

Interleukin-10 Downregulates Anti-Microbial Peptide Expression in Atopic Dermatitis

Michael D. Howell,^{*†} Natalija Novak,[‡] Thomas Bieber,[‡] Saveria Pastore,[§] Giampiero Girolomoni,[§] Mark Boguniewicz,^{*†} Joanne Streib,^{*†} Cathy Wong,^{¶||} Richard L. Gallo,^{¶||} and Donald Y. M. Leung^{*†}

^{*}Division of Allergy and Immunology, Department of Pediatrics, The National Jewish Medical and Research Center, Denver, Colorado, USA; [†]Department of Pediatrics, University of Colorado Health Sciences Center, Denver, Colorado, USA; [‡]Department of Dermatology, University of Bonn, Bonn, Germany; [§]Laboratory of Immunology, Istituto Dermopatico dell'Immacolata, Roma, Italy; [¶]Division of Dermatology, Department of Medicine and Pediatrics, University of California, San Diego, California, USA; ^{||}VA San Diego Health Care System, San Diego, California, USA

Recurrent skin infections in extrinsic atopic dermatitis (EAD) may be because of the suppression of anti-microbial peptide (AMP) expression by interleukin (IL)-4 and IL-13. Twenty to thirty percent of AD, however, are classified as intrinsic atopic dermatitis (IAD). They exhibit normal serum IgE levels, no allergen-specific sensitization, and lower levels of IL-4 and IL-13 than EAD. Both forms of AD have increased propensity to skin infection, suggesting a novel mechanism for infection in IAD. In this study, we observed significantly decreased human β -defensin (HBD)-2 gene expression in the skin of both IAD ($p = 0.010$) and EAD ($p = 0.004$), as compared with psoriasis patients. Conversely, IAD ($p = 0.019$) and EAD ($p = 0.002$) skin lesions exhibited elevated IL-10 gene expression when compared with psoriasis. Using primary keratinocytes, we found that the deficiency in AMP expression is an acquired rather than a constitutive defect. Interestingly, neutralizing antibodies to IL-10 augmented the production of tumor necrosis factor- α and interferon- γ by peripheral blood mononuclear cell from AD patients. Additionally, treatment of AD skin explants with anti-IL-10 augmented the expression of both HBD-2 and LL-37. Thus, increased levels of IL-10 may contribute to the AMP deficiency in both IAD and EAD by reducing cytokines that induce AMP.

Key words: anti-microbial peptides/cytokines/extrinsic atopic dermatitis/intrinsic atopic dermatitis
J Invest Dermatol 125:738–745, 2005

Atopic dermatitis (AD) is a chronic inflammatory skin disorder that is associated with skin infection or colonization with *Staphylococcus aureus* in >90% of patients (Leung and Bieber, 2003; Leung *et al*, 2004). Two subsets of AD have been described: 70%–80% have elevated serum IgE and allergic sensitization, whereas 20%–30% of patients exhibit normal serum IgE levels and lack allergen-specific sensitization (Novak and Bieber, 2003). These latter patients are classified as having non-atopic eczema, non-IgE mediated, or intrinsic AD (IAD). Patients are diagnosed as IAD by the following criteria: (1) the clinical appearance of AD according to the criteria outlined by Hanifin and Rajka (1980), (2) low serum IgE levels, and (3) lack of allergen-specific sensitization (Oppel *et al*, 2000; Johansson *et al*, 2004).

Recent studies have demonstrated that the Th2 cytokines, interleukin (IL)-4 and IL-13, downregulate anti-microbial peptide (AMP) expression in atopic eczema or extrinsic AD (EAD) skin, and this may account for their propensity toward recurrent skin infections (Ong *et al*, 2002; Nomura *et al*, 2003). But patients with IAD, as compared with EAD, express significantly lower levels of IL-4 and IL-13 in their skin and peripheral blood (Kagi *et al*, 1994; Akdis *et al*, 1999;

Jeong *et al*, 2003). This explains the normal levels of serum IgE found in IAD as compared with EAD. It does not explain, however, why both groups of AD suffer from recurrent skin infections, and suggests that a mechanism other than increased expression of IL-4 and IL-13 may account for increased skin infection in IAD.

