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Stimulation of the Intracellular Bacterial Sensor NOD2 Programs Dendritic Cells to Promote Interleukin-17 Production in Human Memory T Cells

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SUMMARY

How the development of antibacterial T helper 17 (Th17) cells is selectively promoted by antigen-presenting dendritic cells (DCs) is unclear. We showed that bacteria, but not viruses, primed human DCs to promote IL-17 production in memory Th cells through the nucleotide oligomerization domain 2 (NOD2)-ligand muramyldipeptide (MDP), a derivative of bacterial peptidoglycan. MDP enhanced obligate bacterial Toll-like receptor (TLR) agonist induction of IL-23 and IL-1, which promoted IL-17 expression in T cells. The role of NOD2 in this IL-23-IL-1-IL-17 axis could be confirmed in NOD2-deficient DCs, such as DCs from selected Crohn's disease patients. Thus, antibacterial Th17-mediated immunity in humans is orchestrated by DCs upon sensing bacterial NOD2-ligand MDP.

INTRODUCTION

Protection against certain bacteria requires the activity of the cytokine IL-17 (Kolls and Linden, 2004) produced by a specialized subset of T helper 17 (Th17) cells (Harrington et al., 2005; Park et al., 2005; Langrish et al., 2005; Aggarwal et al., 2003). Recent studies in mice (Veldhoen et al., 2006a; Mangan et al., 2006; Bettelli et al., 2006) have indicated that the development of Th17 in naive T cells can be induced by the combination of the cytokines TGF-β and IL-6. However, in humans, this finding has not been confirmed thus far. In addition, both in mice and man, Th17 cells can be induced in memory Th cells by IL-23 (Harrington et al., 2005; Park et al., 2005; Langrish et al., 2005; Aggarwal et al., 2003) and IL-1 (Kidoya et al., 2005; Sutton et al., 2006). The importance of this IL-23-IL-17 axis in antimicrobial immunity is underlined by previous studies showing that protection against *Klebsiella pneumoniae* (Happel et al., 2005) and *Streptococcus pneumoniae* (Malley et al., 2006), for example, is impaired in mice deficient in the IL-23-specific subunit p19 or in IL-17. Furthermore, despite the fact that the development of Th17 cells in *Citrobacter rodentium*-infected mice is promoted by the TGF-β-IL-6 pathway, IL-23 is indispensable in these mice for the Th17 response that protects against *C. rodentium*-driven colitis (Mangan et al., 2006). The importance of IL-23 in the development of functional Th17 cells is further illustrated in mouse models of Th17-mediated chronic inflammatory diseases (e.g., experimental allergic encephalomyelitis [EAE] [Langrish et al., 2005], arthritis [Murphy et al., 2003], and colitis [Yen et al., 2006]). More detailed experiments have shown that although TGF-β is essential for the initiation of EAE, progression of EAE is associated with enhanced production of IL-23 (Veldhoen et al., 2006b). Altogether, these data support the concept that the development of IL-17 in T cells in mice can be initiated by the TGF-β-IL-6 pathway, but that the induction of fully functional Th17 cells that are protective against bacterial infection or progressors of chronic inflammatory disease depends on IL-23. In addition to IL-23, protection against bacteria requires IL-1 (Zwijnenburg et al., 2003; Miller et al., 2006), which may be explained by the finding that IL-1β and IL-1α synergize with IL-23 in the induction of protective IL-17 by murine Th cells (Kidoya et al., 2005; Sutton et al., 2006).

In the present study, we have focused on the mechanisms underlying the triggering of the IL-23-IL-17 pathway by bacteria in humans. A well-established concept is that the development of protective Th cell subsets is orchestrated by cytokines produced by dendritic cells (DCs) that differentially sense archetypical structures defining different classes of pathogens (Kapsenberg, 2003; Sousa, 2004). Although DCs have been previously implicated in promoting Th17 cells (Schnurr et al., 2005; Happel et al., 2003), the bacterial compounds that program for Th17-promoting DCs, as well as the type of pattern recognition receptors and soluble mediators involved, have not yet been clearly established.

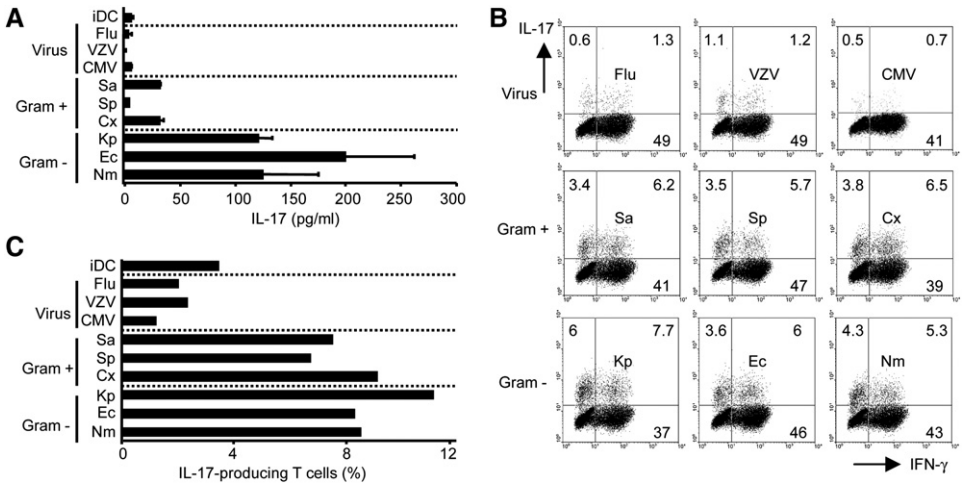

Figure 1. Gram-Positive and Gram-Negative Bacteria Prime DCs to Enhance IL-17 in CD4$^+$ T Cells

Unfractionated CD4$^+$ T cells were cocultured with DCs that were primed for 16 hr with Gram-positive bacteria *S. aureus* (Sa), *S. pneumoniae* (Sp), *C. Xerosi* (Cx), Gram-negative bacteria *K. pneumoniae* (Kp), *E. coli* (Ec), *N. meningitidis* (Nm) (10^7 bacteria/ml), or influenza virus (Flu), *Varicella zoster* (VZV), and cytomegalovirus (CMV) (moi 1:5) in the presence of *Staphylococcus* enterotoxin B (SEB, 10 pg/ml).

(A) After 4 days, IL-17 was determined in supernatant by ELISA. Data are shown as means ± SD of triplicate cultures.

