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IL-22 Increases the Innate Immunity of Tissues

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Summary

Interleukin 22 (IL-22) is mainly produced by activated Th1 cells. The data presented here indicate that neither resting nor activated immune cells express IL-22 receptor, and IL-22 did not have any effects on these cells in vitro and in vivo. In contrast, cells of the skin and the digestive and respiratory systems represent putative targets of this cytokine. The expression of IL-22 receptor in keratinocytes was upregulated by Interferon-γ. In these cells, IL-22 activated STAT3 and directly and transcriptionally increased the expression of β-Defensin 2 and β-Defensin 3. High levels of IL-22 were associated with strongly upregulated β-Defensin expression in skin from patients with T cell-mediated dermatoses. Taken together, IL-22 does not serve the communication between immune cells but is a T cell mediator that directly promotes the innate, nonspecific immunity of tissues.

Introduction

IL-22, together with IL-10, IL-19, IL-20, IL-24, and IL-26, belongs to the IL-10 family of cytokines. IL-22 was first described in 2000 as a gene differentially expressed in IL-9-treated BW5147 murine T lymphoma cells (Dumoutier et al., 2000a). The human counterpart was identified a few months later based on its similarity to mouse IL-22 and human IL-10 (Dumoutier et al., 2000b; Xie et al., 2000). Both human and mouse cDNA encode proteins of 179 amino acids with amino acid positions characteristic of the IL-10 family. They are 22% and 25% identical to human and mouse IL-10, respectively, and 79% identical to each other. The human IL-22-encoding gene is located on chromosome 12q15 near the Interferon-γ (IFN-γ) and the IL-26 locus. It comprises six exons and shows an organization similar to the genes of the other IL-10 family members (Dumoutier et al., 2000c). In mice the IL-22 gene was mapped on chromosome 10 also near the IFN-γ gene. The existence of a second gene copy was found in the genome of some mouse strains, but no evidence for its expression could be demonstrated (Dumoutier et al., 2000c).

Like all members of the IL-10 family, the mature IL-22 protein is a secreted, α-helical molecule (Dumoutier et al., 2000a; Xie et al., 2000). Major sources of IL-22 are activated T and NK cells (Wolk et al., 2002). Polarization of T cells toward the type 1 (T1) phenotype further increases the activation-induced IL-22 expression, whereas polarization toward the type 2 (T2) or the regulatory phenotype reduces it. Highest IL-22 expression has been detected in CD4+ memory cells. In contrast, neither resting nor activated monocytes or B cells express this cytokine (Wolk et al., 2002).

Like all other members of the IL-10 family, IL-22 exerts its biological effects via members of the cytokine receptor family class 2. The cell surface-standing IL-22 receptor complex consists of the receptor chains IL-22R1 and IL-10R2 (Dumoutier et al., 2000b; Xie et al., 2000; Kotenko et al., 2001a). The latter also functions as an accessory receptor chain for the IL-10, IL-26, and the IL-28/IL-29 receptor complex (Langer et al., 2004). Rapid STAT tyrosine phosphorylation upon IL-22 treatment was observed in several tissue cell lines (Dumoutier et al., 2000a, 2000b; Xie et al., 2000; Aggarwal et al., 2001; Lejeune et al., 2002). As demonstrated in the H4IIE rat hepatoma cell line, recombinant mouse IL-22 also induced phosphorylation of Jak1 and Tyk2. Moreover, activation of the three major MAP kinase pathways and serine phosphorylation of STAT3 was suggested to be required for maximum IL-22-induced transactivation of STAT-responsive promoter in these cells (Lejeune et al., 2002).

In addition to the cell surface IL-22 receptor complex, there is a soluble, single-chain IL-22 receptor named IL-22 binding protein (IL-22BP), IL-22RA2, or CRF2s short, which also demonstrates the features of the extracellular domain of the class 2 cytokine receptors. It has been shown to antagonize IL-22 cellular binding and signaling in vitro (Dumoutier et al., 2001; Kotenko et al., 2001b; Xu et al., 2001; Gruenberg et al., 2001).

Although different cell lines have been shown to express functional cell surface-standing IL-22 receptor complex, little is known about the biological role of IL-22. In the HepG2 human hepatoma cell line, IL-22 upregulated mRNA expression of acute phase reactants such as Serum amyloid A (SAA), α1-Antichymotrypsin, and Haptoglobin. Rapid induction of SAA-specific mRNA was also observed in mice upon application of murine IL-22 (Dumoutier et al., 2000b). Additionally, murine IL-22 was shown to induce mRNA expression of Pancreatitis-associated protein-1 in pancreas acinar cells and in pancreas in vivo, a protein of unknown function that has been associated with acute pancreatitis (Aggarwal et al., 2001).

In order to achieve a better insight into the biological importance of IL-22, this study aimed at the characterization of its targets by systematic analyses of cellular

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IL-22 receptor expression and of the biological effects of IL-22 on selected cell populations.

Results

Lack of IL-22 Receptor Expression in Immune Cells

The close protein family relation between IL-22 to IL-10 allowed us to suppose that IL-22 could also have extensive effects on immune cells. However, we have recently shown that freshly isolated human monocytes, T cells, and NK cells do not express IL-22R1, which is the specific subunit of the IL-22 receptor complex. In B cells, IL-22R1 expression levels near the detection limit of the measuring method were detected (Wolk et al., 2002). Simultaneously, clear expression of IL-10R2, the second subunit of the IL-22 receptor that also functions as an R2 chain of the IL-10, the IL-26, and the IL-28/IL-29 receptor complexes, was found in resting monocytes, B, T, and NK cells. As there are Interleukin receptors that are only expressed upon cellular activation (e.g., IL-2Rα), we now asked whether stimulated immune cells would express IL-22R1. Negatively separated monocytes and B, T, and NK cells from the blood of different healthy donors were cultured in the presence or absence of the typical cell-specific stimuli lipopolysaccharide (LPS) (monocytes) and fixed *Staphylococcus aureus* (B cells), immobilized anti-CD3 monoclonal antibody (mAb) (T cells), and IL-2/IL-12 (NK cells), respectively, for 2, 6, and 18 hr. Expression of IL-22R1 and IL-10R2 was quantitatively analyzed by real-time polymerase chain reaction on reverse transcribed mRNA (qPCR). As shown in Figure 1A, no IL-22R1 expression was induced upon stimulation of either of the different cell populations. Interestingly, the expression of IL-10R2 was slightly downregulated at all time points in B, T, and NK cells, and at the first two time points in monocytes. To analyze whether additional costimulation or the presence of cytokines known to induce functional polarization would induce IL-22R1 expression in T cells, the latter were cultured on anti-CD3 mAb/anti-CD28 mAb-coated vessels in culture milieus known to induce polarization toward T1 cells, T2 cells, or regulatory T cells, or let without polarization for 6 and 18 hr like above, and additionally for 42 and 66 hr (Figure 1B). Again, no expression of IL-22R1 was observed in either of these groups. IL-10R2 downregulation was even much more pronounced in case of costimulation but did not vary between different polarization groups. IL-22R1 expression was not detected either in resting and LPS-activated macrophages (Mϕ) and dendritic cells (DCs) derived from primary human monocytes (Figure 1C). In these cells, activation also led to downregulated expression of IL-10R2. In contrast to immune cells, primary human hepatocytes, one of the two cellular targets of IL-22 action known so far, show clear expression of both IL-22R1 and IL-10R2. Interestingly, the expression levels of IL-22R1 and IL-10R2 in these cells were not modulated by IL-6-induced activation (Figure 1D). In summary, our data suggest that IL-22 acts neither on resting nor activated immune cells.

Lacking Regulation of Immune Cell Function by IL-22

To confirm the suggested insensitivity of immune cells toward IL-22 we performed functional studies in vitro and in vivo. First, we assessed the production of soluble mediators in peripheral blood mononuclear cell (PBMC) cultures by ELISA. As shown in Figure 2A, neither induction of Tumor necrosis factor α (TNF-α), IL-10, IFN-γ, IL-6, nor SAA could be detected in a kinetic study until 72 hr. In contrast, LPS induced production of the mentioned cytokines. Additionally, no influence of IL-22 was seen in PBMC cultures with respect to constitutive and LPS-induced IL-1 receptor antagonist (IL-1RA), IL-8, and TNF-α, and anti-CD3-induced IFN-γ production (data not shown).

Second, PBMCs cultured for 24 hr in the presence or absence of cell-specific stimuli as described above were screened for possible IL-22-induced modifications of cell membrane protein expression by means of flow cytometry. No influence was found on monocytes (CD16, CD54, CD64, and HLA-DR expression), B cells (CD25, CD69, CD71, and CD86 expression), T cells (CD25, CD69, CD95, CD45RA, and CD45RO expression), and NK cells (CD54 and CD69 expression). Figures 2B and 2C show examples with respect to monocytes and NK cells. Whereas IL-10 downregulated HLA-DR and upregulated CD16 expression, and IFN-γ upregulated HLA-DR and downregulated CD16 expression, no effect of IL-22 on these monocyte parameters could be detected. Similarly, whereas IL-12 upregulated NK cell expression of CD54 and CD69 that could be prevented by the presence of transforming growth factor (TGF)-β, no influence of IL-22 on constitutive and IL-12-induced CD54 and CD69 expression could be observed in these cells. In contrast to PBMCs, primary human hepatocytes responded to IL-22 by elevated production of SAA, although to a lesser extent than after IL-6 stimulation (Figure 2D).