Two major classes of AMP— β -defensins (Harder *et al*, 1997) and cathelicidins (Gallo *et al*, 2002)—have been identified in mammalian tissue and have been shown to play an essential role in host defense against invading microbes (Nizet *et al*, 2001; Howell *et al*, 2004). Specifically, the antibacterial activities of human β -defensin (HBD)-2 and LL-37 against *S. aureus* have been previously described (Ong *et al*, 2002). Additionally, we have demonstrated that inflamed skin from psoriasis patients exhibits increased levels of AMP expression whereas patients with EAD do not (Ong *et al*, 2002; Nomura *et al*, 2003). These studies were therefore conducted to investigate the expression of HBD-2 and LL-37 in eczematous skin lesions from IAD and EAD patients to determine whether AMP were equally low in these two forms of AD and investigate whether there might be a novel immune pathway that explained the increased propensity to infection for IAD and contribute to reduced AMP in EAD as well.

Results

AMP gene expression levels in psoriasis and AD skin Skin explants were collected from the lesional skin

Abbreviations: ACD, allergic contact dermatitis; AD, atopic dermatitis; AMP, anti-microbial peptide; EAD, extrinsic atopic dermatitis; GAS, Group A *Streptococcus*; HBD, human beta defensin; IAD, intrinsic atopic dermatitis; IFN, interferon; IL, interleukin; PBMC, peripheral blood mononuclear cell; TNF, tumor necrosis factor

of normal subjects psoriasis, IAD, and EAD patients and were evaluated for AMP gene expression using real-time RT-PCR. In order to make comparisons between samples and patient populations, AMP gene expression was normalized to the endogenous control, GAPDH, for all samples. As shown in Fig 1, HBD-2 expression was significantly higher in skin lesions from psoriasis (mean: 6089 ± 1868) as compared with lesions from IAD (mean: 53.50 ± 22.53 ; $p = 0.0003$) or EAD (mean: 35.63 ± 12.92 ; $p < 0.0001$). Similarly, IAD (mean: 6.128 ± 3.64) and EAD (mean: 4.16 ± 1.73) skin lesions expressed significantly lower levels of LL-37 expression than psoriasis (mean: 31.50 ± 9.32 ; $p = 0.009$ and 0.0002 , respectively). Levels of HBD-2 and LL-37 in normal skin were significantly lower (HBD-2: $p = 0.0007$; LL-37: $p = 0.0006$) than psoriasis and were similar to those previously observed (Ong *et al*, 2002). No significant differences in AMP expression were observed between IAD and EAD skin lesions.

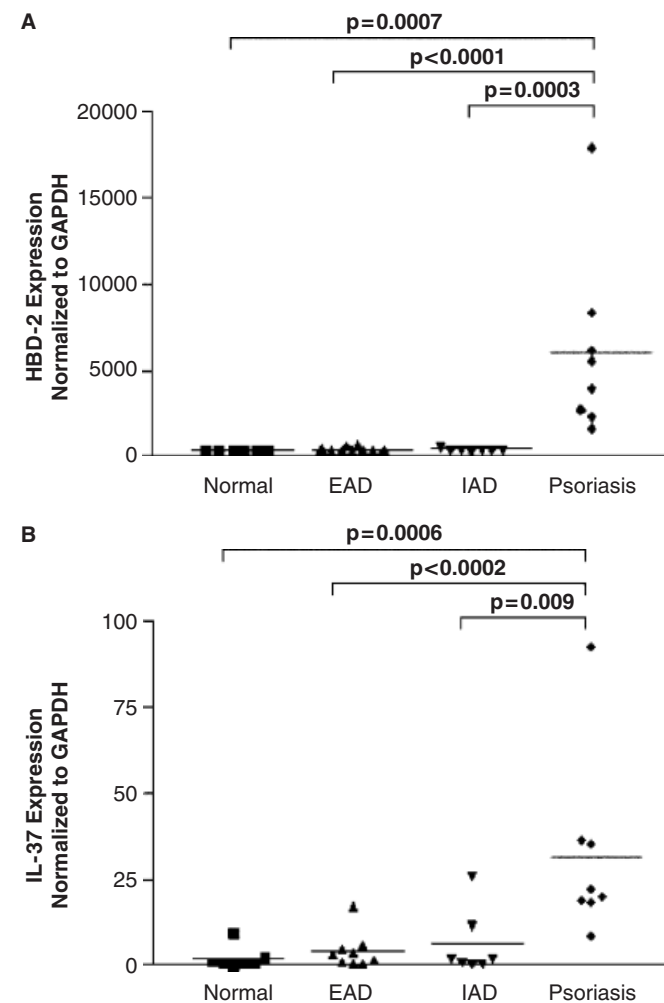


Figure 1
Decreased anti-microbial gene expression in extrinsic atopic dermatitis (EAD) and intrinsic atopic dermatitis (IAD) patients. RNA was isolated from the skin of normal subjects (■, $n = 7$), EAD (▲, $n = 9$), IAD (▼, $n = 7$), and psoriasis (◆, $n = 8$), patients and the levels of human β -defensin (HBD)-2 (A) and LL-37 (B) evaluated by real-time PCR. Significant differences between patient groups were determined by a non-parametric Mann-Whitney test.