(B and C) After 12 days of culture in the presence of IL-2, resting T cells were restimulated with PMA and ionomycin and the frequency of IL-17- and IFN-γ-producing T cells was determined by intracellular FACS staining.

(B) FACS plot showing percentages of positive cells in each quadrant.

(C) Histogram of percentage of IL-17-producing cells (upper left and right quadrants in [B]). Data are representative of five independent experiments.

We report that, in contrast to the murine system, bacteria-primed DCs do not induce IL-17 in human naive Th cells via the TGF-β-IL-6 pathway. However, upon their activation by bacteria, but not viruses, DCs promote the development of human Th17 cells from memory Th cells. Further experiments support the concept that antibacterial Th17-mediated immunity in humans is orchestrated by DCs upon sensing bacterial nucleotide oligomerization domain 2 (NOD2)-ligand muramyldipetide (MDP), which programs these DCs for elevated IL-23 and IL-1 production.

RESULTS

Bacterial Peptidoglycan Programs DCs to Enhance IL-17 Secretion in Th Cells

To evaluate the concept that bacteria prime DCs for the selective induction of Th17 cells, we tested to what extent randomly selected species of Gram-positive (*Staphylococcus aureus, Streptococcus pneumoniae*, and *Corinebacterium xerosis*) and Gram-negative (*Escherichia coli*, *Klebsiella pneumoniae*, and *Neisseria menigitidis*) bacteria program DCs to promote Th cells to produce IL-17. For comparison, we tested influenza virus, *Varicella zoster*, and cytomegalovirus, which cannot be eradicated by neutrophils and are not expected to induce Th17 cells. In these assays, human monocyte-derived DCs were cultured for 16 hr in the presence of whole microorganisms, washed, and subsequently analyzed for their Th17-polarizing activity upon coculture with peripheral blood CD4$^+$ Th cells comprising both naive and memory Th cells. Clearly, both Gram-negative and Gram-positive bacteria, but none of the tested viruses, programmed the DCs to markedly enhance IL-17 production in these T cells after 4 days of coculture (Figure 1A). DCs primed with Gram-negative bacteria induced higher amounts of IL-17 in the T cells than did Gram-positive bacteria, and one (*S. pneumoniae*) of the three Gram-positive bacteria tested consistently failed to enhance IL-17 secretion. Despite the variability in IL-17 levels produced in these short-term T cell cocultures with DCs primed by different pathogens (Figure 1A), all bacterial species (including *S. pneumoniae*) primed DCs for the ability to promote in long-term cultures a Th cell subset with intracellular IL-17 expression upon nonspecific restimulation (Figures 1B and 1C). None of the viruses induced this effect, which was not due to inactivity of the virus, as confirmed by virus-induced IL-6 production by DCs (Figure S1 in the Supplemental Data available online).

To assess which bacterial components are capable of programming Th17-inducing DCs, we tested the effects of various bacterial molecules known to interact with host Toll-like receptors (TLRs), as well as some virus-associated TLR-agonists. These experiments revealed that the capacity of DCs to support IL-17-production (Figure 2A) and Th17 cells (Figures 2B and 2C) resulted from DC activation by the TLR2-ligand peptidoglycan (PGN), a cell-wall component of both Gram-negative and Gram-positive bacteria, and not by the TLR2-TLR1 heterodimer-ligand Pam3CSK4 (a synthetic cell-wall lipopeptide of both Gram-negative and Gram-positive bacteria), the TLR4 ligand lipopolysacchride (LPS, a major cell-wall component of Gram-negative bacteria), the TLR5 ligand

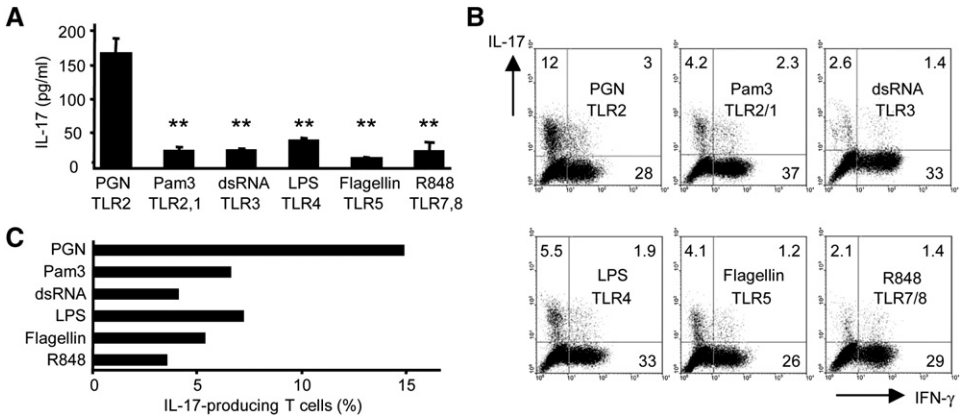

Figure 2. TLR2 Ligand PGN Programs DCs to Promote IL-17 in CD4$^+$ T Cells

CD4$^+$ T cells were cocultured with DCs primed for 16 hr with PGN (10 μg/ml), Pam3CSK4 (5 μg/ml), dsRNA (poly I:C, 20 μg/ml), LPS (100 ng/ml), flagellin (1 μg/ml), and R848 (2 μg/ml) in the presence of SEB.

(A) After 4 days, IL-17 was determined in supernatant by ELISA. Data are shown as means ± SD of triplicate cultures.

(B and C) After 12 days, resting T cells were restimulated with PMA and ionomycin, and the frequency of IL-17- and IFN-γ-producing T cells was determined by intracellular FACS staining.

(B) FACS plot showing percentages of positive cells in each quadrant.

(C) Histogram of percentage of IL-17-producing cells (upper left and right quadrants in [B]). **$p < 0.01$. Data are representative of five independent experiments.

flagellin (a component of flagellated bacteria), or ligands that activate intracellular TLR3 (dsRNA) and TLR7 and TLR8 (R848), which are associated with viruses. In all experimental conditions, Th17 cells consisted of variable frequencies of cells that produce only IL-17 and cells that coproduced IL-17 and IFN-γ (Figures 1 and 2), but not IL-4 (Figure S2), underscoring the relationship between Th17 and Th1 cells (Aggarwal et al., 2003; Mathur et al., 2006).

