

Cytokine Effects Induced by the Human Autoallergen α -NAC

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Autoallergy is a phenomenon found in a subgroup of patients with atopic dermatitis (AD). These patients exhibit serum IgE reactivity toward autoantigens like the alpha-chain of the nascent polypeptide-associated complex (α -NAC; Hom s 2). α -NAC has been shown before to induce T-cell proliferation and secretion of IFN- γ . To elucidate the immune modulating functions α -NAC may exert, we analyzed its effects on cytokine transcription and secretion in peripheral blood mononuclear cells (PBMCs), monocytes, and CD4+ T cells. Transcription and secretion of IFN- γ , IL-17, and IL-22 were increased in α -NAC-stimulated PBMCs. As IL-17 was significantly upregulated by α -NAC, we assessed signal transduction in PBMCs and found signal transducer and activator of transcription 3 phosphorylation in α -NAC-stimulated cells. Furthermore, we could show the importance of monocyte activation by α -NAC, as isolated T cells reacted only weakly toward the stimulation. Inhibition of IL-23 p19 led to lower amounts of IL-17 in the PBMC supernatants after α -NAC stimulation. α -NAC stimulation of PBMCs from non-allergic donors resulted in secretion of IL-10, which was greatly reduced in PBMCs from α -NAC-sensitized AD patients. Our findings provide insights into the mechanisms of autoallergy, investigating the interplay of immune cells, signaling events, and cytokines, which are known to be relevant in atopic skin inflammation.

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INTRODUCTION

The autoallergen Hom s 2 or α -NAC (alpha-chain of the nascent polypeptide-associated complex) belongs to a group of autoallergens without known cross-reactivity to exogenous allergens. These autoallergens (Hom s 1–5) have been identified by screening of a human epithelial complementary DNA library for IgE binding of sera from atopic dermatitis (AD) patients (Natter *et al.*, 1998; Valenta *et al.*, 1998). Besides its allergenic potential, α -NAC is an intracellular protein, which is involved in sorting of newly synthesized polypeptides (Wiedmann *et al.*, 1994), and acts as a transcriptional coactivator (Yotov *et al.*, 1998).

Mossabeh *et al.* (2002) already showed proliferation of peripheral blood mononuclear cells (PBMCs) stimulated with

α -NAC, which was higher in IgE-sensitized patients than in non-sensitized controls. This pointed to the involvement of α -NAC-reactive T cells. In addition, the T-cell cytokine IFN- γ is produced in high amounts by α -NAC-stimulated PBMCs, which has been shown to damage keratinocytes and could thereby lead to further impairment of the epithelial barrier and to worsening of skin inflammation (Mittermann *et al.*, 2008). Previously, we analyzed T-cell responses to α -NAC in IgE-sensitized AD patients (Heratizadeh *et al.*, 2011). We could show that α -NAC induced preferential proliferation in the cutaneous lymphocyte-associated antigen+, i.e., skin-homing, T-cell subsets from PBMCs. CD4+ T-cell clones were generated from blood and skin of the patients, which showed a predominant Th0 and Th1 cytokine profile, respectively, and some skin-derived clones also produced IL-17. From the blood, CD8+ T-cell clones could be generated and these produced mainly IFN- γ .

Although IFN- γ clearly has been shown to have a role in the chronic phase of AD, the situation is less conclusive for IL-17 and IL-22. IL-17 has been shown to be increased in acute AD lesions, whereas IL-22 seems to have a role in chronic AD lesions (Souwer *et al.*, 2010). Cutaneous sensitization through the impaired skin barrier typical of AD could influence the cytokine pattern, which has been shown recently for epicutaneous sensitization with ovalbumin in a mouse model (He *et al.*, 2007). In this model, epicutaneous sensitization favored the development of IL-17-secreting CD4+ T cells. As sensitization to autoallergens relevant in AD is most probably initiated in the skin, where intracellular antigens can be

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Abbreviations: AD, atopic dermatitis; α -NAC, the alpha-chain of the nascent polypeptide-associated complex; HSP, heat shock protein; PBMCs, peripheral blood mononuclear cells; STAT, signal transducer and activator of transcription; TLR, Toll-like receptor

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released from stressed or damaged cells (Schmid-Grendelmeier *et al.*, 2005; Balaji *et al.*, 2011), a similar mechanism could apply for those molecules. Furthermore, autoallergens seem to have intrinsic Th1-favoring properties that distinguish them from exogenous allergens (Aichberger *et al.*, 2005; Mittermann *et al.*, 2008).

In order to investigate the development and the possible consequences of α -NAC autoallergy, this study aimed to further analyze the cytokine response α -NAC elicits in mononuclear cells and the underlying signaling events. In PBMCs, transcription and secretion of T-cell cytokines was induced most efficiently by the combined stimulation with α -NAC and IL-2. This combination was also most effective in triggering signal transducer and activator of transcription 3 (STAT3) phosphorylation in PBMCs. We could also show monocyte dependency of T-cell cytokine induction in PBMCs. Finally, a significant difference was observed regarding IL-10 secretion in PBMCs from control donors and from α -NAC-sensitized AD patients, which could have a role in disease exacerbation in the sensitized patients.

RESULTS

α -NAC induces high amounts of IFN- γ , IL-17, and IL-22

Secretion of IFN- γ and IL-10 has been described for α -NAC-stimulated PBMCs (Mittermann *et al.*, 2008) and there are hints from T-cell clone analyses that α -NAC might promote Th17 responses (Heratizadeh *et al.*, 2011). Here, we stimulated PBMCs with α -NAC in the presence or absence of IL-2 and analyzed cytokines in supernatants and on mRNA expression level. α -NAC alone was not sufficient to upregulate

secretion of the T-cell cytokines IFN- γ and IL-22, but both cytokines were produced after the combined stimulation of PBMCs with α -NAC and IL-2 (Figure 1a). Stimulation with α -NAC alone led to significant secretion of IL-17, albeit in very low levels. Higher amounts of IL-17 were secreted by PBMCs after stimulation with α -NAC and IL-2. In addition, transcription of IFN- γ , IL-17, and IL-22 was upregulated in PBMCs (Figure 1b). Interestingly, transcription of IFN- γ and IL-17 was also induced by stimulation with α -NAC alone, but still less than by the combined stimulation with IL-2. In contrast, transcription of IL-17F was not upregulated by α -NAC (data not shown). As cytokine induction varied considerably between the donors, selected data of Figure 1 and the following figures are plotted in line graphs in Supplementary Figure S3 online.

Monocytes are important for induction of T-cell cytokines by α -NAC

We have recently shown that α -NAC activates monocytes and induces secretion of the proinflammatory cytokines IL-12 p40 and IL-6 in these cells (Hradetzky *et al.*, 2013). To investigate the importance of monocyte activation for the T-cell response, we stimulated PBMCs as well as isolated CD4⁺ T cells with α -NAC. Although the T-cell cytokines IL-17 and IL-22 were strongly induced by α -NAC in PBMCs (Figures 1 and 2a), isolated CD4⁺ T cells reacted only weakly to stimulation with α -NAC (Figure 2a).