In order to exclude disease severity as a means for deficient AMP expression, we investigated the relationship between SCORAD and AMP gene expression using regression analysis. There is no correlation between the severity of disease and AMP expression (HBD-2: $p = 0.25$; LL-37: $p = 0.20$) (data not shown).

Immunostaining for LL-37 in IAD and EAD Previously we have shown that skin sections from psoriasis exhibit more intense HBD-2 and LL-37 staining than EAD (Ong *et al*, 2002). To investigate AMP protein expression in IAD and EAD, explants were stained with a polyclonal antibody directed against LL-37. Allergic contact dermatitis (ACD) skin was used as a control for immunostaining in these studies because of the histological similarities of ACD with AD. Figure 2A illustrates that skin sections from ACD patients exhibited more intense staining for LL-37 than skin from normal subjects, as well as IAD and EAD patients. The composite data for LL-37 immunostaining in all samples are shown in Fig 2B. The intensity of LL-37 staining in normal, EAD, and IAD skin samples was significantly lower than ACD ($p = 0.008$, 0.008 , and 0.03 , respectively). The granular cell layer and stratum corneum of normal, IAD, and EAD skin samples was almost devoid of LL-37 staining, as compared with ACD.

AMP deficiency is acquired in AD To investigate whether the reduced expression of AMP in AD skin is constitutive or acquired, primary keratinocytes were isolated from the skin of IAD, EAD, psoriasis, and normal subjects, and then cultured for three passages to eliminate the *in vivo* effects of inflammation. Differentiated primary keratinocytes were stimulated with tumor necrosis factor (TNF)- α and interferon (IFN)- γ , to optimally induce HBD-2 (Ong *et al*, 2002) or group A *Streptococcus* (GAS), which has previously been shown to induce the expression LL-37 (Dorschner *et al*, 2001). In comparison with non-treated (NT) samples, HBD-2 was upregulated following stimulation with TNF- α and IFN- γ (Fig 3). There were no differences, however, between the different skin types. Additionally, GAS induced greater LL-37 expression in keratinocytes from normal, IAD, EAD, and psoriasis patients; but no differences were observed between study groups (Fig 3).

Downregulatory role of IL-10 on AMP production Since previous studies have demonstrated that IL-4 and IL-13 are decreased in IAD, we sought another mechanism to explain the deficiency of AMP in IAD. IL-10 is known to downregulate the pro-inflammatory response of mononuclear cells and to be increased in AD (Ohmen *et al*, 1995). As depicted in Fig 4, IL-10 was significantly elevated in IAD (mean: 1.34 ± 0.45) and EAD (mean: 0.86 ± 0.11) skin lesions when compared with psoriasis (mean: 0.28 ± 0.06 ; IAD: $p = 0.02$; EAD: $p = 0.003$) and normal skin (mean: 0.30 ± 0.06 ; IAD: $p = 0.02$; EAD: $p = 0.001$). Using linear regression, we investigated whether there was a correlation between the overexpression of IL-10 and reduced expression of AMP. In IAD patients we observed a significant relationship between the expression of IL-10 and HBD-2 ($p = 0.017$) and LL-37 ($p = 0.043$) (Fig 5). In contrast, there was no correlation between the expression of IL-10 and AMP in EAD patients.