PGN-Primed DCs Promote Th17 Cells in Memory but Not in Naive Th Cells

Subsequently, we addressed the question of how PGN-primed human DCs promote Th17 production. Recent mouse model studies have demonstrated that IL-17 production and Th17 cell development can be induced in naive Th cell precursors under the influence of the combination of TGF-β and IL-6 (Veldhoen et al., 2006a; Mangan et al., 2006; Bettelli et al., 2006). Indeed, in our control experiments, Th17 cells did develop from mouse naive T cells in the presence of these cytokines (Figure 3A, left). In these experiments, a low frequency of Th17 cells also arose in response to PGN-primed DCs (Figure 3B, left), which was amplified by the additional presence of either TGF-β or IL-6 (data not shown), and, to a greater extent, by the combination of these cytokines (Figure 3B). In contrast, Th17 cells never arose from human naive T cells stimulated by PGN-primed DCs, in the additional presence of either SEB (Figure 3B, right) or anti-CD3 (Figure S3A) or by anti-CD3 and anti-CD28 with or without TGF-β and IL-6 (Figure 3A, right). SMAD3 and STAT3 phosphorylation confirmed the bioactivity of TGF-β and IL-6, respectively, in the human T cells (Figure S4). Moreover, the addition of neutralizing antibodies against IL-12, IL-4, and IFN-γ, cytokines that inhibit Th17 development (Harrington et al., 2005; Park et al., 2005; Hoeve et al., 2006), did not alter the outcome of the results (Figures S3B and S3C).

It was shown previously that the expression of IL-17 in T cells is promoted by IL-23 in previously activated (memory) Th cells (Aggarwal et al., 2003), which is amplified by the additional presence of IL-1 (Kidoya et al., 2005; Sutton et al., 2006). The critical role of the IL-23 pathway in human cells was suggested by the finding that PGN-primed DCs promoted IL-17 production in human (and mouse) memory T cells (Figure 3D). Control experiments confirmed that the combination of IL-23 and IL-1 readily promoted high percentages of Th17 cells in human (and mouse) memory Th cells (Figure 3F) but not naive Th cells (Figure 3E).

Enhancement of Th17 Cells Requires IL-23 and IL-1 Production by DCs

In order to determine to what extent IL-23 is the major Th17-promoting factor of PGN-primed DCs, we first compared the expression of mRNA encoding the IL-23-specific p19 subunit and mRNA encoding the IL-12-specific p35 subunit in differentially activated DCs. Strikingly, PGN-activated DCs expressed high and sustained amounts of p19 mRNA in the virtual absence of IL-12-specific p35 mRNA (Figure 4A), which is a negative regulator of IL-17 (Hoeve et al., 2006). To confirm the role of PGN-primed DC-derived IL-23 protein in promoting Th17 cells, we made use of a neutralizing polyclonal antibody that binds to the p40 subunit shared by IL-23 and IL-12 and thereby neutralizes both IL-23 and IL-12, and also a monoclonal antibody (20C2) that specifically binds IL-12 p35 and thereby selectively neutralizes IL-12. No antibodies

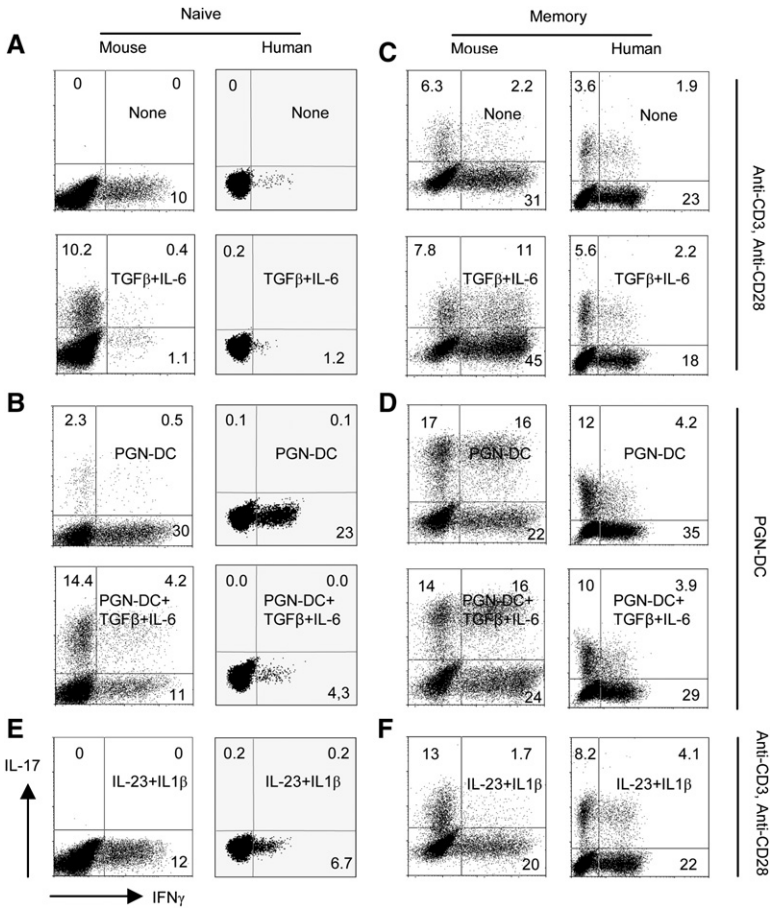

Figure 3. Both PGN-Primed moDCs and the Combination of TGF-β and IL-6 Are Not Capable of Inducing IL-17 in Human Naive CD4$^+$ T Cells

Human naive CD4$^+$CD45RA$^+$ T cells or murine naive CD4$^+$CD62L$^+$CD44$^-$ T cells (A, E) and human memory CD4$^+$CD45RO$^+$ and murine memory CD4$^+$CD62L$^-$CD44$^+$ T cells (C, F) were stimulated with anti-CD3 and anti-CD28 in the absence or presence of TGF-β (10 ng/ml) and IL-6 (50 ng/ml) (A, C) or IL-23 (10 ng/ml) and IL-1β (10 ng/ml) (E, F). Human or murine naive CD4$^+$ T cells (B) and human and murine memory CD4$^+$ T cells (D) were stimulated with PGN-DCs in the presence of SEB (human culture) or PGN-BMDCs in the presence of anti-CD3 (murine culture) in the absence or presence of TGF-β (10 ng/ml) and IL-6 (50 ng/ml). After 12 days (human) or 7 days (mouse), restimulated with PMA and ionomycin and the frequency of IL-17- and IFN-γ-producing T cells was determined by intracellular FACS staining. Data are representative of five independent experiments.

selectively neutralizing IL-23 p19-p40 heterodimer are currently available. In cultures of memory T cells driven by PGN-primed DCs, both antibodies, as expected, inhibited IFN-γ production (Figure 4B, right), which was driven by DC-derived IL-12 and to a lesser extent by DC-derived IL-23. In contrast, IL-17 production (Figure 4B, left) was inhibited only by the polyclonal antiserum that blocks both IL-12 and IL-23, and not by 20C2 that selectively blocks IL-12, indicating that PGN-primed DCs promote IL-17 production via the production of IL-23.