We further investigated whether IL-22 would modify serum levels of cytokines in mice. As shown in Figure 3A, no induction of TNF-α, IL-10, IFN-γ or IL-6 was observed in a kinetic study with BALB/c mice until 72 hr after IL-22 injection, whereas LPS injection had clear inducing effect. Furthermore, IL-22 did not modify LPS-induced plasma levels of TNF-α, IL-10, or Macrophage inflammatory protein 2 (MIP-2) in BALB/c mice 1.5 hr postinjection either (Figure 3B and data not shown). In contrast, IFN-γ and IL-10 were able to counteract LPS-induced IL-10 and TNF-α, respectively. No impact of IL-22 was also found on IFN-γ and IL-6 levels 8 hr postinjection (Figure 3C). In line with published mRNA data (Dumoutier et al., 2000b), IL-22 induced production of SAA, although to a minor extent compared to LPS, and also enhanced LPS-induced SAA levels 1.5 hr postinjection (Figures 3A and 3B). Taken together, the absence of IL-22R1 in the analyzed immune cell populations associated with the absence of IL-22-induced modification of a range of immunological parameters in vitro and in vivo strongly suggests that these cells are not targets of IL-22 action.

IL-22 Receptor Expression in Various Nonimmune Tissues

We then investigated human tissues for the expression of the IL-22 receptor subunits. As demonstrated in Figure 4A, all analyzed tissues expressed the IL-10R2 chain. In contrast, only a limited number of tissues expressed IL-22R1, among them skin, small intestine, liver, colon,

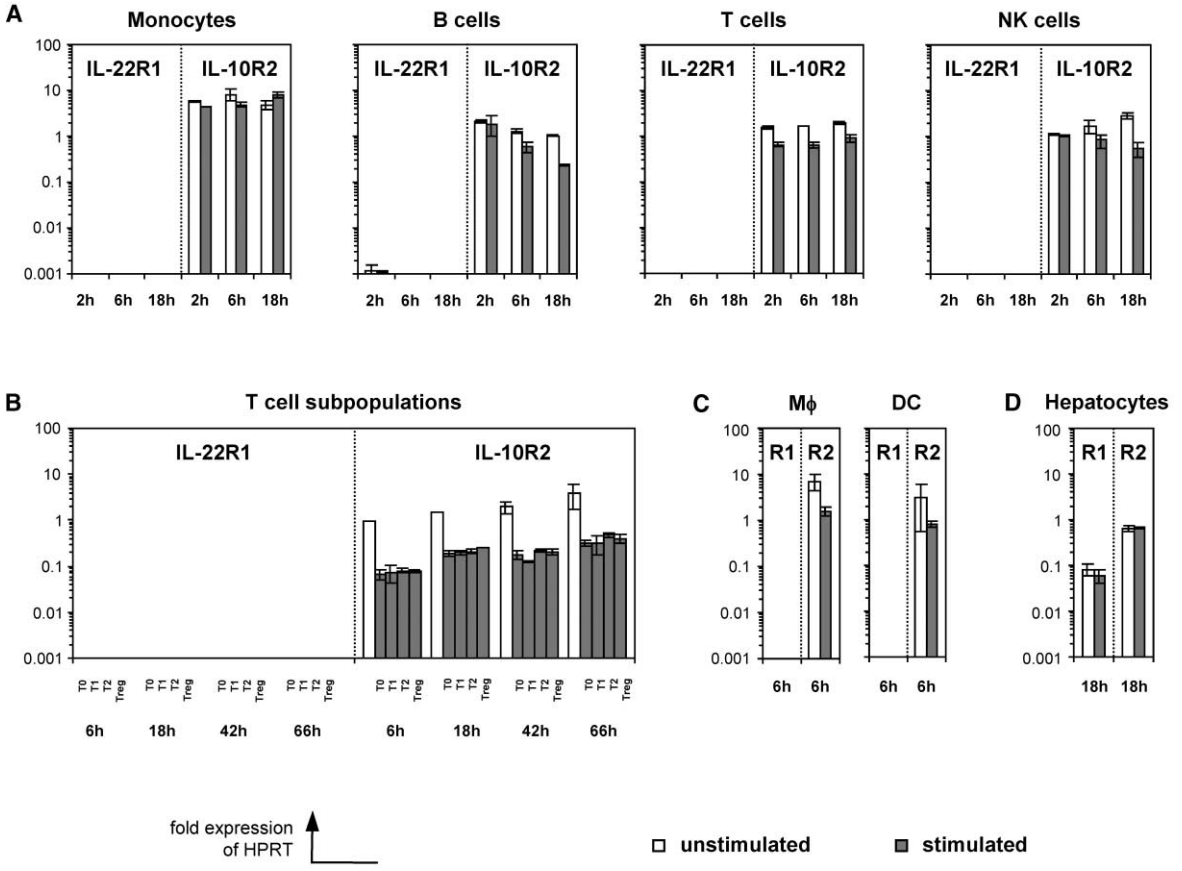


Figure 1. Neither Resting Nor Stimulated Human Immune Cells Express IL-22R1

(A) Isolated blood mononuclear cell populations were stimulated or not (controls) with LPS (monocytes), fixed *Staphylococcus aureus* cells (B cells), IL-2/IL-12 (NK cells), or anti-CD3 mAb coated on culture vessels (T cells) for 2, 6, and 18 hr.

(B) Isolated T cells were stimulated with anti-CD3 and anti-CD28 mAbs coated on culture vessels in the presence of IL-12/anti-IL-4 mAb (polarization toward T1 cells), IL-4/anti-IFN-γ mAb (polarization toward T2 cells), IL-10/TGF-β1,2 (polarization toward regulatory T cells, T reg) or isotypic control mAbs (no polarization, T0), or cultured without stimulation in the presence of isotypic control mAbs (control) for 6, 18, 42, and 66 hr.

(C) Primary monocyte-derived Mϕ and DCs were stimulated or not (controls) with LPS for 6 hr.

(D) Primary human hepatocytes were stimulated or not (control) with IL-6 for 18 hr.

IL-22R1 and IL-10R2 gene expression was analyzed by qPCR. Expression data relative to that of HPRT from two independent experiments are given as mean ± range.

lung, kidney, and, most prominently, pancreas. In line with the results obtained with blood immune cell subpopulations, no IL-22R1 was detected in bone marrow, PBMCs, thymus, and spleen. A pattern similar to that observed in tissues was found in corresponding cell lines (Figure 4B). Whereas C170 colon tumor cells, Hep G2 hepatocyte carcinoma cells, A549 lung carcinoma cells, MDA-MB-231 breast adenocarcinoma cells, BxPC-3 pancreatic adenocarcinoma cells, A3 sub E placenta cell line, and HaCaT keratinocyte cells expressed both IL-22R1 and IL-10R2, the Jurkat T-lymphoblast cell line and the THP-1 monocytic cell line expressed IL-10R2, but not IL-22R1.

IL-22 Receptor Expression in Keratinocytes

Because of the high IL-22 receptor expression levels in skin and HaCaT cell lines, our further studies focused on the main cells of the skin epidermis, the keratinocytes (KCs). mRNA expression analysis of both receptor chains in KCs from seven donors showed a pattern similar to that observed in human skin samples with average expression levels of IL-10R2 of 3.7 (±1.14) times higher than that of IL-22R1 (Figure 4C). To investigate whether these expression levels would be regulated by cytokines, KCs were cultured in the presence or absence of IFN-γ or IL-4 for 2, 6, 18, and 42 hr. As demonstrated in Figure 4D, IFN-γ exposure strongly induced a progressive increase of IL-22R1 and IL-10R2 that reached, at 42 hr, more than 600% and 300% of controls, respectively. Both effects were dose dependent as demonstrated for 42 hr (Figure 4E). IL-4 had marginally, if any, downregulating effect on the expression of IL-22R1 (Figure 4D).

IL-22-Induced STAT3 Activation in KCs

In the next part of our study we asked for the effects of IL-22 on KCs. First, we investigated whether IL-22 would activate Jak-STAT pathways in these cells. 20 min IL-22 exposure induced tyrosine phosphorylation of STAT3,

