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Human Bronchial Epithelium Controls TH2 Responses by TH1-Induced, Nitric Oxide-Mediated STAT5 Dephosphorylation: Implications for the Pathogenesis of Asthma

Urs Eriksson, Ulrich Egermann, Michel P. Bihl, Franco Gambazzi, Michael Tamm, Patrick G. Holt, and Roland M. Bingisser

Increased levels of NO in exhaled air in association with increased NO synthetase (NOS)2 expression in bronchial epithelial are hallmark features of asthma. It has been suggested that NO contributes to asthma pathogenesis by selective down-regulation of TH1 responses. We demonstrate, however, that NO can reversibly limit in vitro expansion of both human TH1 and TH2 CD4+ T cells. Mechanistically, NO induces cGMP-mediated reversible STAT5 dephosphorylation and therefore interferes with the IL-2R activation cascade. Human bronchial epithelial cells (HBEC) up-regulate NOS2 after stimulation with IFN-γ secreted by TH1 CD4+ T cells and release NO, which inhibits both TH1 and TH2 cell proliferation. This reversible T cell growth arrest depends on NO because T cell proliferation is completely restored after in vitro blocking of NOS2 on HBEC. HBEC thus drive the effector end of a TH1-controlled feedback loop, which protects airway mucosal tissues at the potential lesional site in asthma from overwhelming CD4+ TH2 (and potentially TH1) responses following allergen exposure. Variations in the efficiency of this feedback loop provides a plausible mechanism to explain why only a subset of atopics sensitized to ubiquitous aeroallergens progress to expression of clinically relevant levels of airways inflammation.

Materials and Methods

Generation of human TH1 and TH2 cell clones

PBL were isolated by Ficoll gradient centrifugation (Histopaque 1077; Sigma-Aldrich) from healthy donors and maintained in RPMI 1640, 2 mM l-glutamine, 1 mM sodium pyruvate, 20 mM HEPES, 100 U/ml rIL-2 (Hoffmann-LaRoche), antibiotics, and 5% HSA. PBL were either irradiated and used as feeder cells or monoclonally expanded in Terasaki plates following standardized cloning techniques. TH1 and TH2 T cell clones were identified after nonspecific activation using intracellular staining for IL-4 and IFN-γ and FACS analysis (Cytofix/Cytoperm kit; BD Biosciences). Alternatively, cytokines were measured in cell culture supernatants using commercially available ELISA kits (Quantikine; R&D Systems).

Human bronchial epithelial cells (HBEC)

HBEC were derived from specimens of patients undergoing resection of lung cancer. Many of these patients were smokers. Samples were transferred in RPMI 1640, 5% FCS, 20 mM HEPES, 2 mM l-glutamine medium and were kept in air-liquid interfaces with minimal medium at 37°C in a humidified 5% CO2 incubator. Cells were used for cocultures with T cells after achieving 90% of confluence, which was usually the case after 2 wk. For coculture experiments, additional medium was added and 5 × 10^5 T cells were used per 25-cm² tissue culture flask.

Functional T cell analysis

Cells were washed and kept in IL-2-free medium overnight. Cultures were then stimulated by addition of 50 U/ml IL-2 before preparation of cytotoxic...
and nuclear extracts. Depending on the experiment, cells were incubated with 5-nitroso-N-acetylenepicillamine (SNAP), N°-monomethyl-L-arginine (NMMA), 8-bromo-cGMP, and 8-bromo-cAMP (all Calbiochem), or 1 mM orthovanadate (Sigma-Aldrich) (10).

**NOS2 and IFN-γ RT-PCR**

Total RNA was extracted from HBEC and T cells using the RNeasy mini kit. Synthesis of first-strand cDNA was performed with 1 μg of RNA and 200 U of MMLV Reverse Transcriptase RNase (Promega) before amplification. The following primers were used; NOS2, sense 5'-GGGCCTCGGCTCCACGATGTCAC-3', antisense 5'-TGCGGGTCCCAGGTCACATTC-3'; IFN-γ, sense 5'-GTGAGGAGTATACCTATATCATCGGCTTT-3', antisense 5'-GATGCTTCGACCTCAGGTAGCAT-3'; and β-actin, sense 5'-GTACGGTTGCTATCCAGGCCTGGC-3', antisense 5'-CTCTTCTGATGTCCGAGGACT-3'.