IL-1β and IL-1α have been implicated to synergize with IL-23 in the induction of IL-17 by murine Th cells (Kidoya et al., 2005; Sutton et al., 2006). We found that human DCs primed by TLR agonists produced enhanced amounts of both IL-1α and IL-1β, and PGN-induced amounts were significantly enhanced over the other TLR ligands (Figure 4C). Blocking experiments with neutralizing antibodies revealed that the IL-17 production in memory T cells by PGN-primed DCs was dependent on IL-1, in particular IL-1α, produced by these DCs (Figure 4D). Neutralization of both isoforms always almost completely abolished IL-17 production (Figure 4D), stressing the overlapping function of IL-1α and IL-1β in promoting IL-17 by PGN-primed DCs. The synergy between IL-1 and IL-23 in promoting IL-17 production in memory T cells was confirmed in experiments with exogenous recombinant cytokines (Figure S5). Thus, PGN-primed DCs promote IL-17 production in memory CD4 T cells via an IL-23- and IL-1-dependent pathway.

DCs Are Programmed to Promote Th17 Cells upon Sensing of Bacterial Muramypdipetide by NOD2

As is evident from the experiments described in Figures 2A–2C, of all TLR2 ligands tested, only PGN could prime DCs for the capacity to promote Th17 cells. This may be explained by the fact that PGN, after internalization, is metabolized into MDP, which is a ligand for the intracellular NOD2 (Girardin et al., 2003). Recent studies have shown that NOD2 activation by MDP modulates the effects of TLR agonists in various cell types, including DCs (Tada et al., 2005). To analyze the effect of MDP ligation on the IL-17- and Th17-inducing capacity of DCs, these cells were activated with various TLR ligands in the absence or presence of MDP and subsequently tested for their capacity to induce IL-17 production. These experiments revealed that, although MDP by itself was inactive in this respect and did not add to the effect of PGN, MDP together with other TLR agonists enhanced the capacity of DCs to specifically promote IL-17, but not IFN-γ, production in memory Th cells (Figure 5A). Cell proliferation data indicated that these effects were not attributable to changes in cell proliferation (data not shown). To dissect

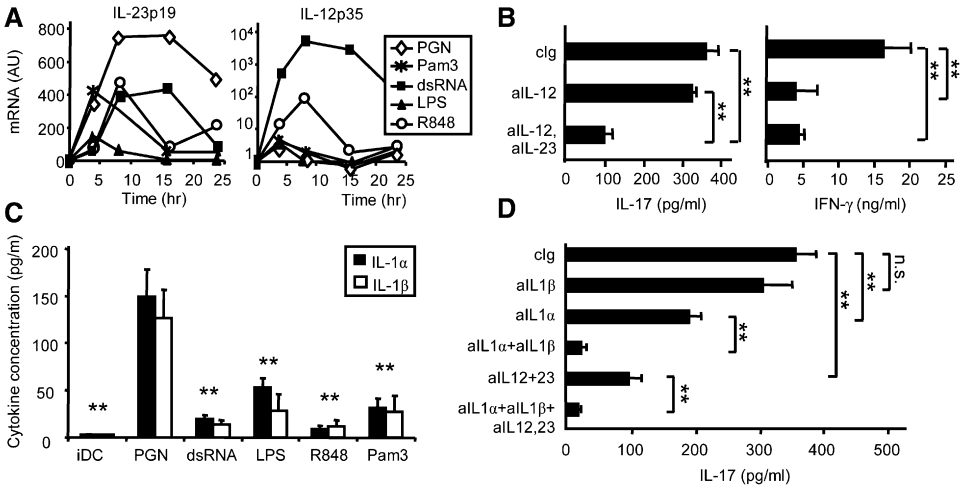

Figure 4. Selective Induction of IL-23p19 mRNA and IL-1 Production by PGN

DCs were stimulated with PGN, Pam3CSK4, dsRNA (poly I:C), LPS, and R848.

(A) After 0–24 hr (indicated in figure), cells were collected and processed for mRNA quantification by RT-PCR. Data are normalized to GAPDH and expressed in arbitrary units (AU), representing mRNA induction compared to unstimulated cells. Left: IL-23p19; right: IL-12p35.

(B) PGN-primed DCs were cocultured with memory $CD4^+CD45RO^+$ T cells and SEB as described in the legend of Figure 1, in the additional presence of an isotype control to both antibodies (clg), a monoclonal αIL-12 antibody (20C2), or a polyclonal antibody that blocks the action of both IL-12 and IL-23. After 4 days, supernatants were harvested and analyzed for IL-17 and IFN-γ production by ELISA. Data are shown as means ± SD of triplicate cultures.

(C) Amounts of IL-1α and IL-1β were measured by ELISA in 24 hr supernatants. Data are shown as means ± SD of triplicate cultures.

(D) PGN-primed DCs were cocultured with memory $CD4^+CD45RO^+$ T cells and SEB as described in the legend of Figure 1, in the additional presence of neutralizing antibodies against IL-1α, IL-1β, IL-12 (20C2), IL-12 and IL-23, or an isotype control to all antibodies (clg). After 4 days, supernatants were harvested and analyzed for IL-17 and IFN-γ production by ELISA. Data are shown as means ± SD of triplicate cultures.

*$p < 0.05$, **$p < 0.01$. Data are representative of three independent experiments.

this mechanism, we used synthetic TLR2 ligand Pam3CSK4 with or without MDP to stimulate DCs. MDP by itself was unable to induce expression of IL-23p19 mRNA, but it upregulated suboptimal Pam3CSK4-induced IL-23p19 expression (Figure 5B). Furthermore, activation of DCs by MDP enhances the production of both IL-1α and IL-1β, which was described previously to occur for IL-1β (Figure 5C; Strober et al., 2006). The critical role of enhanced expression of both IL-23 and IL-1α and IL-1β by DCs in MDP-induced IL-17 production could be confirmed with blocking antibodies against IL-12 and IL-23, IL-1α and IL-1β (Figure 5D). Thus, MDP enhances TLR agonist-induced IL-23 and IL-1, hereby promoting IL-17 expression in T cells.