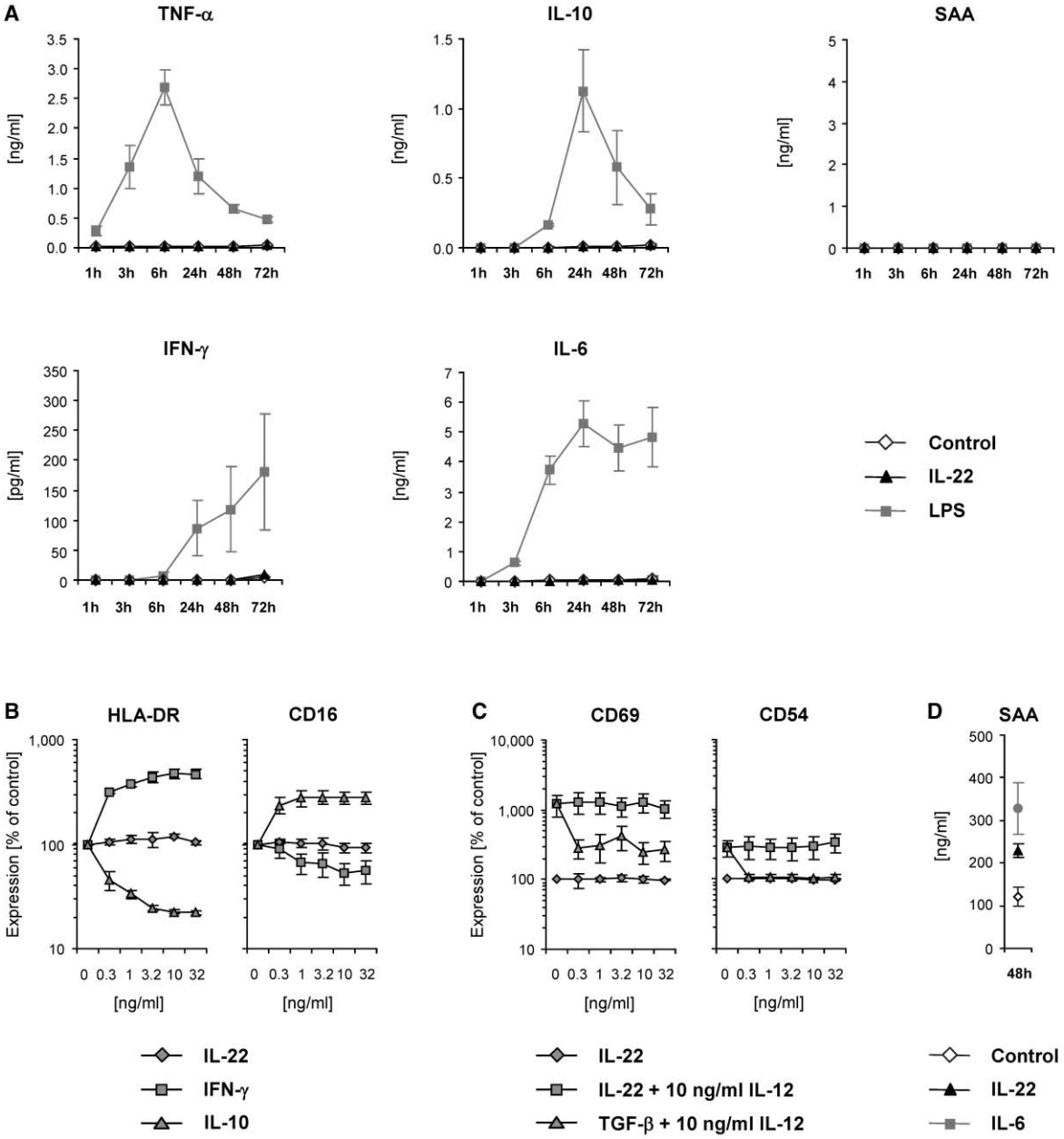


Figure 2. IL-22 Does Not Act on Human Blood Immune Cells In Vitro

(A) PBMCs were cultured in the presence of IL-22, LPS, or without stimulation (control) for 1, 3, 6, 24, 48, and 72 hr. Cell culture supernatant was analyzed for TNF-α, IL-10, IFN-γ, IL-6, and SAA content by ELISA. Data from three independent experiments are given as mean ± SEM.
(B) PBMCs were cultured in the presence of varying concentrations of IL-22, IFN-γ, or IL-10 for 24 hr. Monocyte expression of HLA-DR and CD16 was analyzed by flow cytometry. Data from three independent experiments are given as percent of untreated control calculated based on measured mean fluorescence intensities (mean ± SEM).
(C) PBMCs were cultured in the presence of varying concentrations of IL-22, or IL-22 or TGF-β each in the presence of 10 ng/mL IL-12. NK-cell expression of CD69 and CD54 was analyzed by flow cytometry. Data (mean ± SEM) from three independent experiments are given as percent of untreated control calculated based on measured percentages of positive cells (CD69) or mean fluorescence intensities (CD54).
(D) Primary human hepatocytes were cultured with IL-22, IL-6 or without stimulation (control) for 48 hr. SAA concentration of culture supernatant was determined by ELISA. Data from three independent experiments are given as mean ± SEM.

but not STAT1 and STAT5, demonstrating that the expressed IL-22 receptor was functional in KCs (Figure 4F). This STAT3 phosphorylation was less marked than that observed after exposure to IFN-γ, which additionally induced tyrosine phosphorylation of STAT1 and STAT5. In contrast to KCs, no induction of STAT tyrosine phosphorylation was observed in IL-22-treated PBMCs, further supporting the insensitivity of these cells toward IL-22 (Figure 4F).

IL-22-Induced Upregulation of β-Defensin (hBD)2 and 3 Expression in KCs

Upon screening of a variety of different cellular parameters in IL-22-treated KCs (including proliferation, pro-

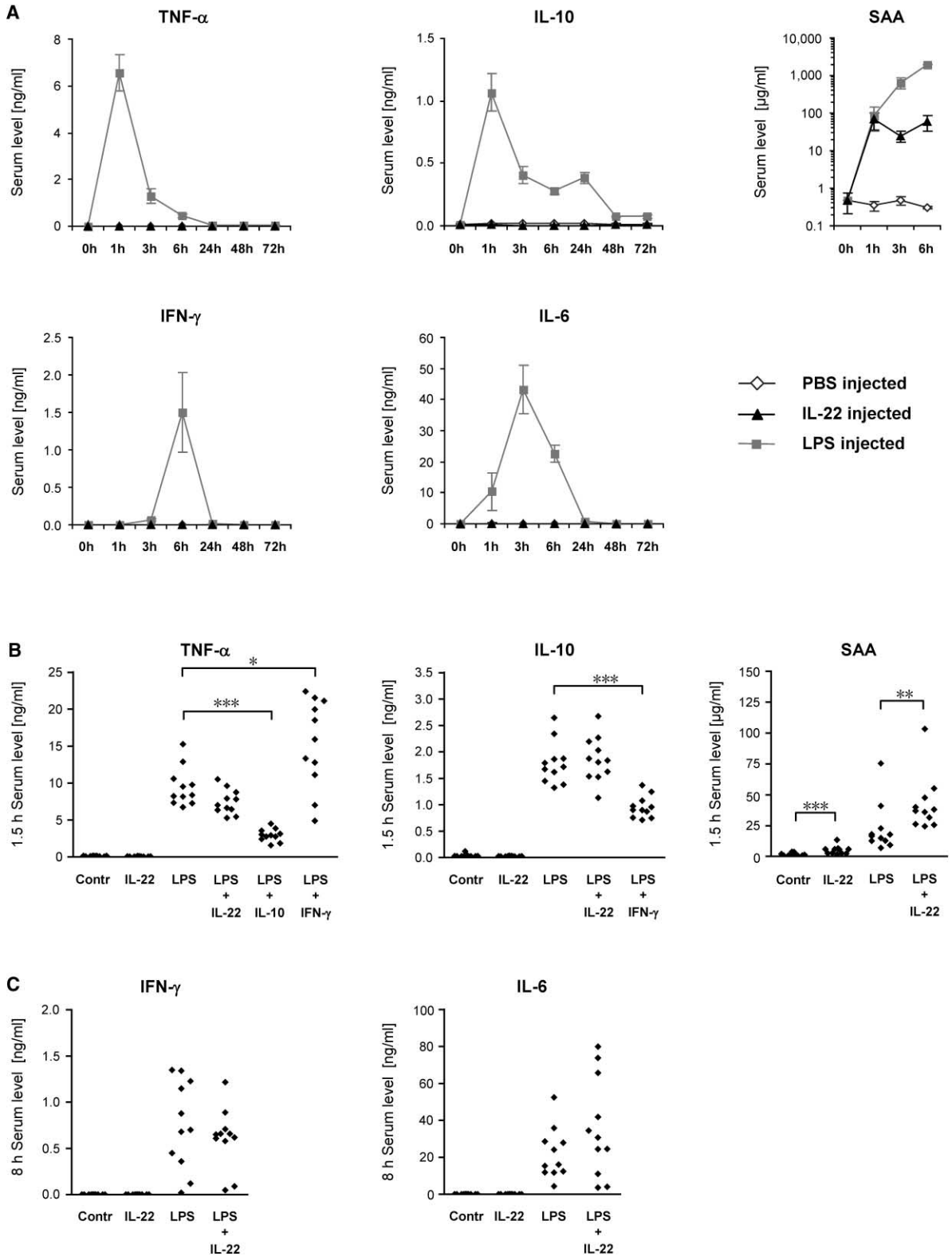
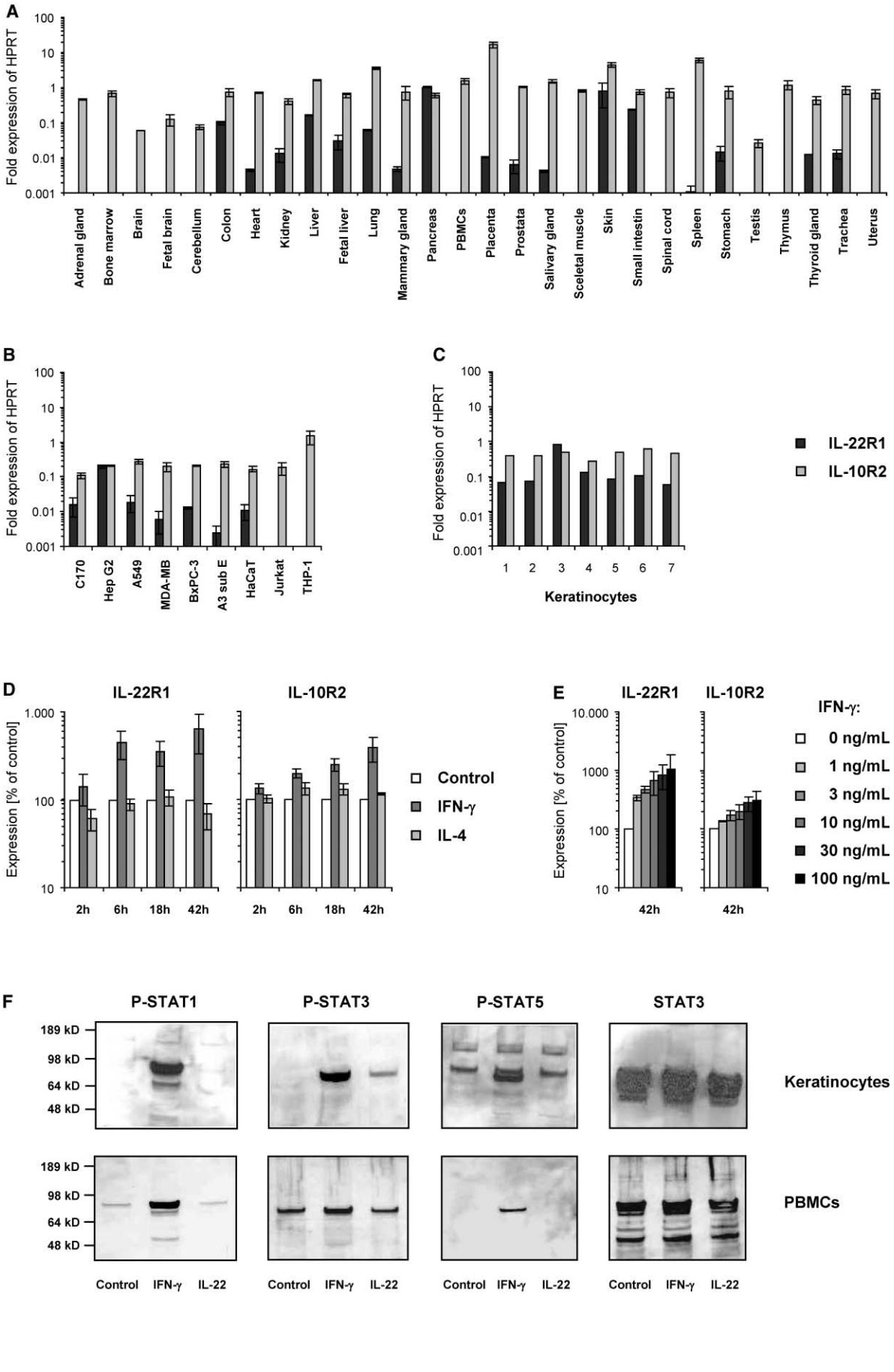