Similarly, CD4⁺ T cells that were incubated with supernatants from monocytes stimulated with α -NAC secreted higher levels of IL-17 and IL-22 compared with directly

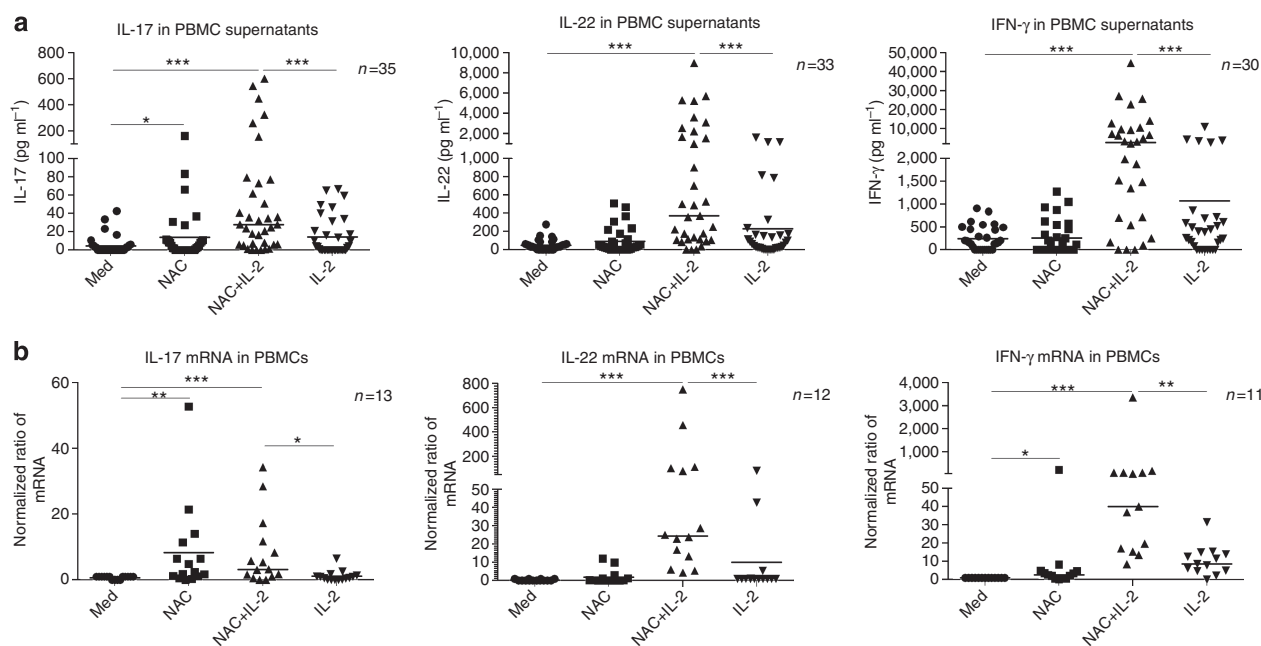


Figure 1. Alpha-chain of the nascent polypeptide-associated complex (α -NAC) induces secretion and transcription of T-cell cytokines. Peripheral blood mononuclear cells (PBMCs) from atopic dermatitis (AD) patients and control donors were stimulated with α -NAC (2.5 μ g ml⁻¹), IL-2 (50 U ml⁻¹), or a combination of both for 72 hours (a) or 8 hours (b). Supernatants were measured for levels of IFN- γ , IL-17, and IL-22 by ELISA (a) and relative levels of mRNA were determined by reverse-transcriptase-quantitative PCR (RT-qPCR) and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (b). Medians are shown. * P <0.05, ** P <0.01, *** P <0.001.

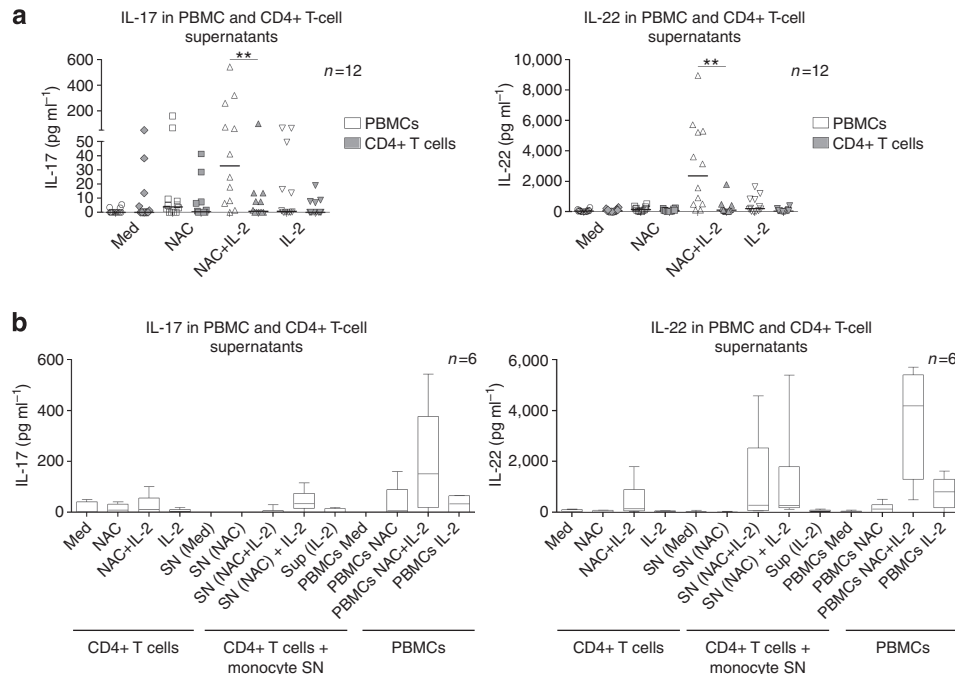


Figure 2. Monocytes are important for induction of T-cell cytokines by the alpha-chain of the nascent polypeptide-associated complex (α -NAC). Peripheral blood mononuclear cells (PBMCs) and CD4+ T cells from the same donors were stimulated with α -NAC (2.5 μ g ml⁻¹), IL-2 (50 U ml⁻¹), or a combination of both for 72 hours (a). Isolated monocytes were stimulated with α -NAC (2.5 μ g ml⁻¹), IL-2 (50 U ml⁻¹), or a combination of both for 72 hours. Isolated CD4+ T cells were incubated with these supernatants for 72 hours. For comparison, PBMCs and isolated CD4+ T cells from the same donors were stimulated with α -NAC (2.5 μ g ml⁻¹), IL-2 (50 U ml⁻¹), or a combination of both for 72 hours (b). Supernatants were measured for levels of IL-17 and IL-22 by ELISA. Medians are shown. ** $P < 0.01$. SN, supernatant.

stimulated CD4+ T cells (Figure 2b). However, cytokine levels were still higher when PBMCs were stimulated with α -NAC, indicating a role for cell contact between monocytes and CD4+ T cells for efficient T-cell stimulation.

IL-23 is a Th17-promoting cytokine produced by monocytes and dendritic cells that binds to the IL-12R β 1/IL-23R complex followed by STAT3 activation. To find out if IL-23 is important in α -NAC-induced upregulation of IL-17, we analyzed mRNA expression and cytokine secretion in PBMCs. Expression of IL-23A was higher in α -NAC-stimulated PBMCs than in medium controls (Figure 3a). Although IL-23 levels in PBMC supernatants were only weakly increased after α -NAC stimulation (Figure 3b), pre-treatment of PBMCs with a blocking antibody against IL-23 p19 led to reduced levels of secreted IL-17 (Figure 3c). IL-22 secretion was not significantly reduced by the IL-23 p19 blockade (Figure 3c). In contrast, pre-treatment of PBMCs with blocking antibody against IL-12 p40, which in addition to IL-23 also blocks IL-12 p70, diminished the secretion of IL-17 and IL-22 (Supplementary Figure S1 online).

STAT3 phosphorylation is essential for α -NAC-induced cytokine effects

STAT3 is a key transcription factor for the differentiation of Th17 cells as well as for IL-17 production, as it regulates retinoic acid-related orphan receptor- γ t expression and binds directly to the *IL17a* locus (Chen et al., 2006). In accordance with this, the combination of α -NAC and IL-2, which

upregulated IL-17 transcription and secretion most efficiently, led to the strongest phosphorylation of STAT3 in PBMCs (Figure 4a and b). Other STATs known to have a role in T-cell activation and differentiation, such as STAT1, STAT4, and STAT5, were not phosphorylated in PBMCs stimulated with α -NAC (data not shown). To elucidate the role of STAT3 regarding the cytokine effects of α -NAC, we pre-treated the cells with the specific STAT3-inhibitor Stattic before stimulation. Stattic, but not the solvent DMSO alone, efficiently reduced STAT3 phosphorylation (Figure 5a and b) and, subsequently, also decreased the levels of the cytokines induced by α -NAC or α -NAC and IL-2 (Figure 5c). 7-Amino-actinomycin D staining of PBMCs after 3 days incubation with Stattic revealed no decrease of cell viability (Figure 5d). Furthermore, PBMCs pre-treated with Stattic or DMSO were still responsive to stimulation with *Staphylococcus enterotoxin B* (Figure 5e). This emphasizes the importance of STAT3 for the effects of α -NAC.