To substantiate the role of NOD2 activation in MDP-mediated induction of IL-17 production, we tested the function of monocyte-derived DCs from patients with Crohn's disease (CD) carrying double-dose *NOD2* mutation in the leucine-rich repeat domain, responsible for ligand binding (double-dose heterozygote or homozygote) (Hugot et al., 2001; Ogura et al., 2001), leading to impaired signaling (Strober et al., 2006). As expected, in contrast to Pam3CSK4-primed DCs from individuals with wild-type *NOD2*, MDP was unable to enhance the IL-17-inducing capacity of Pam3CSK4-primed DCs from *NOD2* mutant CD patients (Figure 6A). At the same time, the capacity to enhance IFN-γ production was not affected by the mutation, underscoring the fact that these cells are not altered in their ability to produce cytokines that are unaffected by NOD2 signaling. In addition, and in agreement with the expectation, MDP-dependent upregulation of IL-23p19 mRNA (Figure 6B), IL-1α, and IL-1β (Figure 6C) was abrogated in DCs from *NOD2* mutant CD patients in this experimental set-up. In addition, PGN induced significantly lower amounts of IL-23p19 mRNA in *NOD2* mutant DCs, as expressed as the ratio between PGN and LPS (which is a NOD2-unrelated reference TLR agonist) per individual (Figure 6D). Concomitantly, PGN induced significantly lower amounts of IL-1α and IL-1β in DCs from *NOD2* mutant CD patients compared to WT controls (Figure 6E). To further confirm the role of NOD2 in IL-17 induction, we used siRNA transfection with a transfection efficiency greater than 90% (Figure S6A), which specifically suppressed NOD2 expression both in resting as well as MDP- or LPS-stimulated wild-type DCs (Figure S6B). The dysfunction of NOD2 was evident in control experiments showing suppression of MDP-induced TNF-α and IL-8 expression by transfection with NOD2-specific siRNA and not with control siRNA (Figure S6C). Further control experiments showed that MDP failed to enhance the PAM3CSK4-induced expression of IL-23p19 mRNA (Figure S7A) in these NOD2 knockdown DCs and the ability of these cells to promote IL-17 production in memory T cells (Figure S7B). The final experiments indicated that PGN-primed NOD2-knockdown DCs, compared to control-knockdown DCs, indeed have reduced capacity to induce IL-17 production in memory T cells (Figure 6F). In addition, these cells show a significantly

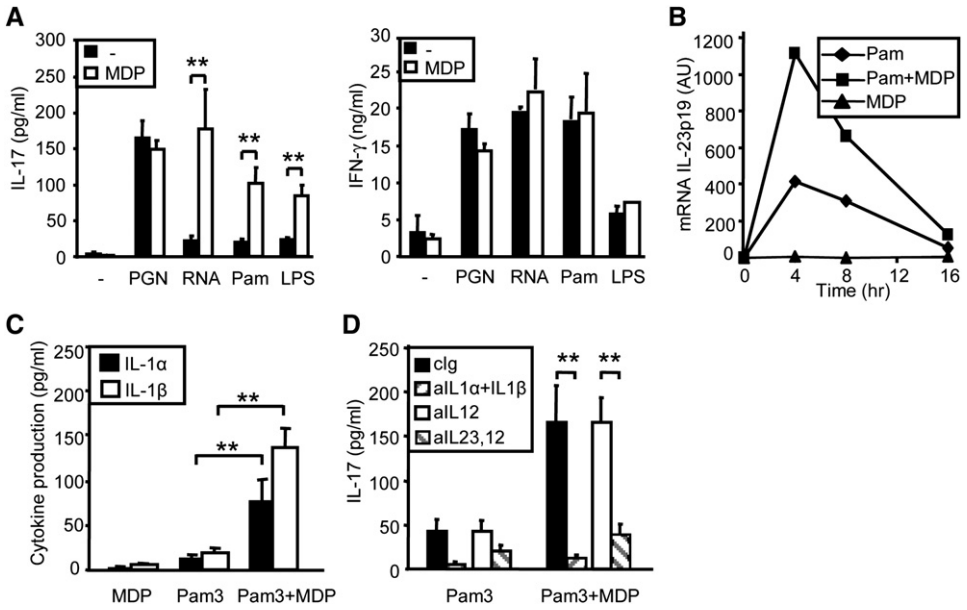

Figure 5. DCs Activated by NOD2 Ligand MDP Induce the Production of IL-17 and Promote Th17 Cells

DCs were primed for 16 hr with PGN, Pam3CSK4, dsRNA, and LPS, with or without MDP (10 μg/ml), and cocultured with $CD4^+CD45RO^+$ Th cells and SEB.

(A) After 4 days, IL-17 and IFN-γ were determined in supernatant by ELISA. Data are shown as means ± SD of triplicate cultures.

(B and C) DCs were activated by MDP, Pam3CSK4, or the combination.

(B) IL-23p19 mRNA expression (0–16 hr) was determined by RT-PCR and normalized to GAPDH.

(C) IL-1α and IL-1β concentrations were measured (24 hr) by ELISA. Data are shown as means ± SD of triplicate cultures.

(D) DCs primed with Pam3CSK4 ± MDP were cocultured with $CD4^+CD45RO^+$ T cells in the presence of neutralizing Ab against IL-1α and IL-1β, IL-12 (20C2), IL-12 and IL-23, or an isotype control to all antibodies (cIg). After 4 days, IL-17 and IFN-γ production were determined in supernatant by ELISA. Data are shown as means ± SD of triplicate cultures.

*$p < 0.05$, **$p < 0.01$. Data are representative of five (A) or three (B–D) independent experiments.

lower expression of IL-23p19 mRNA ratio (Figure 6G), as well as reduced production of IL-1α and IL-1β (Figure 6H). Thus, PGN and MDP promote IL-17 in T cells upon ligation of NOD2.