Figure 3. IL-22 Does Not Act on Immune Cells In Vivo

(A) Male BALB/c mice were i.p. injected with PBS (control), murine IL-22, or LPS. Directly before (0 hr) and 1, 3, 6, 24, 48, and 72 hr after injection blood plasma was collected and analyzed for murine TNF-α, IL-10, IFN-γ, IL-6 (0-72 hr), and SAA (0-6 hr) levels by ELISA. Data are given from three mice per time point as mean ± SEM.

(B) Female BALB/c mice were i.p. injected either with PBS (control), murine IL-22, or LPS in the presence or absence of murine IL-22, IL-10, or IFN-γ. 1.5 hr postinjection blood plasma was collected and analyzed for murine TNF-α, IL-10, and SAA levels by ELISA. Data are given from eleven mice per group.

(C) Female BALB/c mice were i.p. injected either with PBS (control), murine IL-22, or LPS in the presence or absence of murine IL-22. 8 hr postinjection blood plasma was collected and analyzed for murine IFN-γ and IL-6 levels by ELISA. Data are given from eleven mice per group. Significance of cytokine-induced alterations of constitutive and LPS-induced plasma levels (B and C) was investigated by use of Kolmogorov-Smirnov test for two samples (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$).


A
Fold expression of HPRT
100
10
1
0.1
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Adrenal gland
Bone marrow
Brain
Fetal brain
Cerebellum
Colon
Heart
Kidney
Liver
Fetal liver
Lung
Mammary gland
Pancreas
PBMCs
Placenta
Prostata
Salivary gland
Sceletal muscle
Skin
Small intestin
Spinal cord
Spleen
Stomach
Testis
Thymus
Thyroid gland
Trachea
Uterus
B
Fold expression of HPRT
C170
Hep G2
A549
MDA-MB
BxPC-3
A3 sub E
HaCaT
Jurkat
THP-1
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Fold expression of HPRT
1 2 3 4 5 6 7
Keratinocytes
IL-22R1
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Expression [% of control]
1.000
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Control
IFN-γ
IL-4
E
IL-22R1
IL-10R2
Expression [% of control]
10.000
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189 kD
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Keratinocytes
PBMCs
Control IFN-γ IL-22

duction of cytokines, expression of cell membrane proteins) we found an influence of this cytokine on the expression of hBDs. These small, cationic peptides have been known for a long time as broad-spectrum, antimicrobial agents produced by epithelial cell (Lehrer and Ganz, 2002; Zasloff, 2002). As demonstrated in Figure 5A, KCs constitutively expressed high levels of hBD1 and hBD3 and very low levels of hBD2 as deduced from qPCR analysis. 42 hr exposure toward IL-22 increased the expression of hBD2 and hBD3 but had minimal if any influence on hBD1. This increase was specific as it was blocked by the presence of anti-IL-22 antibodies (Figure 5A) and was dose dependent (Figure 5B). IL-22-induced expression of hBD2 was also detected in primary hepatocytes. In contrast, no effect of IL-22 was detected on hBD1, hBD2, and hBD3 expression in monocytes, although hBD1 and hBD2 expression could be upregulated by IFN-γ and LPS in these cells, corresponding to published data (Duits et al., 2002) (see Supplemental Data available online at http://www.immunity.com/cgi/content/full/21/2/241/DC1/). We also tested the time dependency of hBD induction in KCs and compared it to the effect of other cytokines known to modulate hBD levels in epithelial cells (Mathews et al., 1999; Jia et al., 2001; Liu et al., 2003). Cells were cultured in the presence or absence of IL-22, IFN-γ, IL-4, or IL-1β for 2, 6, 18, and 42 hr. Whereas IL-22 did not clearly modulate hBD1 expression at any of the time points, it progressively upregulated the expression of hBD2 and hBD3, reaching at 42 hr, on average, more than 1400% and 500% of control levels, respectively (Figure 5C). Regarding hBD2, IL-22 was comparably potent as IFN-γ but much less potent than IL-1β. IL-4 had a downregulating effect. Regarding hBD3, IL-22 was less effective than IFN-γ but comparably potent as IL-1β although its effect started earlier compared to that of IL-1β. The effect of IL-4 on hBD3 expression strongly fluctuated depending on the donor of the cells.

We subsequently investigated the mechanism of IL-22-induced hBD expression in more detail. Since a previous study emphasized the requirement of cellular differentiation for KC hBD2 production induced by IL-1 (Liu et al., 2002), we investigated the effect of differentiation-promoting elevated calcium concentration on IL-22-induced hBD expression. As shown in Figure 6B, increased calcium concentration induced a clear increase of constitutive, IL-1β- and IL-22-induced expression of hBD1 and hBD2, but not hBD3. However, the fold induction of hBD2 expression by IL-22 did not change.

The observed IL-22 effect could be a direct one or might be mediated by the action of a mediator. Since IL-1 is the only cytokine produced by KCs that is known to induce KC hBD expression, we tested the influence of IL-1RA presence during KC IL-22 stimulation. As shown in Figure 6A, IL-1RA up to 1 μg/mL had no effect on the IL-22-induced hBD expression, whereas IL-22BP, already at 0.2 μg/mL, prevented this IL-22 action. We also treated KCs with IL-22 in the presence of the transcription inhibitor actinomycin D (Act D), the protein synthesis inhibitor cycloheximide (CHX), the nonclassical secretion inhibitor methylamine (MA), or the respective solvent controls for 18 hr. As shown in Figure 6C, Act D completely prevented the IL-22-induced hBD2 and hBD3 expression. CHX and MA did not affect these IL-22 effects, and a slight enhancement of hBD3 expression was actually observed in the presence of CHX (Figures 6D and E). These data indicate that the observed IL-22 effects were generally independent of de novo protein synthesis and alternative protein secretion and were transcriptionally regulated.

We also asked whether IL-22 would upregulate the expression of further antimicrobial proteins. In fact, we found a clear induction of psoriasin (Figure 6F) whose *Escherichia coli*-selective activity was recently described (Gläser et al., 2001, J. Invest. Dermatol., abstract).

Association of Elevated IL-22 Expression with Elevated hBD Expression in Inflamed Skin

The fact that IL-22 induced hBDs in skin cells led to the question about the relevance of this finding. We investigated IL-22 expression in biopsies of healthy as well as of diseased skin by means of qPCR. As shown in Figure 7A, no IL-22 expression was observed in healthy skin. In contrast, high IL-22 levels were detected in skin from patients suffering from inflammatory skin diseases such as psoriasis or atopic dermatitis. This upregulation was even more pronounced than the disease-associated modulation of IFN-γ, IL-1α, and IL-1β expression (Figure 7A). We hypothesized that the observed cytokine expression pattern, particularly the high IL-22 levels, would be associated with elevated hBD levels in these patients. In fact, the expression of hBD2 and hBD3 was strongly upregulated in diseased skin from patients with psoriasis and atopic dermatitis (Figure 7A). At this, hBD2 levels were, on average, 20 times higher in psoriasis than in atopic dermatitis. No modification of constitutive hBD1 levels was observed in either of these skin diseases. A correlation analysis of hBD versus the different cytokine expressions in biopsies from 25 persons (healthy or suffering from psoriasis or atopic dermatitis) revealed high positive correlation of hBD2 and hBD3

Figure 4. Expression of Functional IL-22 Receptor Complex in Different Human Tissues, Cell Lines, and Primary Human KCs

(A and B) IL-22R1 and IL-10R2 gene expression was analyzed in tissues and cell lines by qPCR. Data from two independent analyses are given as relative to HPRT expression (mean $\pm$ range).