Despite the effective IL-17 induction and STAT3 phosphorylation by α -NAC, we could not observe an effect on retinoic acid-related orphan receptor- γ t in terms of changes in mRNA expression (data not shown).

PBMCs of α -NAC-sensitized AD patients fail to secrete IL-10 in response to α -NAC

All experiments described above have been performed with PBMCs and isolated monocytes or CD4+ T cells from AD patients as well as from control donors. For most of the analyzed

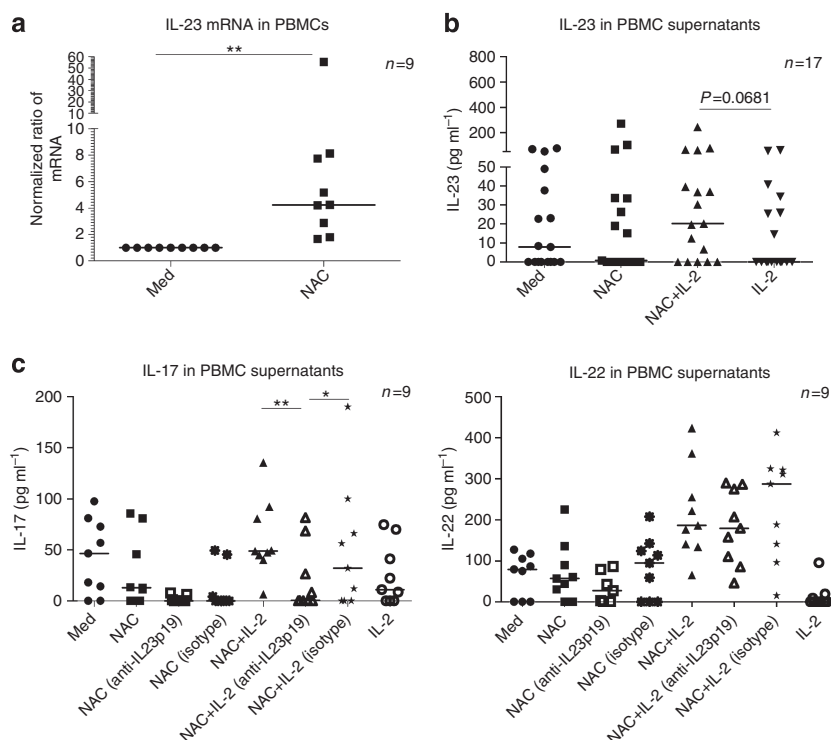


Figure 3. Monocyte-derived IL-23 is important for the alpha-chain of the nascent polypeptide-associated complex (α -NAC)-induced IL-17 secretion. Peripheral blood mononuclear cells (PBMCs) were stimulated with α -NAC ($2.5 \mu\text{g ml}^{-1}$) for 4 hours and relative levels of mRNA were determined by reverse-transcriptase-quantitative PCR (RT-qPCR) and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (a). PBMCs were stimulated with α -NAC ($2.5 \mu\text{g ml}^{-1}$), IL-2 (50 U ml^{-1}), or a combination of both for 72 hours. IL-23 levels were measured by ELISA (b). PBMCs were preincubated with anti-IL-23 p19 antibody or the isotype control, followed by stimulation with α -NAC ($2.5 \mu\text{g ml}^{-1}$), IL-2 (50 U ml^{-1}), or a combination of both for 72 hours. IL-17 and IL-22 were measured by ELISA (c). Medians are shown. * $P < 0.05$, ** $P < 0.01$.

cytokines, there were no significant differences between the AD patients and the controls, however, when we analyzed IL-10 levels in supernatants from PBMCs stimulated with α -NAC, it became evident that the control group secreted high amounts of IL-10, whereas in the group of AD patients sensitized to α -NAC IL-10 levels were significantly lower (Figure 6a). PBMCs from AD patients that were not sensitized to α -NAC released similar levels of IL-10 compared with the control donors. The reduced IL-10 levels in supernatants from cells of AD patients sensitized to α -NAC was not due to their general inability to secrete IL-10, as this cytokine was produced in response to human thioredoxin (median: 195 pg ml^{-1} , range $49\text{--}1,560 \text{ pg ml}^{-1}$). Thioredoxin may act as an autoallergen in some patients with AD, our patients were, however, not sensitized to this protein. To find out which cell subset of the PBMCs is responsible for the observed IL-10 production, we stimulated PBMCs, monocytes, and CD4^+ T cells of the same control donors. As shown in Figure 6b, monocytes secreted IL-10 in comparable amounts to the corresponding PBMCs. In CD4^+ T-cell supernatants, only very low levels of IL-10 were detected. This finding underlines again the impact of monocytes in generating an immune response to α -NAC.

DISCUSSION

AD is a chronic inflammatory skin disease characterized by a dysregulated immune response as well as skin barrier defects.

Allergen-specific T cells are major skin-infiltrating effector cells of the disease, with a Th2 bias in the acute phase and a Th1 dominance in the chronic phase (Grewe *et al.*, 1998). In addition, the Th17 and Th22 subtypes have been described to participate in the pathogenesis of AD (Toda *et al.*, 2003; Koga *et al.*, 2008; Nogales *et al.*, 2009; Eyerich *et al.*, 2011). Although Th17 cells have been found to be elevated in peripheral blood and in acute lesions of AD patients, Th22 (and Tc22) cells were also detected in chronic AD lesions. The exact role of both cell types is still not clear. For example, IL-17 levels are much lower in AD than in psoriasis, which might be due to the inhibitory effect of Th2 cytokines on IL-17 secretion (Toda *et al.*, 2003; Eyerich *et al.*, 2009; Nogales *et al.*, 2009; Gittler *et al.*, 2012). As IL-17 promotes the release of antimicrobial peptides by keratinocytes, the reduced IL-17 levels render AD patients susceptible to bacterial skin infection while this is not common in psoriasis (Guttman-Yassky *et al.*, 2008). On the other hand, Th22 counts and IL-22 mRNA levels are markedly higher in AD than in psoriasis (Nogales *et al.*, 2009). IL-22 inhibits the terminal differentiation of keratinocytes, both directly and by inducing other IL-10 family cytokines, like IL-20, in keratinocytes (Boniface *et al.*, 2005; Tohyama *et al.*, 2009; Wolk *et al.*, 2009a,b). Similar to IL-17, IL-22 promotes the production of antimicrobial peptides and chemokines, although with lower potency than IL-17 (Wolk *et al.*, 2004; Nogales *et al.*, 2008;

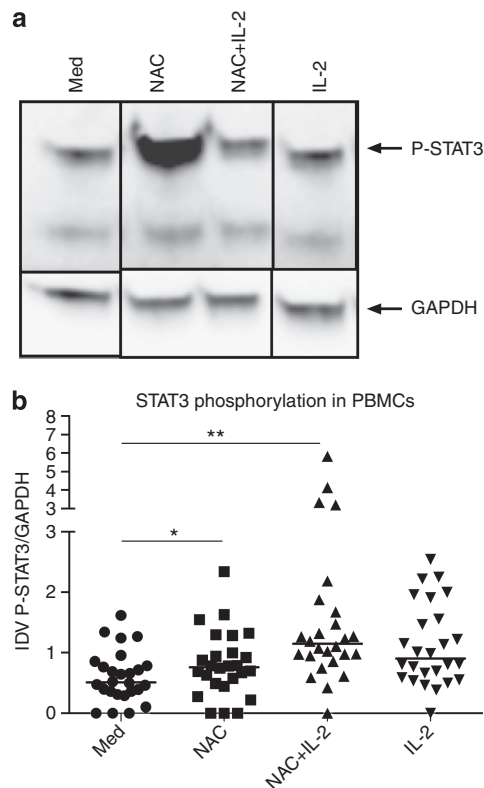


Figure 4. Stimulation with the alpha-chain of the nascent polypeptide-associated complex (α -NAC) leads to signal transducer and activator of transcription 3 (STAT3) phosphorylation in peripheral blood mononuclear cells (PBMCs). PBMCs from 26 donors were stimulated with α -NAC ($2.5 \mu\text{g ml}^{-1}$), IL-2 (50 U ml^{-1}), or a combination of both for 30 minutes. Western blots for phospho-STAT3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were performed. One representative experiment is shown (a). Integrated density values (IDV) of phospho-STAT3 bands were normalized to the corresponding GAPDH bands. Medians are shown (b). * $P < 0.05$, ** $P < 0.01$.

