B proteins (41) but are not affected by the compound. Therefore, similar to the situation with IL-4, a general blockade of NF- κ B signaling by VAF347 appears unlikely. The mode of action of the compound in both cell types is currently under investigation and a search for the molecular target is underway.

In summary, we have identified a novel low molecular weight immunomodulator that acts by a dual mode of action: first, inhibition of DC-mediated T-cell proliferation and cytokine production. *In vivo*, this translated into abrogation of the inflammatory cascade as evidenced by inhibition of lung eosinophilia, goblet-cell hyperplasia, and serum IgE. Because these events are driven by Th2 cells, one can argue that their function was blocked as a result of improper activation/differentiation by VAF347-immunomodulated DCs. Second, isotype-specific inhibition of IgE class switching by human B lymphocytes. To our knowledge this is the first description of a small molecule with such a biological profile. Because initial toxicity studies in rodents look promising, VAF347 is expected to have therapeutic benefits in the clinic for patients suffering from allergic diseases such as asthma, rhinitis, or atopic dermatitis.

Conflict of Interest Statement: P.E. is an employee of Novartis Pharma research. P.M. is an employee of Novartis Pharma. F.K. is an employee of Novartis Pharma Research for more than 3 yr. W.N. is an employee of Novartis Pharma. N.H. is an employee of Novartis Pharma. G.H. is an employee of Novartis Pharma. M.M.E. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. V.B. is an employee of Novartis Pharma. C.H. is fully employed by Novartis Pharma AG, Switzerland, as a research scientist and manager, and does not receive any other financial compensation (from any sources) except salary from Novartis Pharma AG. M.W. is an employee of Novartis Pharma.

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