**EMSA**

T cells were incubated for 10 min on ice in low-salt buffer (20 mM HEPES, 10 mM KCl, 0.1 mM NaVO4, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 0.2% Nonidet P-40, pH 7.9), centrifugated, and supernatants taken as cytosolic extracts. Pellets were incubated for 30 min on ice in high-salt buffer (420 mM NaCl, 20 mM HEPES, 10 mM KCl, 0.1 mM NaVO4, 1 mM EDTA, 1 mM EGTA, 20% glycerol, pH 7.9) and centrifugated to obtain nuclear extracts. A total of 2 μg of the nuclear extracts were incubated with 1 ng of [α-32P]dGTP and [α-32P]dCTP labeled oligonucleotide probes (βCAS, 5'-GATTCTCTAGGAATTCAATCC-3') in 20 mM HEPES, 4% Ficoll, 1 mM MgCl2, 40 mM KCl, 0.1 mM EGTA, 0.5 mM DTT, pH 7.9, for 20 min at room temperature. A total of 1 μg of poly(dI-dC)-poly(dI-dC) (Pharmacia Biotech) was added as a nonspecific DNA competitor. To test the specificity of formed complexes we added 1 μl of either anti-STAT5a or anti-STAT5b (Santa Cruz Biotechnology) to the gel-shift binding reactions. Reaction products were displayed using a Molecular Dynamics PhosphorImager (Amersham Biosciences) after a 4% PAGE.

**Proliferation assays, metabolic activity of T cells, and NO production**

T cell proliferation was measured by [3H]thymidine incorporation. In parallel, T cells were taken for assessment of their metabolic activity by measurement of the tetrazolium salt reduction of the tetrazolium salt XTT as readout (Cell Proliferation kit II; Roche Diagnostics). Nitrite levels reflecting NO production were measured using a colorimetric assay.

**Statistics**

Dichotomous data were analyzed using Fisher's exact test. Proliferation responses and cytokine levels were compared using ANOVA and the t test. A value of \( p < 0.05 \) was considered significant.

**Results**

**NO inhibits proliferation of TH1 and TH2 CD4⁺ T cells**

We have recently shown that NO induces a reversible growth arrest of activated murine T cells (10, 11). To test the hypothesis that NO also reversibly limits human CD4⁺ T cell expansion, we generated TH1 and TH2 CD4⁺ T cell clones (9) from healthy volunteers and assessed T cell growth in the presence or absence of various concentrations of the NO donor SNAP in vitro.

As illustrated in Fig. 1A, NO dose-dependently inhibited both TH1 (Fig. 1A, blue) and TH2 (Fig. 1A, red) CD4⁺ T cell growth to the same extent. This effect was not due to NO-mediated dose-dependent induction of apoptosis because the overall proportion of apoptotic and dead T cells was very low in our cell culture system and remained constant within the dose range of the NO donor SNAP (data not shown). Most importantly, NO had no effect on the viability and mitochondrial activity because T cell cultures showed unaltered reduction of the tetrazolium salt XTT despite the presence of NO (Fig. 1B). Furthermore, NO-mediated T cell growth arrest was fully reversible (Fig. 1C).

**Figure 1.** NO dose-dependent inhibition of TH1 and TH2 CD4⁺ T cell growth. A, NO induces reversible growth arrest of TH1 and TH2 CD4⁺ T cells. T cells were cultured with 50 U/ml rIL-2, before measurement of [3H]thymidine incorporation. Proliferative responses are expressed as percentages compared with an average standard value representing proliferation in the absence of the NO donor SNAP. B, Preserved mitochondrial enzymatic activity of CD4⁺ T cells (XTT activity, blue bar) despite NO-mediated inhibition of proliferation ( ). Each value corresponds to a percentage of proliferation or XTT activity ± SD of five culture wells relative to an average standard value representing proliferation in the absence of the NO donor SNAP. One of several experiments with similar results is shown. C, Reversibility of NO-mediated suppression of T cell proliferation. T cells were grown with 50 U/ml IL-2 without ( ) or with (triangles) 500 μM NO donor SNAP. After 16 h, all T cells were transferred into fresh culture wells. SNAP pretreated T cells (triangles) were further grown in either SNAP free medium (upward triangle) or again in medium containing 500 μM SNAP (downward triangle). Thymidine uptake was assessed daily. Each CPM value corresponds to 5 culture wells ± SD. One of several experiments with different TH1 and TH2 clones is shown.