Tohyama *et al.*, 2009). Although CD4⁺ T cells outnumber CD8⁺ T cells in AD lesions, the latter have been shown to initiate skin inflammation (Hennino *et al.*, 2007), to infiltrate the epidermis of lesional skin in AD and to contribute to secretion of cytokines involved in atopic skin pathology like IL-13, IL-22, and IFN- γ (Hijnen *et al.*, 2012).

Although it is typical for exogenous allergens to induce Th2 responses, this might not be true for autoallergens. Autoallergens are self-proteins that elicit IgE responses in a subgroup of AD patients and they have been discussed to exaggerate disease activity just as exogenous allergens can do (Werfel and Kapp, 1998; Valenta *et al.*, 2000, 2009). Autoallergens may be released in inflamed skin or after scratching and may thus be accessible to immune cells. For human manganese superoxide-dismutase, upregulation in inflammatory skin diseases has been shown, which was not restricted to AD. Nevertheless, IgE sensitization toward human manganese superoxide-dismutase was specific for AD patients (Schmid-Grendelmeier *et al.*, 2005). Thioredoxin, another autoallergen and homologue of the *Malassezia sympodialis* allergen Mala

s 13, is released from keratinocytes after stimulation with IFN- γ (Balaji *et al.*, 2011).

α -NAC has been shown to induce IFN- γ secretion in PBMCs from AD patients and healthy donors. This could in turn damage epithelial cells treated with the PBMC supernatants (Mittermann *et al.*, 2008). Here, we could show that α -NAC induces the T-cell cytokines IL-17 and IL-22 in addition to IFN- γ . mRNA expression of IFN- γ and IL-17 was also upregulated with α -NAC alone and cytokine secretion was amplified when α -NAC was combined with IL-2. Stimulation of isolated CD4⁺ T cells yielded no or very low cytokine secretion, while the stimulation of PBMCs was much more effective. This could be explained by the strong Toll-like receptor (TLR)-2-dependent activation of monocytes and dendritic cells by α -NAC (Hradetzky *et al.*, 2013), which may be needed for a full activation of T cells. As monocytes and inflammatory dendritic epidermal cells were equally efficient in terms of IL-12 secretion and, subsequently, IFN- γ induction in CD4⁺ T cells (unpublished data and Hradetzky *et al.*, 2013), we used monocytes as easily available model cells for further experiments.

In addition to the importance of IL-12 for the IFN- γ response to α -NAC, we have observed before, we could now show upregulation of IL-23 expression by α -NAC in PMBCs as well as reduced IL-17 secretion when PBMCs were pre-treated with a blocking IL-23 antibody. In contrast, IL-22 secretion was not significantly affected by this pre-treatment, which is in agreement with former studies showing that Th2-derived IL-22 is not dependent on IL-23 (Zenewicz and Flavell, 2011), although there are some studies pointing to an involvement of IL-23 in IL-22 secretion *in vivo* (Siegemund *et al.*, 2009; Graham *et al.*, 2011). The different impact of other monocyte-derived cytokines such as IL-1 β , IL-6, and tumor necrosis factor- α on IL-17 and IL-22 production by CD4⁺ T cells could explain this difference (Volpe *et al.*, 2008; Duhon *et al.*, 2009). These findings further underline the importance of monocytes for cytokine induction by α -NAC.

Another finding of our study is the STAT3 dependency of the effects of α -NAC on PBMCs. STAT3 phosphorylation was most pronounced when T-cell cytokine secretion was induced most efficiently. Conversely, if cells were pre-treated with the specific STAT3 inhibitor Stattic (Schust *et al.*, 2006; Samavati *et al.*, 2009), cytokine secretion was greatly reduced. STAT3 is an essential transcription factor for Th17 cells, it is important for the differentiation of this cell type and also for IL-17 expression, as it directly binds to the *IL17a* locus and regulates expression of the master transcription factor of Th17 cells, retinoic acid-related orphan receptor- γ t (Chen *et al.*, 2006; Yang *et al.*, 2007). In agreement with this, the treatment of PBMCs with Stattic led to reduced IL-17 expression and secretion and further to a decrease in IL-22 and IFN- γ secretion. The inhibitory effect of Stattic may be due to STAT3 signaling downstream of TLR-2, which has been shown for TLR-2 activation by heat shock protein 60 (HSP60; Zanin-Zhorov *et al.*, 2005). This is interesting, as HSP60 is an intracellular chaperone and autoantigen like α -NAC and T-cell reactivity to HSP60 has been shown in AD patients (Kapitein *et al.*, 2013). In addition, there are other