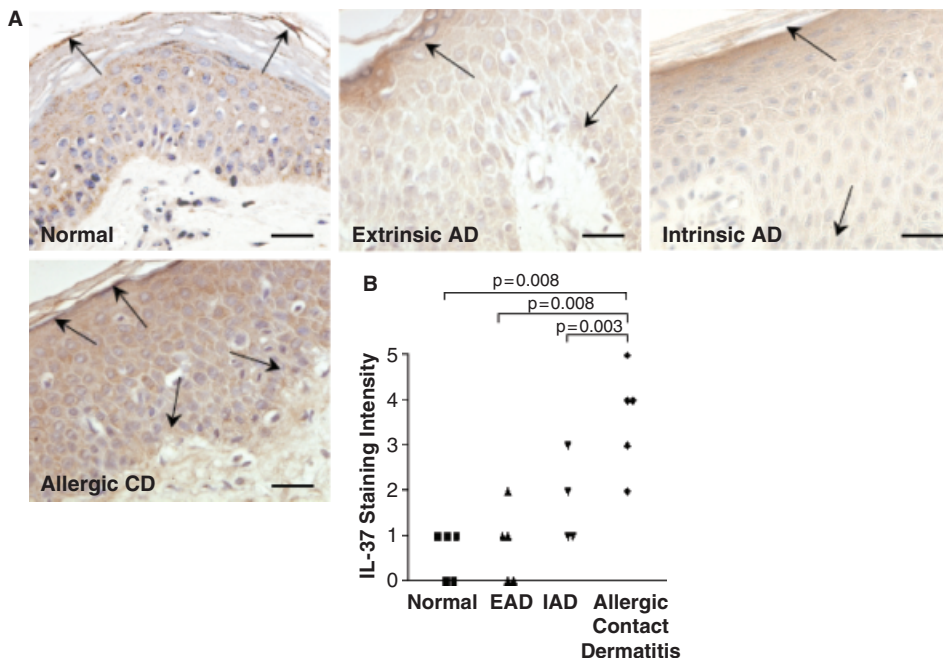


Figure 2
Expression of LL-37 is increased in allergic contact dermatitis (EAD) but not extrinsic atopic dermatitis (IAD). (A) Paraffin-embedded skin explants from EAD (n = 5), IAD (n = 4), and allergic contact dermatitis (n = 5) patients were cut into 5 μ m sections and stained for LL-37. Scale bars represent 25 μ m. (B) The intensity of the immunostaining was visually scored on a scale from 0 to 5, with 0 indicating no staining and 5 the most intense staining. Significant differences between patient groups were determined by a non-parametric Mann-Whitney test.

Interestingly, however, the expression of both IL-10 and IL-13 in lesional skin from EAD patients correlated with a decrease in both HBD-2 ($p = 0.05$) and LL-37 ($p = 0.03$).

The direct effect of IL-10 on AMP expression in keratinocytes was assessed by stimulating the HaCaT human keratinocyte cell line with TNF- α and IFN- γ in the presence and absence of IL-10 or IL-13. Stimulation with TNF- α and IFN- γ significantly increased HBD-2 ($p = 0.0002$) expression in keratinocytes (data not shown). This induction was significantly inhibited by the addition of IL-13 ($p = 0.001$) but not IL-10, suggesting that IL-10 does not act directly on keratinocyte AMP expression.

To investigate the possibility that IL-10 acts indirectly on AMP expression by reducing generation of pro-inflammatory cytokines by mononuclear cells, peripheral blood mononuclear cell (PBMC) were collected from normal subjects and stimulated with anti-CD3 in the presence or absence of increasing concentrations of IL-10. Using real-time PCR, we evaluated the levels of TNF- α and IFN- γ gene expression in PBMC following stimulation. Additionally, the Bio-Plex assay (Bio-Rad, Hercules, California) was used to evaluate the levels of pro-inflammatory cytokines in the supernatants of media or anti-CD3-stimulated PBMC. Analysis revealed that PBMC stimulated with anti-CD3 secreted high levels of IL-1, IL-6, TNF- α , and IFN- γ (data not shown), i.e. cytokines that have been shown to induce AMP expression (Erdag and Morgan, 2002; Liu *et al*, 2002; Ong *et al*, 2002; Nomura *et al*, 2003). TNF- α ($p < 0.01$) and IFN- γ ($p < 0.01$) mRNA levels were also elevated in anti-CD3-stimulated PBMC (data not shown). The induction of both cytokines was inhibited more than 10-fold by adding 5–50 ng per mL (TNF- α : $p < 0.01$; IFN- γ : $p < 0.05$) of IL-10 to anti-CD3-stimulated PBMC.

To test the effects of IL-10 on the downregulation of the pro-inflammatory cytokines known to induce AMP in human keratinocytes, supernatants from anti-CD3-stimulated PBMC, cultured in the presence of increasing concentra-

tions of IL-10, were added to HaCaT cell cultures. Figure 6 illustrates that, in the absence of IL-10, supernatants from anti-CD3-stimulated PBMC significantly induce the expression of HBD-2 ($p = 0.022$) and LL-37 ($p = 0.002$). Fifty nanograms per milliliter of IL-10, however, reduced the expression of HBD-2 by approximately 75%. Even greater inhibition of AMP expression was observed in keratinocytes exposed to a combination of exogenous IL-13 and supernatant from anti-CD3- and IL-10-stimulated PBMC. The combination of the indirect effect of IL-10 on the pro-inflammatory cytokines and the direct effect of IL-13 on keratinocytes significantly reduced AMP expression in comparison with the effect of IL-10 (HBD-2: $p = 0.067$; LL-37: $p < 0.001$) or IL-13 (HBD-2: $p = 0.002$; LL-37: $p = 0.050$) alone. Direct stimulation of HaCaT cells with anti-CD3 did not induce HBD-2 or LL-37 (data not shown).