Taken together, these findings suggest that NO provides downregulatory signals to reversibly limit the in vitro proliferation of activated TH1 and TH2 CD4⁺ T cells.

**NO induces cGMP-mediated STAT5 dephosphorylation in activated Th cells**

T cell proliferation largely depends on the activation of a downstream IL-2R signaling cascade that involves JAK1- and JAK3-mediated STAT5 phosphorylation (12–16). Interestingly, we have
previously shown that NO inhibits JAK3 and STAT5 phosphorylation and reversibly disrupts T cell activation in mice (10).

To address the mechanisms mediating NO-induced reversible T cell growth arrest of activated human TH1 and TH2 clones, we first assessed STAT5 phosphorylation in the presence or absence of various concentrations of the NO donor SNAP using EMSA. As illustrated in Fig. 2A, phosphorylated STAT5 accumulated after only 30 min in stimulated human TH1 and TH2 T cells. In the presence of the NO donor SNAP, however, STAT5 becomes dose-dependently dephosphorylated. This effect was obvious within 4 h and even more pronounced after 24 h of ongoing NO exposure (Fig. 2A).

Furthermore, NO-mediated STAT5 dephosphorylation in activated human T cells could, at least partly, be prevented by the addition of the phosphatase inhibitor orthovanadate to cell culture wells (Fig. 2B). Of note, the reduced availability of phosphorylated STAT5 in activated human T cells in the presence of NO is not only explained by active dephosphorylation, but is also due to reduced STAT5 rephosphorylation (data not shown).

Given the active role of phosphatases mediating NO-induced STAT5 dephosphorylation in activated T cells, and the observed lag time between NO exposure and dephosphorylation, we hypothesized that second messengers such as cAMP or cGMP might play a critical role in the signaling cascade (10). Indeed, the cell-diffusible cGMP analogue bromo-cGMP dose-dependently promoted STAT5 dephosphorylation and simultaneously impaired the proliferation of activated T cells (Fig. 2C). In contrast, bromo-cAMP mediated neither STAT5 dephosphorylation nor T cell growth arrest.

Taken together, our findings suggest that NO limits the proliferation of activated human TH1 and TH2 helper cells by interference with STAT5, a crucial transcription factor in the IL-2R cascade. Mechanistically, we found that these effects depended on NO-induced cGMP-mediated activation of phosphatases.

**Activated TH1 cells up-regulate NOS2 on HBEC**

Inflammation of airways in patients with asthma is associated with release of NO in exhaled air (1, 4, 5). In fact, bronchial epithelial cells had been recognized as the quantitatively most important source of NO in the human airways (17, 18). Given the fact that NO suppresses the proliferation of TH2 helper cells, we hypothesized that HBEC might confine the local expansion of activated T cells in a setting of allergic airway inflammation. To test this hypothesis, we assessed NOS2 expression in cocultures of HBEC and human TH2 cells. As shown in Fig. 3A, activation with PHA did not up-regulate NOS2 mRNA in HBEC in the absence of T cells (Fig. 3A, lane a). Furthermore, HBEC did not express NOS2, if cocultured with nonstimulated TH1 or TH2 T cells (data not shown). In contrast, PHA activation of TH1 helper cells in HBEC T cell cocultures resulted in marked NOS2 mRNA expression in HBEC.
and significant NO release into the culture supernatants (Fig. 3A, lane b).

Given our findings that activated TH1 T cells but not TH2 T cells induce NOS2 in HBEC, we first asked whether this effect is related to direct interactions between T cells and HBEC. However, this is not the case, because the addition of cell-free supernatant from PHA-stimulated TH1 T cells also induced NOS2 expression in HBEC (Fig. 3A, lane c). Thus, activation of TH1 T cells induces NOS2 expression and NO release in cocultured HBEC. Importantly, we found that in our coculture system, the release of NO from HBEC parallels a marked reduction in T cell proliferation. Fig. 3B shows this effect indeed depended on NOS2 activity, because the addition of the NOS2 inhibitor NMMA (10) to cocultures restored T cell proliferation and STAT5 phosphorylation (Fig. 3B, upper and lower panels, respectively).