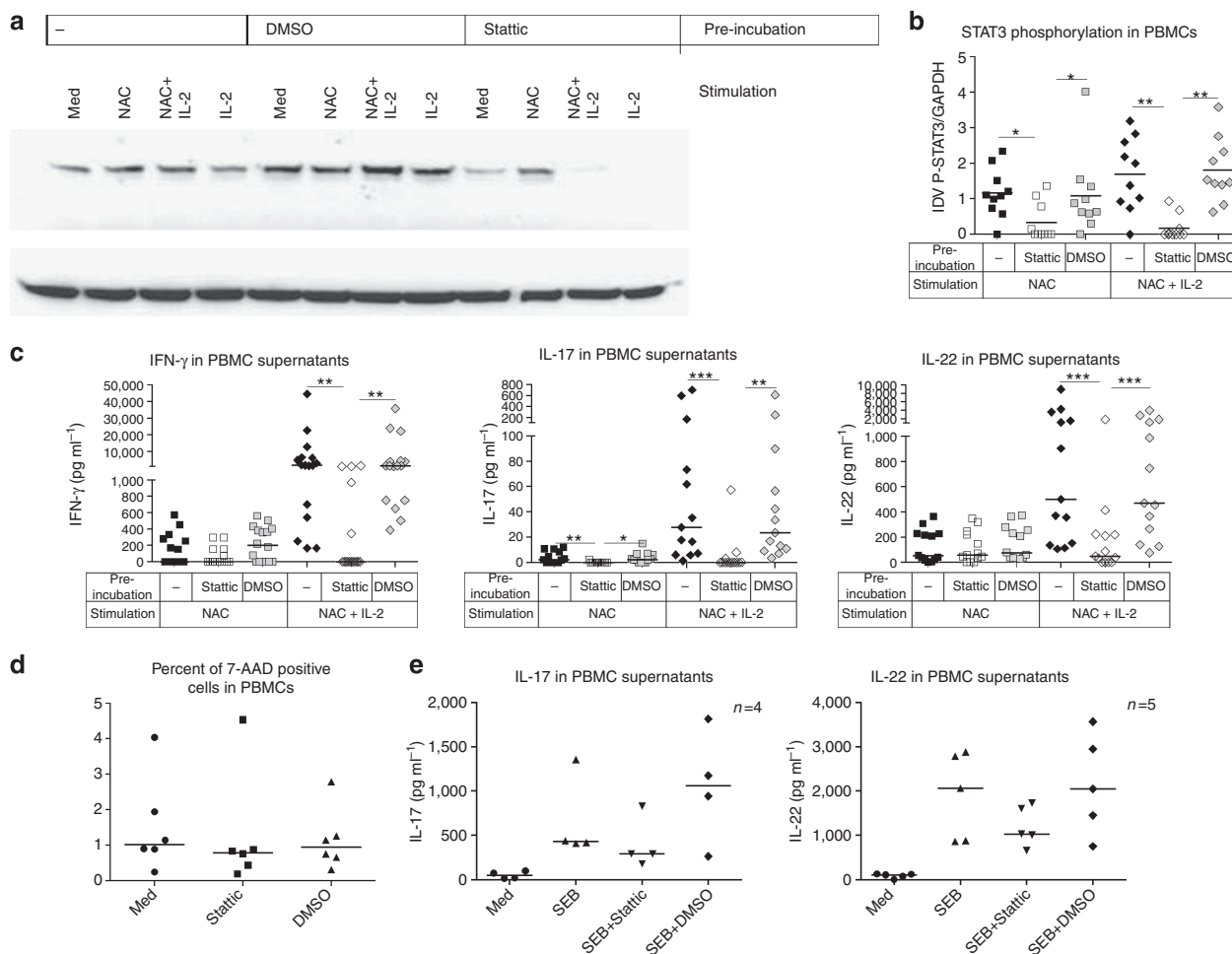


Figure 5. Signal transducer and activator of transcription 3 (STAT3) is essential for the α -NAC-induced cytokine secretion. Peripheral blood mononuclear cells (PBMCs) were preincubated with 20 μ M STAT3-inhibitor (Stattic) or the solvent DMSO alone and then stimulated with α -NAC (2.5 μ g ml⁻¹), IL-2 (50 U ml⁻¹), or a combination of both for 30 minutes and blotted as described above. One representative experiment of 10 independent experiments from different donors is shown in (a) and integrated density values (IDVs) of phospho-STAT3 normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are shown in (b). PBMCs were preincubated with 5 μ M Stattic or DMSO for 30 minutes and then stimulated with α -NAC (2.5 μ g ml⁻¹), IL-2 (50 U ml⁻¹), or a combination of both for 72 hours. Supernatants were measured for levels of IFN- γ ($n = 16$), IL-17 ($n = 13$), and IL-22 ($n = 15$) by ELISA (c) and 7-AAD-staining was performed ($n = 6$) (d). PBMCs were pre-treated with Stattic as described above and stimulated with *Staphylococcus enterotoxin B*. Supernatants were analyzed by ELISA (e). Medians are shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. 7-AAD, 7-aminoactinomycin D.

findings pointing to the fact that autoantigens or danger-associated molecular patterns may bind different epitopes on TLRs than exogenous antigens or pathogen-associated molecular patterns do. For example, TLR-4 mutations, which make the receptor less responsive to the pathogen-associated molecular pattern, lipopolysaccharide (LPS) resulted in enhanced activation of TLR-4 downstream signaling molecules by the danger-associated molecular pattern fibrinogen (Hodgkinson *et al.*, 2008). Other danger-associated molecular patterns need additional co-receptors, e.g., for receptor activation by hyaluronic acid, a complex of TLR-4, MD-2 and, unlike for LPS, CD44 is required (Taylor *et al.*, 2007). Consequently, autoantigens may activate distinct signaling pathways and cause different outcomes compared with exogenous antigens (Piccinini and Midwood, 2010).

As many of the studies on endogenous TLR ligands have been performed with recombinant proteins derived from

Escherichia coli (Erridge, 2010), the issue of contaminants has to be taken into account. We have used a highly purified α -NAC preparation, containing 1.9 ng LPS per mg protein, and performed control experiments to rule out cytokine induction by the remaining LPS (see Supplementary Figure S2 online). Nevertheless, we cannot exclude interaction of α -NAC with bacterial components. In fact, such an interaction has been suggested as a factor promoting sensitization to different aeroallergens, such as Fel d 1 and Can f 6 (Herre *et al.*, 2013), and for some of the proposed endogenous TLR ligands, such as HSPs and serum amyloid A (Erridge, 2010). Such a pathogen-associated molecular pattern-sensitizing function of α -NAC might provide a link between cell damage in atopic skin inflammation and microbial colonization of the skin. Furthermore, α -NAC has the ability to form aggregates with HSPs or DNA, which might further enhance its immunogenic potential (Mossabeh *et al.*, 2002; Wang *et al.*, 2010).

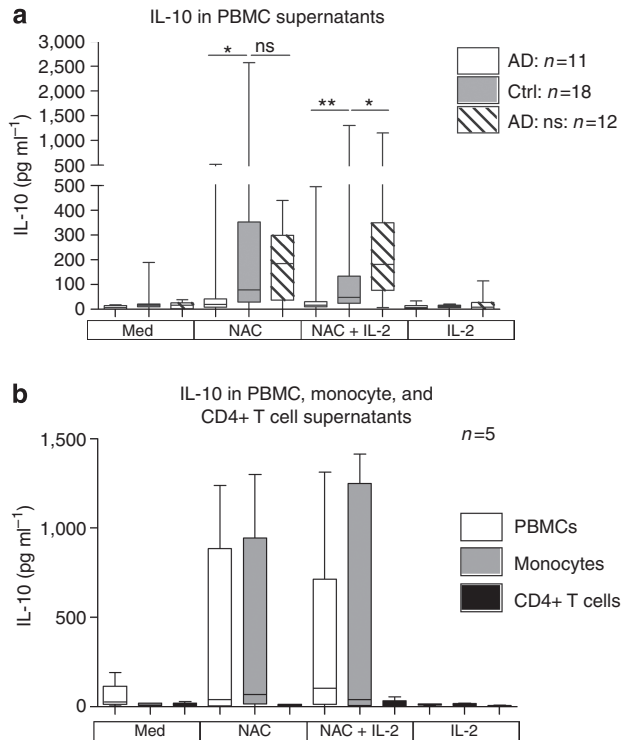


Figure 6. Peripheral blood mononuclear cells (PBMCs) of the alpha-chain of the nascent polypeptide-associated complex (α -NAC)-sensitized atopic dermatitis (AD) patients fail to secrete IL-10 in response to α -NAC. PBMCs from AD patients and non-allergic donors were stimulated with α -NAC ($2.5 \mu\text{g ml}^{-1}$), IL-2 (50 U ml^{-1}), or a combination of both for 72 hours. AD, α -NAC-sensitized: open boxes ($n=11$), controls: grey boxes ($n=18$), AD, not α -NAC-sensitized: striped boxes ($n=12$) (a). PBMCs, monocytes, and CD4+ T cells from non-sensitized donors were stimulated with α -NAC ($2.5 \mu\text{g ml}^{-1}$), IL-2 (50 U ml^{-1}), or a combination of both for 72 h (b). Supernatants were analyzed for levels of IL-10 by ELISA. Medians are shown. Groups were compared by Mann-Whitney test. * $P<0.05$, ** $P<0.01$. ns, not significant.

Although the pro-inflammatory cytokines IFN- γ , IL-17, and IL-22 could be induced in PBMCs from control donors and AD patients in comparable amounts, a clear difference was observed for IL-10 secretion. PBMCs from α -NAC-sensitized AD patients produced significantly lower levels of this anti-inflammatory cytokine compared with PBMCs from control donors and non-sensitized AD patients. As monocytes were the main producers of α -NAC-induced IL-10, this again points to the involvement of innate immune cells in initiation or perpetuation of autoallergic reactions. In fact, there are hints on genetic and functional differences between innate immune receptors from AD patients and from healthy subjects (Novak *et al.*, 2007; Niebuhr *et al.*, 2008, 2009). A possible mechanism, linking sensitization to α -NAC and the monocyte response toward the autoallergen, could lie in the high-affinity IgE receptor-mediated activation of these cells. Novak *et al.* (2001) have shown high-affinity IgE receptor-dependent IL-10 secretion by monocytes, which was, however, higher in cells from atopic patients than from non-atopic donors. On the other hand, a higher number of IL-10-secreting allergen-specific T cells from non-allergic

compared with allergic donors has been shown (Akdis *et al.*, 2004). Yet, the mechanisms and the role of IL-10 induction by autoallergens have to be further investigated in future studies.

The α -NAC-induced pro-inflammatory cytokines IFN- γ , IL-17, and IL-22 are all known to act on keratinocytes and can in turn lead to increased apoptosis or reduced differentiation of these cells. This may start a vicious circle of even more autoallergen release, which again promotes the ongoing inflammation. Therefore, understanding the mechanisms by which autoallergens influence the immune response are important for future studies and therapeutic approaches and we provided a further piece of the picture.