(C) IL-22R1 and IL-10R2 gene expression was analyzed in cultured KCs by qPCR. Data from seven individual donors are given as relative to HPRT expression.

(D) KCs were cultured in the presence and absence (control) of IFN-γ and IL-4 for 2, 6, 18, and 42 hr. IL-22R1 and IL-10R2 gene expression was analyzed by qPCR. Data from three independent experiments are given as percent of controls (mean $\pm$ SEM).

(E) KCs were cultured in the presence of different concentrations of IFN-γ for 42 hr. IL-22R1 and IL-10R2 gene expression was analyzed by qPCR. Data from two independent experiments are given as percent of control (0 ng/mL) (mean $\pm$ range).

(F) KCs and PBMCs were stimulated with IL-22, IFN-γ, or without additives (control). After 20 min, levels of tyrosine-phosphorylated STAT1, STAT3, and STAT5, and total STAT3 were assessed by Western blot analysis.

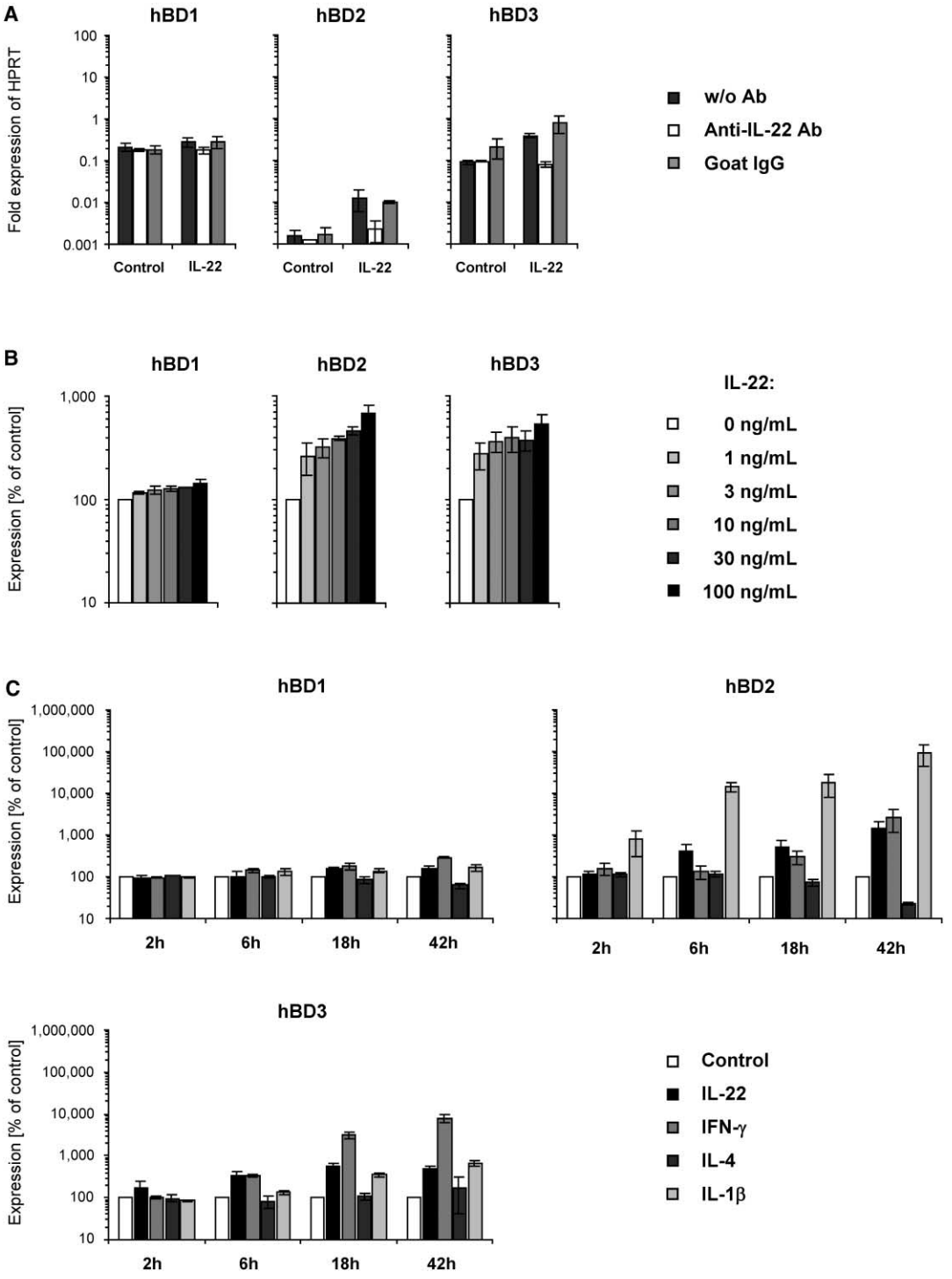


Figure 5. IL-22 Upregulates the Expression of hBDs in Human Primary KCs

(A) KCs were stimulated with IL-22 or not (control) each in the presence or absence of either neutralizing anti-IL-22 antibodies or control goat IgG for 42 hr. Expression of hBD1, hBD2, and hBD3 was analyzed by qPCR. Data from two independent experiments are given as relative to HPRT expression (mean ± range).

(B) KCs were stimulated with varying concentrations of IL-22 for 42 hr. Expression of hBDs was analyzed by qPCR. Data from three independent experiments are given as percent of control (0 ng/mL) (mean ± SEM).

(C) KCs were stimulated with IL-22, IFN-γ, IL-4, IL-1β, or let without additives (control) for 2, 6, 18, and 42 hr. Expression of hBDs was analyzed by qPCR. Data from three independent experiments are given as percent of controls (mean ± SEM).

expression with IL-22 expression (see Table in Figure 7A) that might support the relevance of IL-22 to hBD expression in inflamed skin.

Cellular Sources of IL-22

Since elevated IL-22 expression in diseased skin was detected at the mRNA level, it must result from local producers. This led to the question about the cellular source of this cytokine. Inflammatory skin diseases are characterized by activated KCs as well as by infiltration of activated immune cells. As known, activated T and NK cells, but not monocytes and B cells, are able to produce IL-22 upon appropriate in vitro stimulation (Figure 7B and Wolk et al., 2002). To investigate whether KCs are also able to express IL-22, primary KCs were cultured in the presence or absence of IFN-γ, IL-4, or IL-1β for 2, 6, and 18 hr. The expression of IL-22 and IL-8 was analyzed by qPCR. As shown in Figure 7B, no IL-22 expression could be detected under any of the tested conditions. As expected (Larsen et al., 1989), KCs expressed high levels of IL-8, which was rapidly upregulated in the presence of IL-1β (Figure 7B). These data suggest that local IL-22 production in skin diseases should be derived from infiltrating lymphocytes rather than resident KCs. This also matches data from a murine model of inflammation. In fact, systemic application of LPS known to activate not only resident monocytes/macrophages and dendritic cells (DCs) but also tissue cells in the different organs (including KCs) induced cutaneous MIP-2, but not IL-22 mRNA (Figure 7C).