Taken together, these findings suggest that activated TH1 but not TH2 cells induce NOS2 expression and NO release on HBEC. NOS2 induction and release of NO directly accounts for the reversible growth arrest of Th cells in the HBEC-TH1 T cell coculture system.

IFN-γ mediates NOS2 induction in HBEC

Given our findings that activated TH1 cells but not TH2 cells induced cell contact-independent NOS2 expression in HBEC, we next hypothesized that soluble mediators, most likely TH1 cytokines, might be responsible for NOS2 induction and subsequent NO release from HBEC. Indeed, stimulation of bronchial epithelial cells with recombinant human IFN-γ but not recombinant human TNF-α resulted in up-regulation of NOS2 mRNA and accumulation of nitrite/nitrate in cell culture supernatants (data not shown).

To assess more specifically the role of IFN-γ-mediated NO release from HBEC on the proliferation of activated TH1 and TH2 helper cell clones, we cocultured TH1 or TH2 clones together with HBEC in the presence or absence of rIFN-γ and an IFN-γ blocking Ab. As shown in Fig. 4, proliferation of TH2 clones was still markedly increased compared with TH1 clones after PHA activation in the presence of HBEC. Addition of IFN-γ, however, clearly suppressed the proliferative response of activated TH2 cell clones, suggesting that IFN-γ is involved in HBEC-mediated suppression of T cell responses. In accordance with the decreased proliferation, no NO2/NO3 representing NO release in the medium was detectable. Addition of the NOS2 inhibitor NMMA to PHA-activated TH2 cell clones, however, prevented the IFN-γ-induced suppression of proliferative responses (data not shown). To finally prove that IFN-γ is crucial in HBEC-mediated growth arrest of Th cells, we cocultured activated T cell and HBEC in the presence of an IFN-γ blocking Ab. As illustrated in Fig. 4, the presence of the IFN-γ blocking Ab restored proliferative responses of activated TH1 helper cells in the presence of HBEC. Restoration of proliferative responses was associated with impaired NO release in to the cell culture supernatant (Fig. 4).

Taken together, our findings clearly suggest that IFN-γ from activated TH1 cells but not TH2 cells mediates NO production in HBEC and therefore reversible feedback suppression of TH1 and TH2 cell growth.

Discussion

We describe in this study that NO reversibly suppresses the proliferation of activated human TH1 and TH2 cells. Furthermore, HBEC, the most important source of NO in the airways (17, 18), up-regulates NOS2 expression in the presence of activated TH1 but not TH2 cells. This effect is independent from cell-cell contact and largely mediated through the TH1 cytokine IFN-γ.

Our findings provide in vitro evidence that HBEC are part of an NO-mediated anti-inflammatory mechanism triggered by IFN-γ released from TH1 cells (Fig. 5). This idea challenges the widely accepted concept that the role of NO in allergic asthma is restricted to shifting the TH1/TH2 balance toward a disease-promoting TH2 response (7, 8). Focusing on Th cells as important mediators of the adaptive allergic immune response, the marked NO production in the airways of patients with severe asthma may reflect the operation of a counterregulatory feedback mechanism that limits the expansion of TH1 and TH2 helper cells. Importantly, this mechanism requires the presence of activated IFN-γ-producing TH1 cells that mediate NOS2 induction and NO release from the airway epithelium. This mechanism might additionally be boosted in the presence of TH2 cells producing the cytokine IL-4, which has been shown to potentiate IFN-γ-triggered NOS2 induction (19). From this viewpoint, it appears possible that the lack of appropriate feedback via IFN-γ or IFN-γ-producing T cells, and not just the overproduction of TH2 cytokines like IL-4 alone, may determine progression of allergy per se to expression of clinically relevant levels of airway inflammation. In this context it is pertinent to note that only a relatively small subset of human subjects who are sensitized to common aeroallergens actually progress to the development of asthma (20), which indicates that mechanisms specific to the local

FIGURE 4. PHA-stimulated TH1 cells (blue bars) but not TH2 T cells show both a decrease in proliferation and detectable (+) nitrite levels reflecting NO release in a HBEC/T cell coculture. Addition of the NOS2 inhibitor NMMA increases proliferation rates of TH1 T cells in TH1 T cell/HBEC, but not in TH2 T cell/HBEC cocultures. NO is released and therefore no nitrite is detectable (−). The addition of IFN-γ to both PHA-stimulated TH1 and TH2 cells confines T cell proliferation. Addition of a blocking anti-IFN-γ Ab results in marked proliferation and no detectable nitrite levels (−) in cell cultures.