DISCUSSION

This study is focused on the mechanism underlying Th17-mediated immunity against bacteria in humans. Our data indicated that bacteria prime human DCs to promote IL-17 expression in memory Th cells through NOD2 ligand MDP via enhanced expression of IL-23 and IL-1. Ligation of NOD2 by MDP selectively enhanced IL-17, where IFN-γ was not affected. In contrast to what has been reported for mice (Harrington et al., 2005; Park et al., 2005), a substantial frequency of the human IL-17-producing cells coexpress IFN-γ, but not IL-4, underscoring the relationship between Th17 and Th1 responses (Aggarwal et al., 2003; Mathur et al., 2006). Also, in contrast to the murine system, we found that the combination of TGF-β and IL-6 could not induce development of Th17 cells from human naive T cells. Our current studies focused on the alternative transcriptional control of induction of Th17 cells in human naive T cells. The finding that DCs promoted IL-17 production exclusively in memory T cells, through IL-23 and IL-1, is in agreement with several studies that have shown that IL-23 is predominantly involved in Th17 development from previously activated cells (Aggarwal et al., 2003; Langrish et al., 2005; Sutton et al., 2006). Our data are in line with the finding that IL-23 and IL-1 are crucial in Th17-mediated protection against various bacteria in mice (Mangan et al., 2006; Happel et al., 2005; Malley et al., 2006; Zwijnenburg et al., 2003; Miller et al., 2006). In addition, to our knowledge, this is the first demonstration that antibacterial Th17 cells can be promoted by human DCs upon sensing of NOD2 ligand MDP, a PGN derivative expressed by many bacterial species.

This crucial role of NOD2 in adaptive antibacterial immunity may not be surprising when taking into consideration that NOD2 has also a role in the natural defense against bacteria by mediating the induction of the antimicrobial peptide cryptdins in the intestine (Kobayashi et al., 2005). Napolitani et al. (2005) previously reported that certain TLR-agonist combinations synergistically trigger a Th1-polarizing program in DCs, introducing the concept that the type of immune response is boosted by the combinational activation of DCs via selected pattern recognition receptors. We extend the significance of this concept by showing that bacteria selectively trigger a protective type of Th17-polarizing program by activating DCs through MDP in combination with obligate bacterial-derived TLR agonists, resulting in enhanced TLR

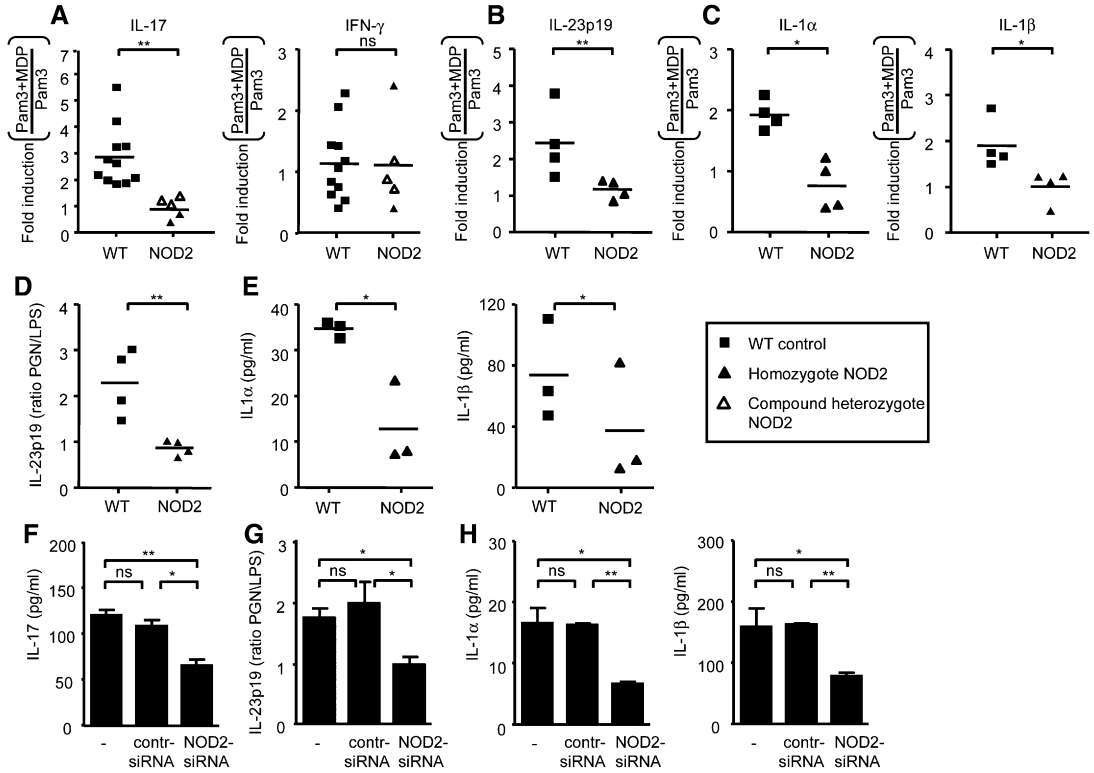

Figure 6. DCs Defective in Their NOD2 Have Reduced Capacity to Induce Enhanced Levels of IL-17 when Primed with Pam3CSK4 and MDP or with PGN

(A) DCs from NOD2-deficient CD patients (n = 5) (double-dose heterozygotes [n = 3] or homozygotes [n = 2]) and healthy WT controls (n = 11) were primed with Pam3CSK with or without MDP and cocultured with memory T cells as described in the legend of Figure 1. Supernatants were harvested (day 4) and analyzed for IL-17 and IFN-γ production. Data are shown as induction of cytokine production induced by MDP + Pam3CSK4 over Pam3CSK4 alone.

(B and C) DCs from NOD2-deficient CD patients (homozygotes [n = 4] and healthy controls [n = 4]) were stimulated with Pam3CSK with or without MDP.

(B) IL-23p19 mRNA expression (6 hr) was determined by RT-PCR and normalized to GAPDH.

(C) The concentration of IL-1α and IL-1β was determined by ELISA in 24 hr supernatants.

(D and E) Alternatively, cells were stimulated with PGN.

(D) The expression of IL-23p19 mRNA (normalized to GAPDH) was established as a ratio with LPS as control value.

(E) IL-1α and IL-1β were measured in supernatant (24 hr).

(A–E) Results are shown as individual donor data (each value measured in triplicate). Significant differences were determined by a paired t test comparing values of experiments conducted the same day of NOD2-deficient donors with healthy WT control donors.

(F–H) DCs were transfected with siRNA specific for NOD2 or scrambled control siRNA and after 48 hr were activated by PGN. Data are shown as means ± SD of triplicate cultures. Data are representative of three independent experiments.

(F) Transfected cells were stimulated with PGN and cocultured with $CD4^+CD45RO^+$ T cells. IL-17 concentration in supernatant (day 4) was determined by ELISA.

(G) Prepared for RT-PCR (6 hr) for PGN-induced IL-23p19. Data are expressed similar as in [D].

(H) Supernatants (24 hr) were measured for PGN-induced IL-1α and IL-1β production.