MATERIALS AND METHODS

Generation of recombinant α -NAC

Recombinant α -NAC was expressed in *Escherichia coli* as a soluble protein and purified by means of nickel affinity chromatography as described previously (Mossabeh *et al.*, 2002; Mittermann *et al.*, 2008). The LPS concentration determined by LAL-Assay (Haemochrom Diagnostica, Essen, Germany) was 2.6 ng ml^{-1} in the stock solution, corresponding to 1.9 ng per mg protein, which results in 5 pg ml^{-1} LPS in the working concentration of α -NAC.

Blood donors

Blood was obtained from AD patients that were recruited from the outpatient clinic from the Department of Dermatology, Allergy and Venerology, Hannover, Germany, after having received written informed consent. Patients had a mean age of 40, mean total IgE values of $3,766 \text{ kU l}^{-1}$ and mean SCORAD of 38 and were not under any immunosuppressive treatment. Patients were tested for IgE sensitization to α -NAC by ELISA. Optical density values at least 2.5 times higher than the negative control sera were regarded as positive. Buffy coats from healthy voluntary blood donors provided by the institute of transfusion medicine, Hannover Medical School, were used as control donors. The study has been approved by the local ethic committee of Hannover Medical School and was conducted according to Declaration of Helsinki principles.

Preparation of cells

PBMCs, monocytes, and CD4+ T cells were isolated as described in the Supplementary Materials and Methods online.

Stimulation of the cells

The following stimulants were used: α -NAC ($2.5 \mu\text{g ml}^{-1}$), IL-2 (50 U ml^{-1} , Roche Applied Science, Mannheim, Germany), LPS (5 pg ml^{-1} , Sigma-Aldrich, Deisenhofen, Germany). Cells were stimulated 30 minutes for protein extraction, 8 hours for RNA isolation, and 3 days for generation of supernatants.

Blocking experiments

STAT3 inhibition was performed by pre-treating the cells with the specific inhibitor Stattic (Calbiochem, Merck, Darmstadt, Germany). Owing to the short incubation time, $20 \mu\text{M}$ Stattic were used for protein extraction. For longer incubations (RNA and supernatants), $5 \mu\text{M}$ Stattic were used. To exclude toxicity of the inhibitor, PBMCs were treated 3 days with $5 \mu\text{M}$ Stattic and viability was assessed by 7-aminoactinomycin D staining (BD Biosciences, Bedford, MA).

Quantitative real-time PCR, ELISA, western blot

These methods are described in the Supplementary Materials and Methods online.

Statistical analyses

Statistical analysis was performed using Wilcoxon signed rank test or Mann–Whitney test for comparison of different groups (GraphPad Prism, San Diego, CA).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at <http://www.nature.com/jid>

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Supplemental data

Cytokine effects induced by the human autoallergen α -NAC

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Supplemental results

Fig. S1

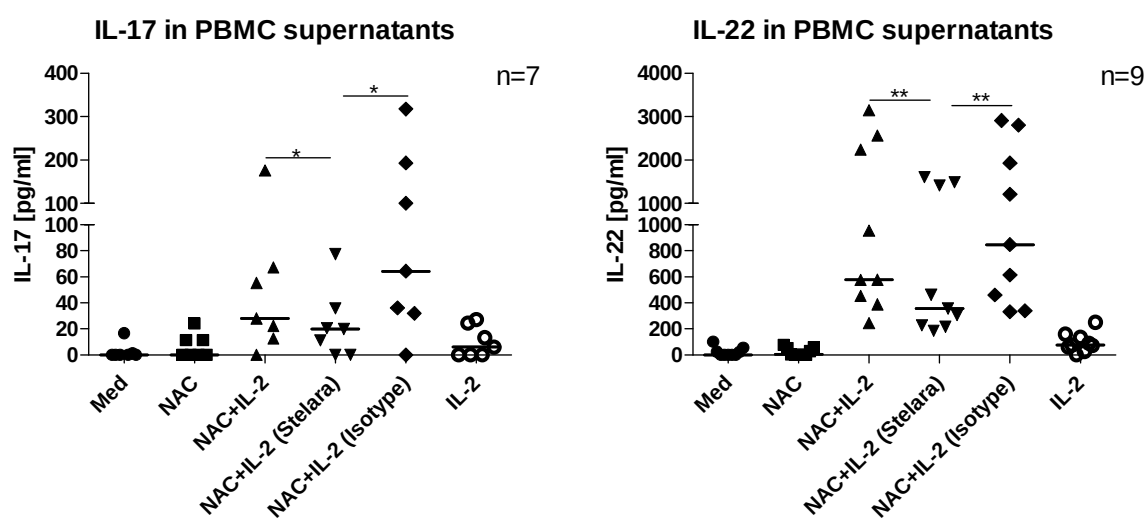


Fig. S1 Blockade of IL-12 p40 reduces secretion of IL-17 and IL-22 in PBMCs

PBMCs from control donors were pretreated with a blocking antibody against IL-12 p40 (Stelara, 10 μ g/ml) or a matching isotype control (10 μ g/ml) and stimulated with α -NAC (2.5 μ g/ml), IL-2 (50 U/ml) or a combination of both for 72 h. Supernatants were measured for levels of cytokines by ELISA. Medians are shown. * $p < 0.05$, ** $p < 0.01$.

Cytokine effects depend on α -NAC, not on LPS in the α -NAC preparation

In all experiments, we used α -NAC as a recombinant protein, expressed in *E. coli*. Therefore we wanted to exclude LPS effects as cause of the induced cytokines. The α -NAC preparation used for the experiments contained 5 pg/ml LPS in the working concentration. We used the same LPS concentration as a control, mimicking these LPS background levels. Stimulation of PBMCs with 5 pg/ml LPS induced no significant secretion of IFN- γ , IL-17 and IL-22 into the supernatants (Fig. S2).

Fig. S2

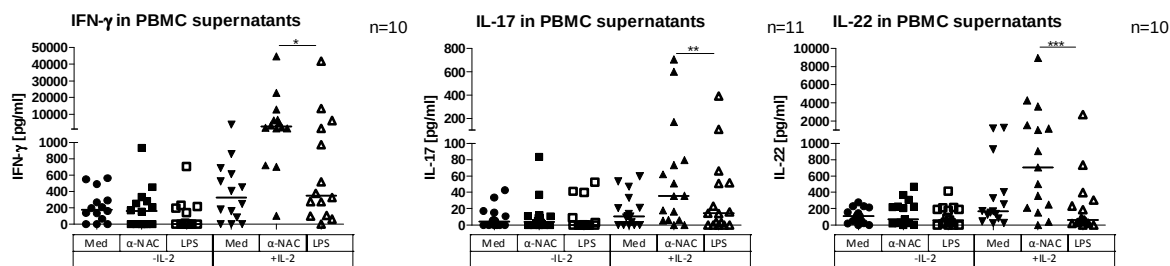
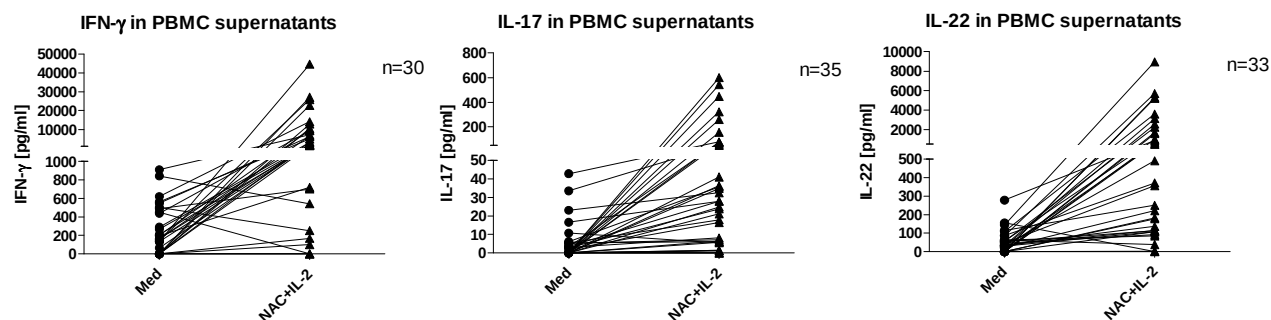