FIGURE 5. Proposed down-regulatory feedback loop confining TH1 and TH2 cell expansion in asthma. Activated TH1 T cells release IFN-γ that induces up-regulation of NOS2 in HBEC and NO release. NO conversely reversibly inhibits T cell proliferation.
tissue microenvironment, in addition to those associated with Th2 cytokine-dependent atopy, are necessary for focusing allergic disease specifically to the airway mucosa, and the mechanism in our study is a plausible candidate. It is also relevant to note that although IFN-γ production is a common component of allergen-specific CD4+ Th memory among atopics (21, 22), excessive levels of allergen-specific IFN-γ production in the airways can itself contribute to asthma pathogenesis (21, 23, 24), and hence feedback mechanisms are also required to ultimately limit sensitization of this cytokine within the airway epithelial microenvironment. The mechanism described in this study also fulfills this function, thus providing a robust mechanism for local control of the exuberance of all forms of local T cell responses.

In relation to asthma pathogenesis, NO has thus far generally been considered only as a proinflammatory free radical inhibiting tissue damage similar to superoxide (O2·−) anion (6). O2·− strongly reacts with NO to form peroxynitrite. Accordingly, inflamed airways from patients with asthma show increased tyrosine nitrosylation, and several clinical studies suggest that the severity of allergic asthma correlates positively with levels of NO released into the airways (1, 4, 5). However, the role of NO in immune-mediated diseases in general is controversial (7, 8, 11, 25). Information from a variety of experimental autoimmune disease models suggests that NO-dependent feedback mechanisms regulating T cell expansion are not limited alone to our human in vitro cell culture system. In mice, for example, NO has been suggested to limit autoreactive T cell expansion in autoimmune myocarditis (26), myasthenia gravis (27), and experimental allergic encephalomyelitis (28), and in a mouse model of pulmonary graft-vs-host disease (29). Strictly speaking, the fact that a reactive free radical like NO is protective against T cell-mediated immune responses does not contradict the widely accepted view that NO and other highly reactive radicals contribute to nonspecific tissue damage. Accordingly, mice lacking the NADPH oxidase, which are protected from experimental autoimmune encephalomyelitis (30). However, these mice still develop severe disease after adoptive transfer if NO production during ex vivo donor cell reactivation is inhibited. Exogenous inhibition of superoxide production, in contrast, enhanced NO activity (31). Thus it has been concluded that extracellular superoxide contributes to the development of autoimmune disease because it inhibits NO and not because it contributes to oxidative stress. Of note, physiological levels of NO inhibit T cell proliferation in vitro reversibly, without induction of apoptosis or affecting the capacity of the cells to produce cytokines (11, 32).

In conclusion, our study adds to a growing literature indicating a potential negative feedback role for NO in immunoinflammatory diseases. In the context of atopic asthma, this feedback loop could involve IFN-γ-releasing TH1 cells functioning in concert with HBEC, which serve as the major source of NO at the potential lesional site in asthma in the airway mucosa. Variations in the efficiency of this feedback loop might explain one of the central enigmas of asthma, which is why only a subset of atopics with Th2-mediated allergy to aeroallergens develop airway inflammation of sufficient intensity to provoke asthma, whereas the majority of their atopic counterparts with equivalent levels of TH2-associated immunity to the same aeroallergens, remain asthma free.

The perception of NO as an important anti-inflammatory rather than a disease-promoting mediator warrants a careful reassessment of current pathophysiological concepts and might have important implications for the development of novel treatment strategies against allergic asthma. Meanwhile, our data are a major step forward in understanding the regulatory mechanisms underlying inflammatory airway diseases.

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Disclosures

The authors have no financial conflict of interest.

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