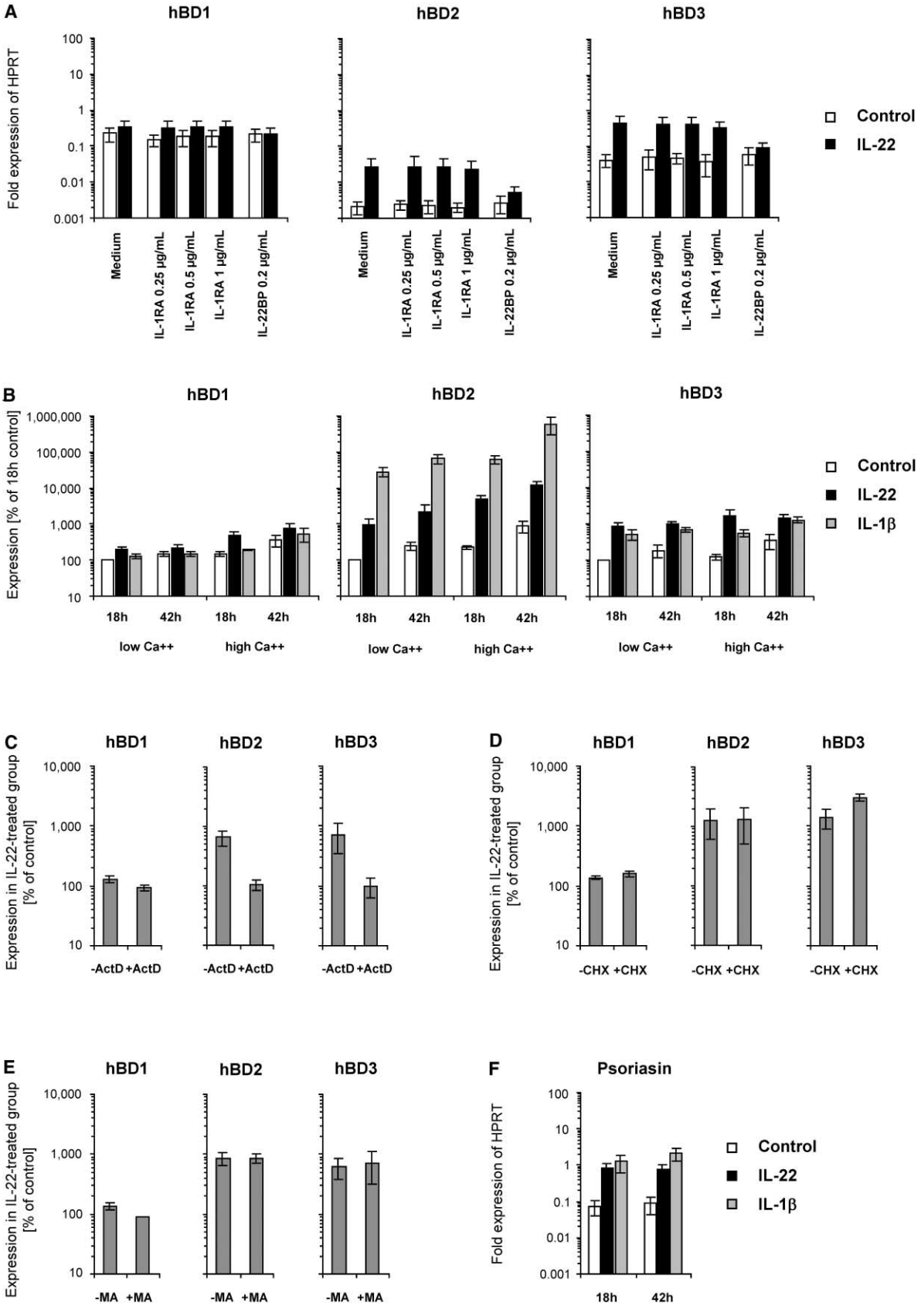
Discussion

The aim of this study was to expand the knowledge about the biological role of the recently discovered cytokine IL-22. Despite its relation to IL-10, which represents one of the most important Interleukins (Moore et al., 2001), IL-22 does not appear to affect immune cells. This conclusion is based on the following observations. First, neither resting nor stimulated human monocytes, Mϕ, DCs, B, T, and NK cells showed expression of IL-22R1. In contrast, the second chain of the IL-22 receptor complex, IL-10R2, was highly expressed and regulated in these cells. The "isolated" expression of IL-10R2 is not surprising since this molecule functions as accessory chain also for the receptor complexes for IL-10, IL-26, and IL-28/IL-29 (Langer et al., 2004). A similar expression pattern was observed in Jurkat and THP-1 cell lines as well as in human bone marrow, PBMCs, thymus, and spleen. Second, no STAT tyrosine phosphorylation was induced by IL-22 in PBMCs. Third, no influence of IL-22 on any of the tested immune cell populations was found in vitro with respect to the release of cytokines and SAA, the expression of a variety of surface proteins, and the hBD mRNA expression. Fourth, the systemic application of murine IL-22 in mice did not modify basal or LPS-induced plasma cytokine levels. Lacking IL-22 sensitivity with respect to constitutive and LPS-induced TNF-α and IL-6 production by murine splenic adherent cells and induction of immunoglobulins in human splenic and tonsillar B cells was already indicated in earlier studies (Xie et al., 2000; Aggarwal et al., 2001; Lecart et al., 2002). However, despite all these data demonstrating unresponsiveness of immune cells toward IL-22, they cannot definitely exclude that a certain immune cell subpopulation under certain conditions might be responsive toward this cytokine.

In contrast to immune cells, expression of IL-22R1 was detected in a range of human tissues including skin and tissues of the digestive and the respiratory system as well as in their corresponding cell lines. This is in line with data previously published by Aggarwal et al. (2001) and Parrish-Novak et al. (2002). Interestingly, in most cases the IL-10R2 expression was much higher than that of IL-22R1. Assuming the association of equal amounts of R1 and R2 chain in functional receptor complexes, excessive IL-10R2 might be present here to mediate effects of another ligand (IL-10, IL-26, IL-28, or IL-29). Apart from pancreas, human skin demonstrated the highest IL-22R1 expression among all tested tissues. This expression pattern was reflected by that found in human primary KCs and the HaCat cell line and could be regulated. In fact, IFN-γ-induced upregulation of both receptor chains suggests an elevated sensitivity of KCs toward IL-22 under T1 conditions.

The IL-22 receptor complex appeared to be functional in KCs as IL-22 induced tyrosine phosphorylation of STAT3, but not STAT1 and STAT5, in these cells. Interestingly, STAT3 has been shown to be important for skin homeostasis as deduced from the phenotype of K5-Cre:STAT3$^{flox/-}$ transgenic mice (Sano et al., 1999). Regarding other tissue cells, IL-22 has been shown to induce activation of all three STAT molecules in TK10 human renal carcinoma as demonstrated by gel shift assay and Western blot analysis (Xie et al., 2000). In HepG2 human hepatoma cells, however, nuclear translocation of STAT3 and also STAT1, but not STAT5, was detected upon IL-22-stimulation (Dumoutier et al., 2000b). Regarding the murine system, the group of Renauld demonstrated nuclear translocation of STAT3 and, to a lesser extent, of STAT5, but not STAT1, in MES13 mesangium cells (Dumoutier et al., 2000a). In another study about downstream events of IL-22 receptor engagement, the same group demonstrated in H4IIE rat hepatoma cells tyrosine phosphorylation of STAT1, STAT3, and STAT5 (Lejeune et al., 2002). Whether the differences in all these studies are due to cell specificity and/or species specificity needs to be clarified by further analyses.

To elucidate the kind of action that IL-22 exerts on KCs, we screened these cells in preliminary studies for IL-22-induced modifications without any positive result. However, the observed IL-22 receptor expression in a range of physiologically outer body barriers (besides skin, also tissues of the gastrointestinal and respiratory system) prompted us to address the question whether IL-22 could regulate immunoprotection at these barriers. In fact, a specific, time- and concentration-dependent induction of hBD2 and hBD3 expression was observed in KCs in response to IL-22. hBDs are very small, highly positively charged proteins that kill bacteria, fungi, and viruses. They are distinguished from α-Defensins on the basis of their size, the three characteristic intramolecular disulfide bonds, and their expression sites. hBDs are mainly expressed in skin and mucosal epithelia, where they form a barrier against microbial infections (Lehrer and Ganz, 2002; Zasloff, 2002). hBD1 was shown to be


A
hBD1
hBD2
hBD3
Fold expression of HPRT
100
10
1
0.1
0.01
0.001
Medium
IL-1RA 0.25 µg/mL
IL-1RA 0.5 µg/mL
IL-1RA 1 µg/mL
IL-22BP 0.2 µg/mL
Control
IL-22
B
hBD1
hBD2
hBD3
Expression [% of 18h control]
1,000,000
100,000
10,000
1,000
100
10
18h
42h
low Ca++
high Ca++
Control
IL-22
IL-1β
C
hBD1
hBD2
hBD3
Expression in IL-22-treated group [% of control]
10,000
1,000
100
10
-ActD
+ActD
D
hBD1
hBD2
hBD3
Expression in IL-22-treated group [% of control]
10,000
1,000
100
10
-CHX
+CHX
E
hBD1
hBD2
hBD3
Expression in IL-22-treated group [% of control]
10,000
1,000
100
10
-MA
+MA
F
Psoriasin
Fold expression of HPRT
100
10
1
0.1
0.01
0.001
18h
42h
Control
IL-22
IL-1β

highly and stably expressed whereas hBD2 and hBD3 are induced by bacteria and proinflammatory cytokines (Mathews et al., 1999; Liu et al., 2002, 2003; Jia et al., 2001). Recent studies also proposed the contribution of hBDs to other immune responses. hBD2 was shown to induce activation and degranulation of mast cells and chemoattracts immature DCs and CD4+ memory T cells through interaction with the chemokine receptor CCR6 (Yang et al., 1999; Niyonsaba et al., 2001). The IL-22-induced hBD2 and hBD3 upregulation in KCs observed in our study was dependent on cellular differentiation, was transcriptionally regulated, and was independent of protein de novo synthesis and alternative protein secretion, indicating a direct effect of this cytokine. The fold induction of hBD2 and nBD3 expression in KCs by IL-22 was comparable to that observed by other hBD inducers.

The analysis of the promoter region down to 1000 base pairs revealed putative STAT binding sites in the hBD2 and the hBD3 gene (data not shown). These could be involved in the regulation of these genes by IL-22 and IFN-γ. Further studies are needed to clarify whether these sites are active and whether STAT3 activation is essential for the IL-22 effects demonstrated here. Interestingly, we have found putative binding sites for NF-κB, AP-1, and NF-IL-6 in the hBD2 gene promoter but no NF-κB sites in the hBD3 gene promoter that may explain the more slightly regulation of hBD3 compared to hBD2 by IL-1β (data not shown).