Fig. S2 Cytokine effects depend on α -NAC, not on LPS in the α -NAC preparation

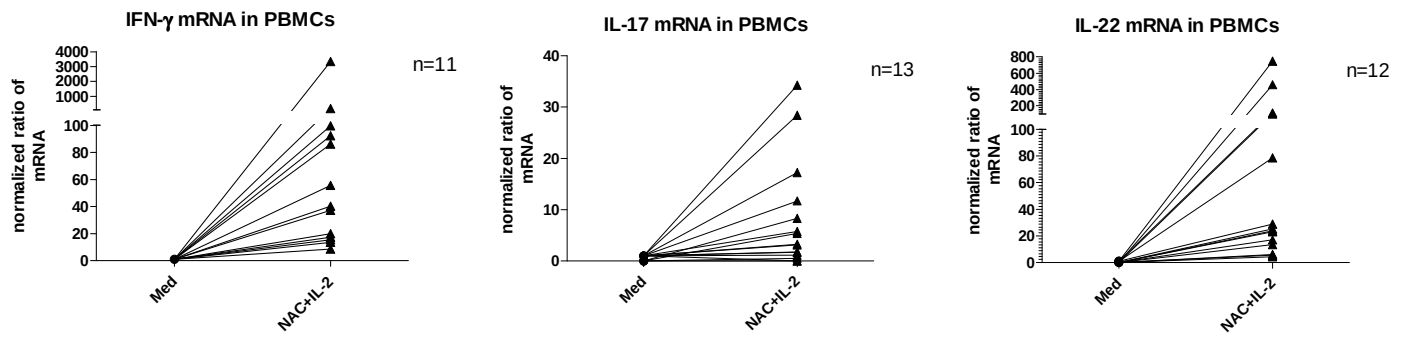
PBMCs from control donors were stimulated with α -NAC (2.5 μ g/ml) or LPS (5pg/ml), respectively, IL-2 (50 U/ml) or a combination of both for 72 h (A). Supernatants were measured for levels of cytokines by ELISA. Medians are shown. * p <0.05, ** p <0.01, *** p <0.001

Fig. S3

a

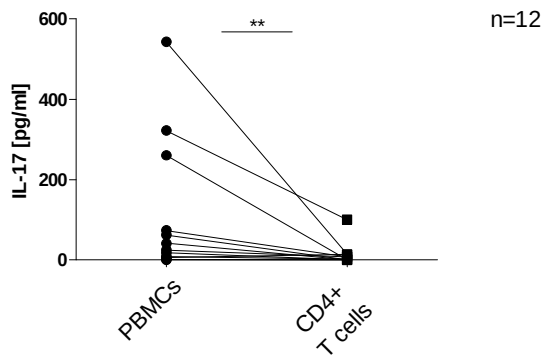


b

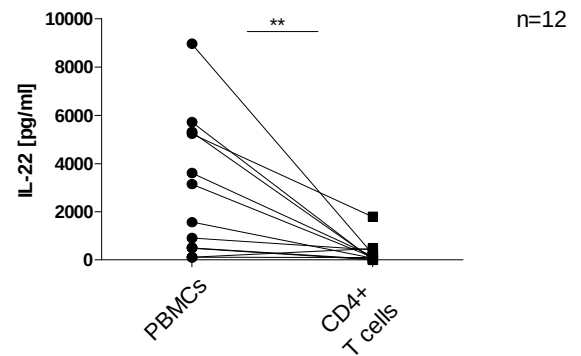


c

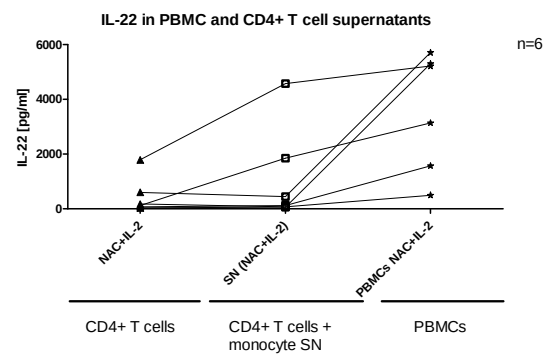
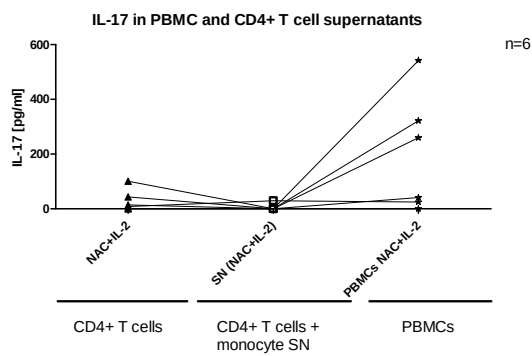
IL-17 in PBMC and CD4+ T cell supernatants



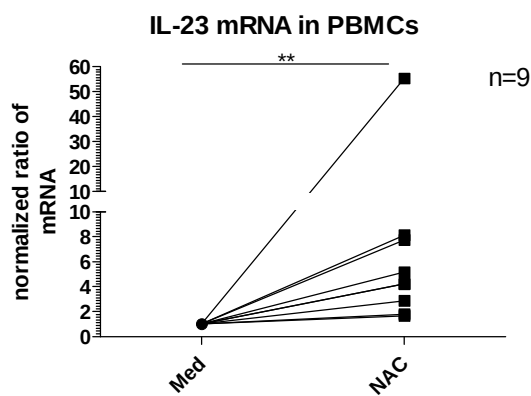
IL-22 in PBMC and CD4+ T cell supernatants



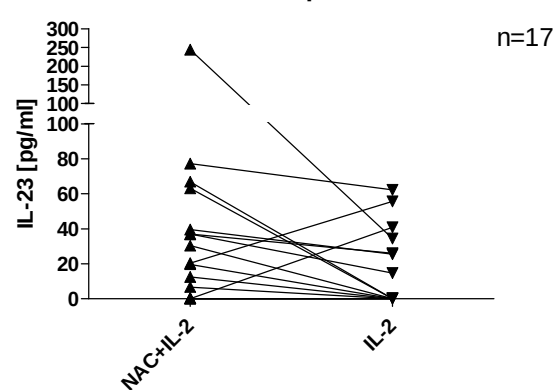
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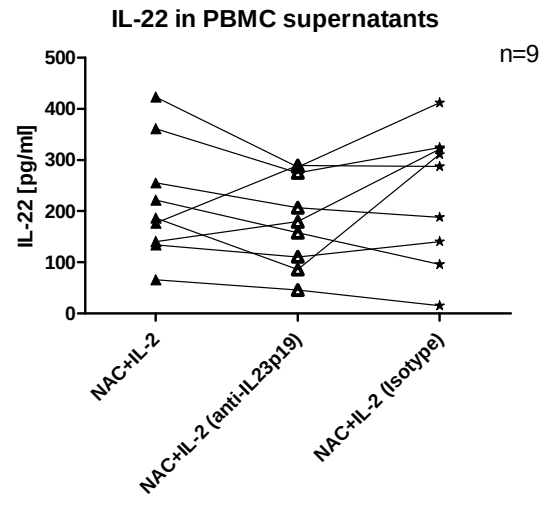
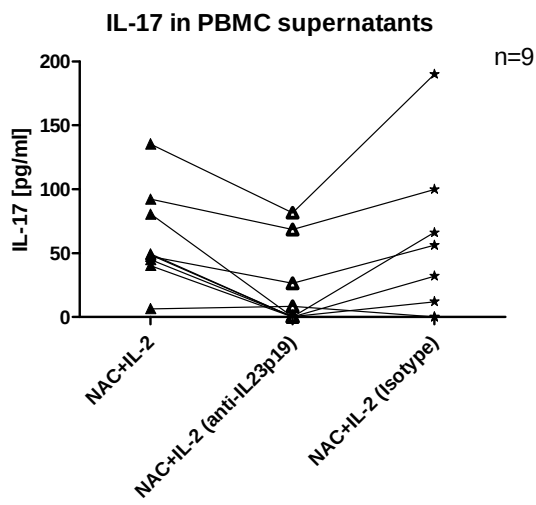
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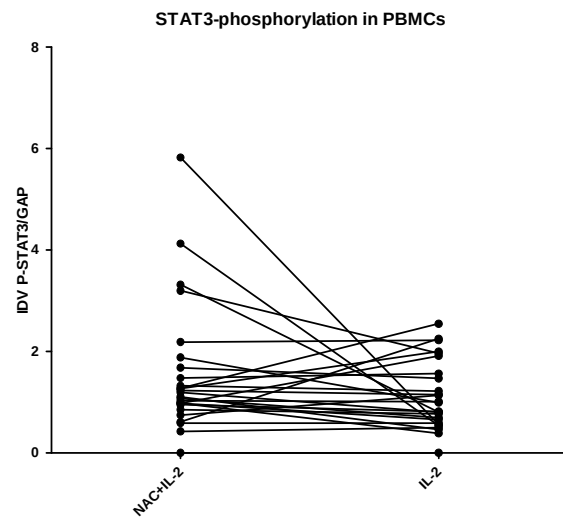
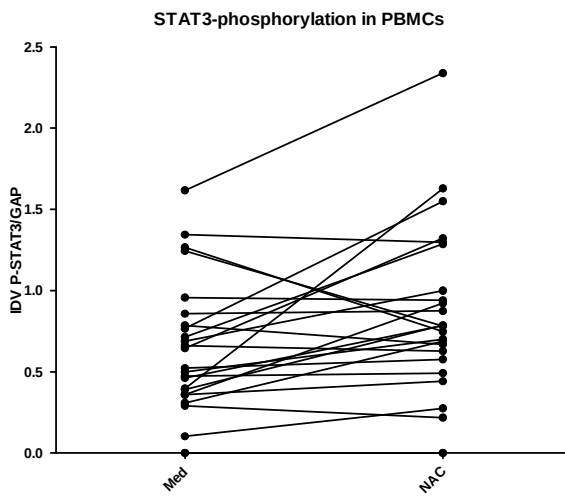
IL-23 in PBMC supernatants