*$p < 0.05$, **$p < 0.01$.

agonist-mediated expression of the IL-17-inducing cytokines IL-23 and IL-1. This modulatory effect of MDP on the activation of DCs by TLR agonists may also explain why TLR4 was implicated in the priming of DCs promoting Th17 (Happel et al., 2003; Higgins et al., 2006). TLR4 ligand LPS is dominantly expressed by Gram-negative bacteria and could very well be crucial in the combinational MDP-TLR-mediated activation of DCs by Gram-negative bacteria.

Our data revealed that DCs derived from NOD2-deficient CD patients have an impaired capacity to induce IL-17 expression upon MDP triggering, whereas their capacity to induce IFN-γ expression was intact. This can be attributed to the loss of the synergistic effects of MDP on TLR-mediated triggering of IL-1α and IL-1β, and IL-23. Indeed, IL-23p19 expression was not enhanced by MDP or reduced by PGN in NOD2-deficient DCs from patients, as well as production of IL-1α and IL-1β. The latter finding is in line with reports on reduced MDP-mediated production of IL-1β in monocytes (Netea et al., 2005) and PBMC (van Heel et al., 2005) from NOD2-deficient CD patients compared to healthy controls. Our findings are further supported by experiments in DCs that do not express NOD2 upon siRNA treatment that show reduced

capacity to promote IL-17 in T cells and reduced expression of IL-23p19 and IL-1α and Il-1β.

It is currently not entirely clear how NOD2 deficiency contributes to the pathogenesis of CD. A strongly advocated concept is that the abnormal adaptive immune responses as observed in CD results from initially ineffective bacterial clearance (Strober et al., 2006; Kelsall, 2005), which in *NOD2* mutant patients may result from reduced expression of antibacterial peptides (Kobayashi et al., 2005) and other antibacterial responses, including IL-8 (van Heel et al., 2005) and induction of Th17 cells (this study). Paradoxically, both in mice and in humans, it is suggested that the IL-23-IL-17 axis is instrumental in the effector phase of active inflammatory bowel disease (Fujino et al., 2003; Zhang et al., 2006; Yen et al., 2006; Elson et al., 2007). It may be hypothesized that these Th17 cells result from a secondary phase of IL-23 production driven by factors that are generated during the uncontrolled inflammation after deficient bacterial clearance in *NOD2* mutant patients. The association for CD with the IL-23-Th17 axis is further supported by a recent study identifying *IL-23R* as an inflammatory bowel disease gene (Duerr et al., 2006). We did not reveal a clear-cut reduction of the frequency of Th17 cells in lesions of NOD2-deficient CD patients compared to NOD2-efficient CD patients (data not shown). This finding, however, is in line with the concept that CD, irrespective of the presence of a mutation in the *NOD2* gene, is mainly caused by dysfunctional Th17 cell responsiveness, regardless of whether this is due to a failure of *NOD2*, *IL-23R*, or any of the many other gene(s) initiating and regulating in the NOD2-IL-23-IL-1-IL-17 pathway. Thus, the link between NOD2 and IL-23 in antibacterial immunity may help to understand why polymorphisms in either *NOD2* (Hugot et al., 2001; Ogura et al., 2001) or *IL-23R* (Duerr et al., 2006) genes may contribute to the development of Crohn's disease.

EXPERIMENTAL PROCEDURES

Reagents

Neutralizing polyclonal rabbit IgG to human IL-12 and IL-23 and neutralizing monoclonal Ab to human IL-12 (20C2) were purchased from U-cytech (Utrecht, The Netherlands). Neutralizing Ab against IL-1α was purchased from R&D and neutralizing Ab against IL-1β were a kind gift from L. Aarden (Sanquin research Amsterdam, The Netherlands). Recombinant human IL-1α was purchased from R&D, rhIL-1β from Bioscource, rhIL-23 from R&D, rmIL-23 from R&D, IL-6 from Sanquin, and TGF-β from R&D. All human cultures were done in IMDM (Life Technologies) containing 10% FCS (HyClone) supplemented with gentamycin (86 μg/ml, Duchefa), all mouse cultured were performed in RPMI (GIBCO) 10% FCS (HyClone) supplemented with L-glutamin (200 mM, Life), 2-mercaptoethanol (50 μM, Sigma) and gentamycin (86 μg/ml, Duchefa). TLR ligands (PGN, LTA, Pam3CSK4, dsRNA [Poly I:C], flagellin [*S. thyphimurium*], FSL-1, and R848) were purchased at Invivogen; LPS (*E. coli*) and MDP at Sigma.

Bacteria and Viruses

Bacteria were cultured to the logarithmic phase in trypticase soy broth (TSB; Difco) at 37°C. *S. pneumoniae* was grown in the presence of 5% CO_2. Bacteria were washed in PBS, resuspended in PBS to a concentration of 10^9 CFU per ml, killed by a 30–240 min exposure to UV light, and stored at −80°C. Killing of the bacteria was confirmed by negative plate cultures of the UV-exposed bacteria. The following bacteria were used: Gram-negative: *K. pneumoniae*, clinical isolate, *E. coli* ML35, *N. meningitidis* H44/76 (Vanderende et al., 1995); Gram-positive: *S. aureus* 42D (Smits et al., 2005), *S. pneumoniae* D39 (NCTC 7466), *C. Xerosis*, clinical isolate. Clinical isolates of VZV and CMV were kindly provided by H.W.M. van Eijk (Amsterdam Medical Center, Department of Clinical Virology, Amsterdam, The Netherlands); and influenza A/PR/8/34 was a kind gift of G. Rimmelzwaan (Erasmus Medical Center, Department of Virology, Rotterdam, The Netherlands). All viruses were inactivated at 56°C for 30 min prior to use.

Isolation of Human and Murine Memory and Naive Th Cells

Human PBMC were isolated from heparinized human peripheral blood by density gradient centrifugation on Lymphoprep (Nycomed), and PBMC were subsequently seperated in monocytes and PBL by density gradient centrifugation on Percoll (Pharmacia). Untouched $CD4^+$ cells were isolated from the PBL fraction to high purity (>98% as assessed by flow cytometry) with a MACS Isolation kit for $CD4^+$ cells (Miltenyi). Naive $CD4^+$ T cells were FACS sorted by gating on $CD4^+CCR7^+CD45RA^+CD45RO^-$ at >99% purity. Memory $CD4^+$ T cells were isolated with $CD45RO^+$-PE (Dakopatts) and anti-PE-beads (MACS). Untouched mouse $CD4^+$ T cells were isolated from a C57BL/6 spleen cell suspension with a MACS Isolation kit for $CD4^+$ cells (Miltenyi). Effector memory ($CD4^+CD62L^-$), central memory ($CD4^+CD62L^+CD44^+$), and naive ($CD4^+CD62L^+CD44^-$) cells (CD62L APC and CD44-PE from Becton) were isolated by sort. C57BL/6 mice were bred under SPF conditions and kept in conventional but pathogen-free animal facilities in accordance with local guidelines.