IL-22-induced hBD expression might be relevant in T cell-mediated inflammatory skin diseases. Indeed, high levels of IL-22 expression were found in diseased skin from patients suffering from psoriasis or atopic dermatitis that are probably derived from activated T cells. Moreover, whereas the expression of hBD1 was not changed in these patients compared to healthy controls, the expression of hBD2 and hBD3 was highly increased. Comparison of cutaneous expression of IL-22 and other hBD inducers (IL-1α, IL-1β, IFN-γ, TNF-α) in patients versus healthy controls and the fact that IL-1 activity is reduced in diseased skin (Hammerberg et al., 1992) might suggest a role of IL-22 in the upregulated expression of hBD2 and hBD3. Unfortunately, we did not succeed in establishing a detection method for IL-22 protein to substantiate these suggestions. It is also possible that additional mediators such as other members of the IL-10 family (e.g., IL-20) contribute to the elevated skin hBD expression. Interestingly, whereas expression of hBD3 was similar in both diseases, the expression of hBD2 was much higher in psoriasis than in atopic dermatitis. Significantly lower hBD2 expression in atopic dermatitis compared to psoriasis was recently also demonstrated by Ong et al. (2002). This may be due to the inhibitory effect of IL-4 that has been described to be upregulated in atopic dermatitis (Leung and Bieber, 2003). Accounting for the defect skin barrier in both diseases, the difference in hBD2 expression matches very well investigations showing high frequency of cutaneous infections in atopic dermatitis and a relative resistance to cutaneous infection in psoriasis (Christophers and Henseler, 1987).

Regarding all data together, a hypothesis about the role of IL-22 may be advanced. Upon local, e.g., bacterial skin infection, specifically activated memory Th cells produce IL-22, which, via induction of hBDs, contributes to the clearance of skin infection by killing invading pathogens as well as by activating local mast cells. Simultaneously, the hBDs prevent the invasion of further pathogens. This may be particularly important for the prevention of a second invasion of a different pathogen that no specific immune response has been established for. This can be important because the first infection provokes activation, maturation, and migration to the lymph nodes of tissue-resident immature DCs (Roake et al., 1995). This leads to the lack of immature DCs in local skin and therefore the lack of the prerequisite for establishing a specific immune response toward the second pathogen. Without the T cell-dependent protection, these (second) pathogens would easily invade the body. Additionally, IL-22, via induction of hBDs, chemoattracts immature DCs to the site of infection, thereby reducing this "defenseless" phase. hBDs also chemoattract CD4+ memory cells, the major source of IL-22, suggesting a positive feedback mechanism. Upon systemic inflammation, systemically present IL-22, via the mentioned mechanisms, might provide protection in all outer barriers. Our further, preliminary data suggest that IL-22 particularly plays a unique role in chronic systemic inflammation. Taken together, IL-22, a cytokine produced mainly by activated Th1 but also by other activated T cells and by activated NK cells, has many targets among tissue cells and seems to directly increase the innate, nonspecific immunity against pathogens (induction of acute phase protein and hBD expression) without (directly) affecting immune cells.

Experimental Procedures

Cell Culture

Human total PBMCs, monocytes, NK, B, and T cells were isolated from healthy donors and cultured as described previously (Wolk et

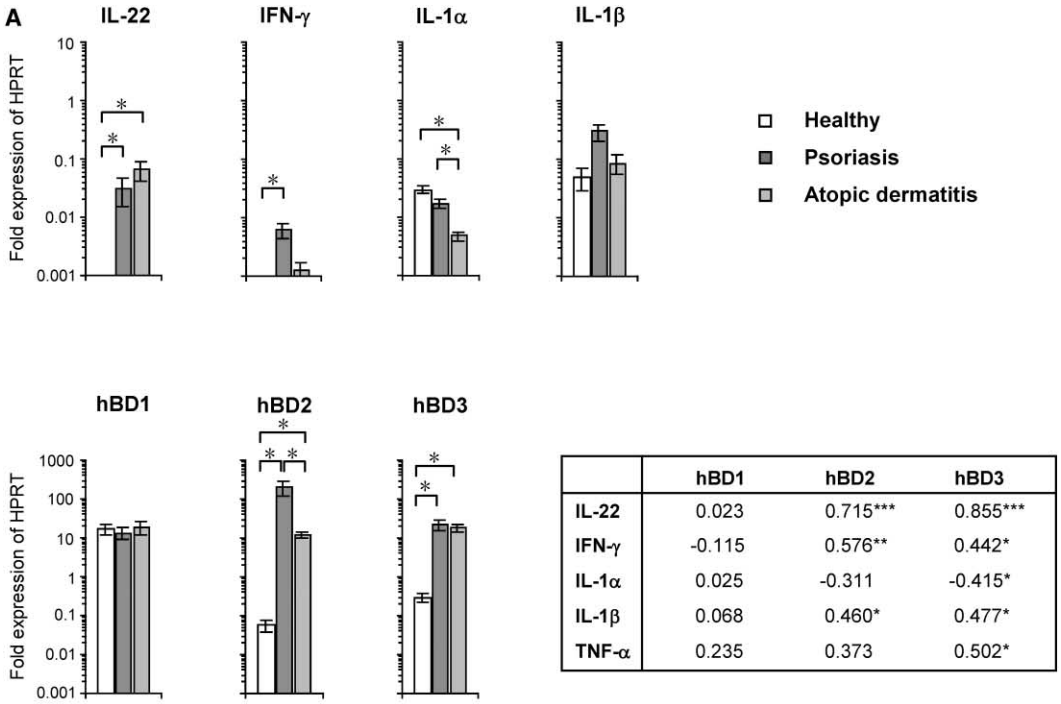
Figure 6. The IL-22-Induced Upregulation of hBD2 and hBD3 Expression in Human Primary KCs Is a Transcriptionally Regulated, Direct Effect that Increases Upon Elevated Medium Calcium Concentration

(A) KCs were stimulated or not (control) with IL-22 in the presence of IL-1RA, IL-22BP, or medium for 18 hr. Expression of hBDs was analyzed by qPCR. Data from three independent experiments are given as relative to HPRT expression (mean ± SEM).

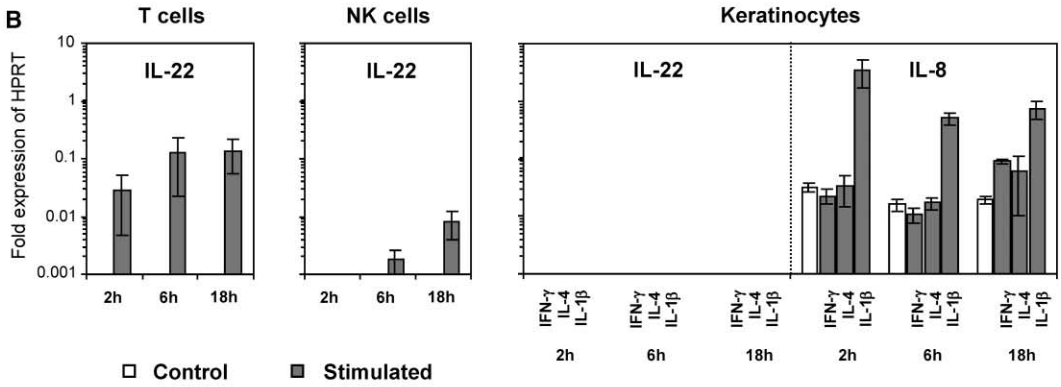
(B) KCs were stimulated with IL-22, IL-1β, or let without cytokine (control) in the absence (low Ca^{2+}, 0.15 mM) or presence (high Ca^{2+}, 1.3 mM) of elevated calcium concentration for 18 and 42 hr. Expression of hBDs was analyzed by qPCR. Data from three independent experiments are given as percent of 18 hr controls with low Ca^{2+} (mean ± SEM).

(C–E) KCs were stimulated or not (control) with IL-22 in the presence of Act D, CHX, MA, or the respective control solvent for 18 hr. Expression of hBDs was analyzed by qPCR. Data of IL-22-stimulated samples from three independent experiments are given as percent of controls (mean ± SEM).

(F) KCs were stimulated or not (control) with IL-22 or IL-1β for 18 and 42 hr. Psoriasin expression was analyzed by qPCR. Data from three independent experiments are given as relative to HPRT expression (mean ± SEM).



	hBD1	hBD2	hBD3
IL-22	0.023	0.715***	0.855***
IFN-γ	-0.115	0.576**	0.442*
IL-1α	0.025	-0.311	-0.415*
IL-1β	0.068	0.460*	0.477*
TNF-α	0.235	0.373	0.502*



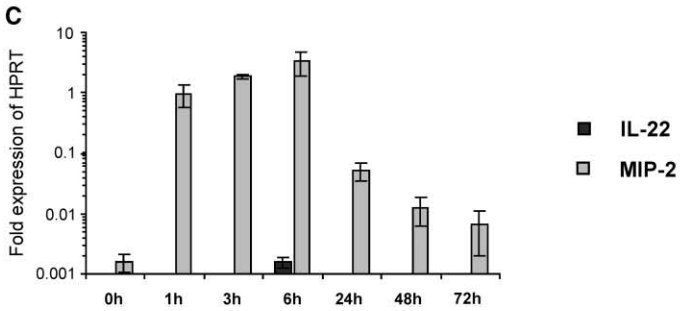


Figure 7. High Levels of IL-22 Seem to Derive From Infiltrating Immune Cells and Are Associated With Upregulated hBD Expression in Human Psoriatic and Atopic Skin