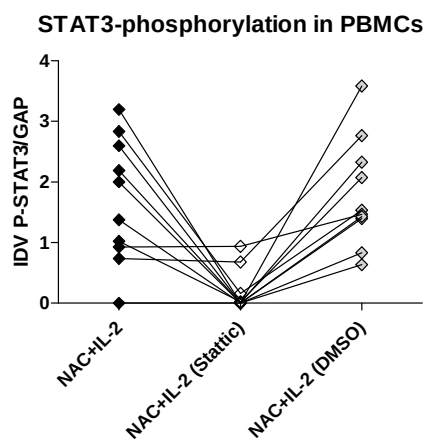
f



g



h



j

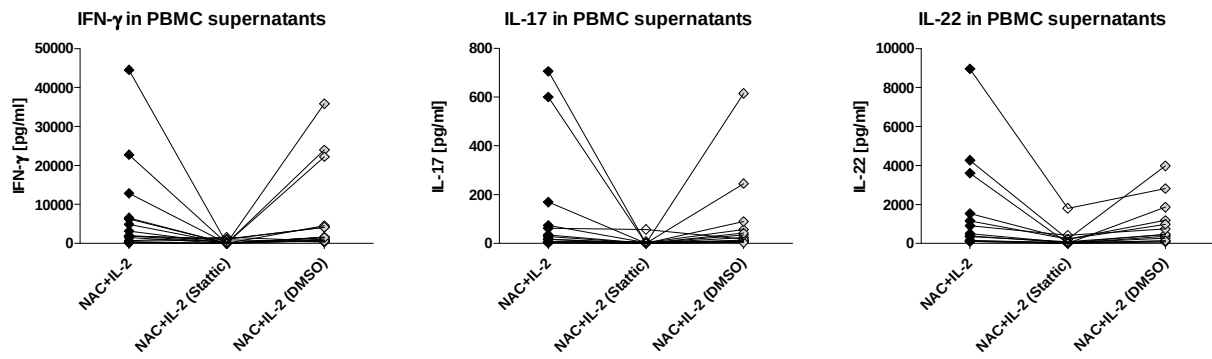


Fig. S3 Line graphs for the clarification of selected results

Paired line graphs are shown for the same data of Fig. 1a and b (a,b, only data from cells not stimulated and stimulated with α -NAC and IL-2 are shown), Fig. 2a (c), Fig. 2b (d), Fig. 3a and b (e, only data sets stimulated with α -NAC and IL-2 or IL-2 alone are shown in the right graph), Fig. 3c (f), Fig. 4b (g), Fig 5b (h), Fig 5c (j). In figures c, d, f, g, h, j only data from cells stimulated with α -NAC and IL-2 are shown.

Supplemental Materials and Methods

Preparation of cells

PBMCs were isolated by density-gradient centrifugation on Ficoll (Pan Biotech, Aidenbach, Germany). Cells were cultured in Iscove medium (Biochrom KG, Berlin, Germany) supplemented with 4% human heat-inactivated AB serum, 2 mM glutamine, 50 mg/ml of gentamicin, 100 mg/ml penicillin and streptomycin, and nonessential amino acids (designated as culture medium). For some experiments, monocytes and CD4⁺ T cells were isolated from the PBMCs by MACS (Monocyte isolation kit II, CD4⁺ T cell isolation kit, Miltenyi, Bergisch Gladbach, Germany). Cells were plated at a density of 1×10^6 /ml for generation of RNA and supernatants and 4×10^6 /ml for preparation of protein extracts, respectively.

Quantitative real time-PCR

RNA was isolated using the ZR RNA MiniPrep kit (Zymo Research, Irvine, USA) and RNA quantity/quality was assessed with the NanoDrop (Thermo Fisher). Reverse transcription was performed with Fermentas RevertAid First strand synthesis kit (Thermo Fisher Scientific, Rockford, US). For Real Time quantitative Light-Cycler PCR, QuantiTect SYBR Green PCR Kit and QuantiTect Primer Assays from Qiagen were used, PCR was run in a Roche Light Cycler. Levels of target mRNA relative to the reference, GAP-DH mRNA, was calculated with the Relative Quantification Software (Roche Molecular Biochemicals).

ELISA

Culture supernatants were taken 3 days after stimulation, centrifuged and the supernatants stored at -20 °C. ELISAs were performed according to the manufacturers' instructions and measurement was done with the FluoStar Reader. ELISAs from R&D (IL-17, IL-22) and eBioscience (IFN- γ , IL-23, IL-10) were used.

Western Blot

Whole cell protein was isolated with M-PER (Thermo Fisher, Rockford, USA) supplemented with proteinase and phosphatase inhibitors. Western Blots were performed using following primary antibodies: anti-Phospho-STAT1 (Tyr701), anti-Phospho-STAT3 (Tyr705), anti-Phospho-STAT4 (Tyr693), anti-Phospho-STAT5 (Tyr694) and anti-GAPDH rabbit antibodies, secondary antibody was anti-rabbit IgG, HRP-linked goat antibody (all from CellSignaling Technology, Danvers, USA).

Protein extracts were diluted in 5x reducing sample buffer, heated at 95 °C for 5 min and applied onto 4-20% Pierce Precise Protein gels (Thermo Fisher, Rockford, USA). Gels were run at 120 V and blotted on nitrocellulose membranes (pore size 0.45 µm, Roth GmbH, Karlsruhe, Germany). The blots were blocked in Roti-Block (Carl Roth, Karlsruhe, Germany) for 1 hour. Primary antibodies were diluted in 5% bovine serum albumin in TBST (TBS with 0.1% Tween), incubation of the blots was done at 4 °C over night. Secondary antibodies were diluted in 5% milk powder in TBST for at least one hour at room temperature. Pierce SuperSignalWest was used as a chemiluminescent substrate and detection and analysis was performed with the ChemiImager software (Genetic Technologies, Miami, USA).