In Vitro Generation and Activation of Human DCs and T Cell Stimulation

Immature DCs were generated by culture of peripheral blood monocytes as described previously (de Jong et al., 2002). Bone-marrow-derived DCs were generated from C57BL/6 mice by culturing bone-marrow cells for 7 days in the presence of GM-CSF (5% culture supernatant of X63 hybridoma, kind gift of K. Mahnke, Heidelberg) as described initially by Inaba et al. (1992). To stimulate T cells, DCs were activated by bacteria, viruses, or TLR ligands for 16 hr and subsequently cocultured with $CD4^+$ T cells (human DCs, 20,000 T cells/well with 2,000 DCs in the presence of *Staphylococcus aureus* enterotoxin B [SEB, 100 pg/ml; Sigma-Aldrich]; murine DCs, 50,000 T cells/well with 5,000 BMDCs in the presence of anti-CD3). In the DC-independent system (used in Figure 3), human T cells were stimulated with plate-bound anti-human-CD3 (16A9; Sanquin, 1 μg/ml) and anti-human-CD28 (5E8, Sanquin, 1 μg/ml), murine T cells were stimulated with anti-mouse-CD3 (10 μg/ml, BD PharMingen) and anti-mouse-CD28 (1 μg/ml, BD PharMingen). From human cultures, after 4 days supernatants were harvested and analyzed for IL-17 (Biosource) and IFN-γ (Ucytech) production. Cells were further cultured in the presence of 10 U/ml IL-2 (Chiron) and resting cells were restimulated at day 12 with PMA (100 ng/ml) and ionomycin (1 μg/ml) for 6 hr, the last 4 hr in the additional presence of brefeldin A (10 μg/ml) as described previously (de Jong et al., 2002) and analyzed for the intracellular production of IFN-γ (PharMingen), IL-4 (PharMingen), and IL-17 (R&D), second Ab α-mouse-PE (Jackson). Murine T cells were cultured in the presence of 10 U/ml IL-2 (R&D) and restimulated at day 7 and analyzed for intracellular IFN-γ (BD) and IL-17 (BD). In several experiments, DCs (40,000 cells/well) were also stimulated for product analysis by PCR (IL-12p35, IL-23p19) or ELISA (IL-1β from Endogen, Rockford, IL, and IL-1α from R&D).

Real-Time Quantitative RT-PCR Analyses

Quantitative RT-PCR (iCycler iQ Multi-Color Real Time PCR Detection System; Bio-Rad) was performed with specific primers general SYBR green (iQ SYBR Green supermix, 2×, Bio-Rad) fluorescence detection. RNA extraction was done by NucleoSpin RNA Isolation Kit, Macherey-Nagel, and for cDNA synthesis the kit of MBI Fermentas was

used. Expression was normalized to GAPDH expression. Primers used: IL-23p19: 5′-GTGGGACACATGGATCTAAGAGAAG, 3′-TTTGCAAGCAGAACTGACTGTTG; IL-12p35: 5′-AGATGTACCAGGTGGAGTTCAAGAC, 3′-AAATTCAGGGCCTGCATCAG; GAPDH (Magner et al., 2000): 5′-GAAGGTGAAGGTCGGAGTC, 3′-GAAGATGGTGATGGGATTTC. Product size: IL-23p19, 124 bp; IL-12p35, 121 bp; and GAPDH, 225 bp. The reaction protocol was identical for all PCR products: first a 3 min incubation at 94°C, followed by 45 cycles of sequential incubations at 94°C (30 s), 61°C (30 s), and finally 72°C (1 min) for data collection. A bulk cDNA sample of CD40L-stimulated human moDCs was used as a standard, and normalization to GAPDH was performed for each sample.

siRNA

siRNAs specific for NOD2 and random controls (100 nM, Dharmacon) were transfected with a 1:250 dilution of lipofectamin 2000 (Invitrogen) for 4 hr. Subsequently, after 48 hr, DCs were stimulated as indicated in Figure 6. Transfection efficiency was tested after 48 hr with FAM-labeled siRNA (Figure S5).

NOD2 Mutant Patients

Patients with Crohn's disease carrying double-dose *NOD2* mutation, in the leucine-rich reapeat domain that is responsible for ligand binding, double-dose heterozygotes (n = 3), or homozygotes (n = 4) (Braat et al., 2005), were selected from the IBD database at the Department of Gastroenterology and Hepatology, Academic Medical Center, Amsterdam, The Netherlands. Patients were confirmed to be negative for mutations *TLR4* 299 (Child et al., 2003). At the moment of the inclusion in the study, all patients were in remission for at least 1 year. Patient characteristics are detailed in Table S1. All patients and healthy volunteers were included according to the Helsinki convention after informed consent had been obtained.

Genotyping of *NOD2*

Variants Crohn's disease patients and healthy controls were genotyped for three with CD-associated variants of *NOD2* gene (R702W, G908R, and 3020Cins) as described previously (Braat et al., 2005). In brief, genomic DNA was amplified in polymerase chain reaction with specific primers creating different restriction sites in the wild-type and mutant alleles, respectively. The amplified products were digested overnight with the restriction enzymes, and the digestion products were separated by electrophoresis on 3% agarose (Eurogentec s.a.) gels containing 0.5 μl/ml ethidium bromide and viewed with the GeneGenius (Syngene, Cambridge, UK).

Intracellular Staining of Phosphorylated Protein

Cells were fixed and permeabilized with cytofix/cytoperm reagent (BD Biosciences) for 10 min at 37°C, then incubated with cold methanol (90%) for 30 min. Staining of the cells was done with anti-phospho-STAT3-APC (BD PharMingen) or anti-phospho-SMAD3 (Calbiochem) for 1 hr, the latter followed by goat-anti-rat-ALEXA 488 (Molecular Probes).

Statistical Analyses

Data were analyzed for statistical significance (GraphPad InStat) by Student's t test. A p value < 0.05 was considered to be significant. Unless stated otherwise, data show a representative out of at least three experiments.

Supplemental Data

Seven figures and one table are available at http://www.immunity.com/cgi/content/full/27/4/660/DC1/.

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