(A) Cutaneous expression of IL-22, IFN-γ, IL-1α, IL-1β, and hBDs in patients with psoriasis, atopic dermatitis, and healthy probands was analyzed by qPCR. (Bar chart) Data from five donors per group are given as relative to HPRT expression (mean $\pm$ SEM). Significance of alterations was investigated by use of Kolmogorov-Smirnov test for two samples (*$p < 0.05$). (Table) Spearman's rang correlation coefficients for correlation between skin hBD and cytokine expression calculated from a total of 25 persons are indicated. Significance of correlation is marked (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

al., 2002). For analyses of IL-22 receptor and IL-22 gene expression, cells were stimulated or not (controls) with LPS (monocytes); fixed *Staphylococcus aureus* cells (B cells); IL-2/IL-12 (NK cells); anti-CD3 mAb (total T cells) for 2, 6, and 18 hr; or, for costimulation and functional polarization of T cells, cultured either in the presence of control mAbs (controls) or stimulated with anti-CD3 and anti-CD28 mAbs in the presence of control mAbs (T0), IL-12/anti-IL-4 mAb (T1), IL-4/anti-IFN-γ mAb (T2), or IL-10/TGF-β1,2 (T reg) for 6, 18, 42, and 66 hr, as described previously (Wolk et al., 2002). Mϕ and DCs were generated by culturing isolated human monocytes for 5 days in the presence of M-CSF and for 7 days in the presence of GM-CSF/IL-4. For analysis of STAT tyrosine phosphorylation in PBMCs, isolated cells precultured for 24 hr, were incubated with IL-22 or IFN-γ or let without additives for 15 min, and washed afterwards for 5 min. To screen for possible IL-22-induced effects in immune cells, PBMCs were cultured in the presence or absence of IL-22 or control mediators (IFN-γ, IL-10, TGF-β, or LPS) for, if not indicated otherwise, 3, 6, 24, or 48 hr. These studies were partially (see Results section) performed in the presence of cell-specific stimuli (LPS, *S. aureus*, IL-12 $\pm$ IL-2, anti-CD3 mAb) as described above. For analysis of monocytic hBD expression, isolated monocytes were cultured in the presence or absence of IL-22, IFN-γ or LPS for 18 and 42 hr. Primary human hepatocytes were obtained from CellSystems (St. Katharinen, Germany), cultured on thin layer Biocoat Matrigel (BD Biosciences, Heidelberg, Germany) in HCM medium, and exposed or not to IL-6 or IL-22 for 18 and 48 hr. The cell lines C170, Hep G2, A549, MDA-MB-231, BxPC-3, A3 sub E, Jurkat, and THP-1 were purchased from the European Collection of Cell Cultures (Salisbury, UK). HaCaT cells kindly provided by N.E. Fusenig (Deutsches Krebsforschungszentrum, Heidelberg, Germany). Primary human KCs were obtained from CellSystems, cultured in KGM medium, and exposed to IL-22, IFN-γ, IL-4, IL-1β, or medium (controls) for the indicated times and in case of STAT phosphorylation assays, for 20 min. These studies were partially performed in the presence of polyclonal anti-IL-22 antibodies or goat IgG, IL-1RA (0.25, 0.5, 1 μg/mL), IL-22BP (0.2 μg/mL), Act D (0.25 μg/mL; Sigma-Aldrich, Deisenhofen, Germany), CHX (1 μg/mL; Sigma-Aldrich), MA (1mM; Sigma-Aldrich), ethanol (solvent control for Act D and MA; 0.02%), DMSO (solvent control for CHX; 0.003%), or, as previously described by Liu et al. (2002), in the presence of elevated calcium concentration (1.3 mM). All cytokines and antibodies mentioned above, as well as IL-1RA and IL-22BP, were purchased from R&D Systems (Wiesbaden-Nordenstadt, Germany) with the exception of anti-CD3 mAb, which was purchased from Cilag (Orthoclone; Sulzbach, Germany). If not indicated otherwise, all cytokines and soluble mAbs were applied at 10 ng/mL and 5 μg/mL, respectively. LPS derived from *Escherichia coli* 0127:B8 was purchased from Sigma-Aldrich and used at 100 ng/mL.

Flow Cytometry

Assessment of composition of isolated cell populations and confirmation of cellular activation were performed by flow cytometry as previously described (Wolk et al., 2002). For functional assays the following fluorescence-labeled mAb clones were used: UCHT1 (CD3), RM052 (CD14), 3G8 (CD16), N901 (CD56), NC1 (CD57), 22 (CD64), BMA031 (TCR$\alpha\beta$), IMMU510 (TCR$\gamma\delta$), 679.1 Mc7 (IgG1) (all from Coulter Immunotech, Hamburg, Germany), SK1 (CD3), SK3 (CD4), S-HCL-3 (CD11c), 4G7 (CD19), 2A3 (CD25), HI100 (CD45RA), UCHL1 (CD45RO), L78 (CD69), Lo1.1 (CD71), 2331 (CD86), DX2 (CD95), L243 (HLA-DR), 338 15X (IgG1/κ), X40 (IgG1/κ), X39 (IgG2a/κ), G155-178 (IgG2a/κ), 27-25 (IgG2b/κ) (all from BD Biosciences).

Gene Expression Analysis

Total RNA from human tissues was obtained from Clontech Laboratories (Heidelberg, Germany). Skin biopsies, snap frozen in Invisorb lysing solution (Invitek, Berlin, Germany), were homogenized during thawing by means of Ultraturrax tissue homogenizer (Jahnke and Kunkel, Staufen, Germany). Murine samples were additionally treated afterwards with 4 mg/mL proteinase K for 1 hr (Clontech Laboratories). Isolation of total cellular RNA was done by use of Invisorb RNA kit II (Invitek). mRNA was reverse transcribed and analyzed by TaqMan PCR with the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Weiterstadt, Germany) as described previously (Wolk et al., 2002). For detection of human IL-22R1, IL-10R2, hBDs, IL-22, IL-4, and housekeeping gene hypoxanthine phosphoribosyl-transferase 1 (HPRT), as well as of murine IL-22 and HPRT, we established detections systems with amplification efficiencies of 100% using exon-exon boundaries spanning 6-carboxy-fluorescein/6-carboxy-tetramethyl-rhodamine double-labeled probes. All other detections systems used were purchased from Applied Biosystems. Expressions were calculated relative to the data for HPRT obtained with the every matching assay.

Mice

To study the effect of IL-22 in vivo, three different experiments were conducted: First, 14-week-old male BALB/c mice were intraperitoneally (i.p.) injected with either phosphate-buffered saline (PBS), 1 μg murine IL-22 (R&D Systems), or 100 μg LPS from *Escherichia coli* 0111:B4 (Sigma-Aldrich). Before or after 1, 3, 6, 24, 48, and 72 hr mice were sacrificed, and blood was drawn for the recovery of plasma. Second, 12-week-old female BALB/c were i.p. injected with either PBS, 1 μg murine IL-22, or 300 μg LPS (see above) in the presence and absence of murine IL-22, IL-10, or IFN-γ (each 1 μg; R&D Systems). After 1.5 hr mice were sacrificed, and blood was drawn for the recovery of plasma. Third, 12-week-old female BALB/c were i.p. injected with either PBS, 1 μg murine IL-22, or 300 μg LPS (see above) in the presence and absence of 1 μg murine IL-22. After 8 hr mice were sacrificed, and blood was drawn for the recovery of plasma. Age and sex had influence on the induced serum SAA and IFN-γ levels in these mice. For IL-22 gene expression analysis, 14-week-old male BALB/c were i.p. injected with 100 μg LPS (see above). Before and after 1, 3, 6, 24, 48, and 72 hr mice were sacrificed, and skin samples were taken. These studies have been approved by the regional authorities for provisions on labor, health, and technical safety, Berlin.

ELISA

Human SAA and murine SAA and TNF-α were quantified by ELISA from Biosource (Camarillo, CA). Human IFN-γ and mouse IL-10, IFN-γ, IL-6, and MIP-2 were quantified by using ELISAs from R&D Systems. Detection of human TNF-α, IL-10, and IL-6 was realized by Immulite system (DPC Biermann, Bad Nauheim, Germany).

Western Blot Analysis

Cell lysing, protein electrophoresis, and Western blotting was performed as described previously (Wolk et al., 2003). Blotted samples were incubated with polyclonal antibodies against phospho-STAT1 (Tyr 701), phospho-STAT3 (Tyr 705), phospho-STAT5 (Tyr 694), or total STAT3 (all from Cell Signaling Technology, Beverly, MA) and peroxydase-conjugated AffiniPure goat anti-rabbit IgG (H and L) (Dianova, Hamburg, Germany), followed by ECL detection (Amersham Pharmacia Biotech, Freiburg, Germany).

Patients' Skin Biopsies

Punch biopsies were obtained from diseased skin from adult patients either with chronic plaque psoriasis, with atopic dermatitis, or from healthy skin. These biopsies were approved by the Clinical Institutional Review Board of the medical faculty Charité Berlin.

(B) Blood T and NK cells and KCs were stimulated or not with either anti-CD3 mAb (T cells), IL-2/IL-12 (NK cells), or IFN-γ, IL-4, or IL-1β (KCs) for 2, 6, and 18 hr. Expression of IL-22 (T and NK cells, KCs) and IL-8 (KCs) was analyzed by qPCR. Data from three independent experiments are given as relative to HPRT expression (mean $\pm$ SEM).

(C) BALB/c mice were i.p. injected with LPS. Expression of murine IL-22 and MIP-2 was analyzed by qPCR in skin samples taken before (0 hr) and 1, 3, 6, 24, 48, and 72 hr after injection. Data referring to three mice per time point are given as relative to HPRT expression (mean $\pm$ SEM).

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