Neutralization of IL-10 and IL-13 augments AMP expression in AD skin Previously we have demonstrated that TNF- α and IFN- γ are potent inducers of AMP expression in keratinocytes (Ong *et al*, 2002) and that IL-10 downregulates the production of these cytokines by normal PBMC. In these studies, we additionally demonstrated that PBMC from AD patients stimulated with anti-CD3 secrete higher levels of IL-10 protein than PBMC from normals treated under the same conditions (data not shown). Therefore, PBMC were collected from AD patients to determine whether the downregulatory effect of IL-10 could be blocked by neutralizing antibodies. PBMC were stimulated with anti-CD3 in the presence or absence of anti-IL-10 and the levels of TNF- α and IFN- γ in the culture supernatants were determined by ELISA. Table I illustrates that levels of TNF- α and IFN- γ were less than 15.6 pg per mL in PBMC stimulated, with media alone or media plus anti-IL-10. PBMC treated with anti-CD3 plus anti-IL-10, however, released greater levels of TNF- α ($p = 0.05$) and IFN- γ ($p < 0.05$) protein into the supernatant than anti-CD3-stimulated cells.

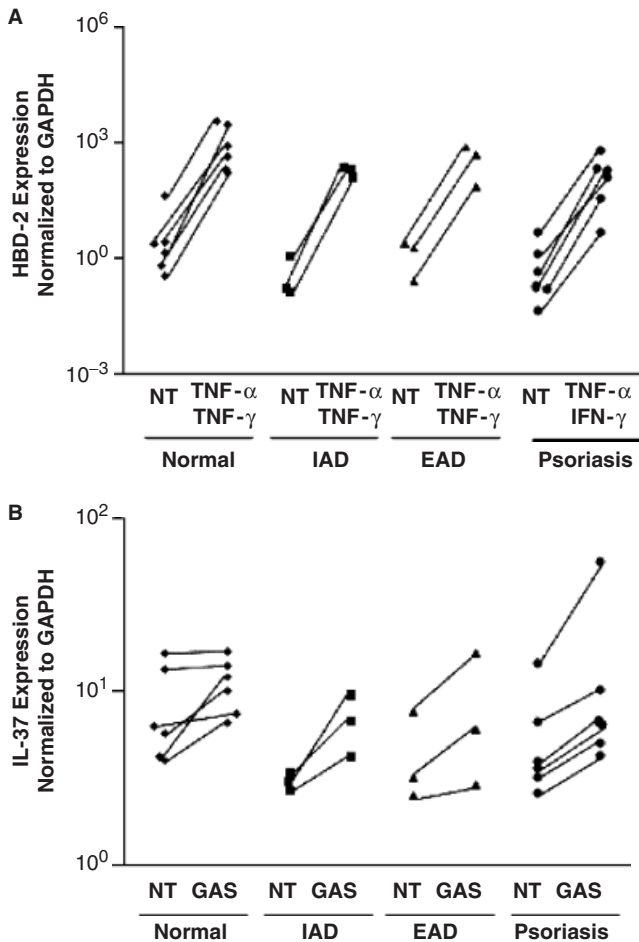


Figure 3
Reduced anti-microbial peptide expression is an acquired defect in primary keratinocytes from atopic dermatitis (AD) patients. The levels of human β -defensin (HBD)-2 (A) and LL-37 (B) were evaluated by real-time RT-PCR in differentiated keratinocytes obtained from normal subjects (\blacklozenge , $n=6$), extrinsic AD (EAD) patients (\blacktriangle , $n=3$), intrinsic AD (IAD) patients (\blacksquare , $n=3$), and psoriatic patients (\bullet , $n=6$). HBD-2 was induced by 24 h stimulation with tumor necrosis factor- α (TNF- α) plus interferon- γ (IFN- γ), and LL-37 with heat-inactivated group A *Staphylococcus* extract (GAS). Samples treated with media alone are indicated by non-treated (NT).

To investigate whether low AMP in AD skin could be reversed, lesional skin from EAD patients was cultured with neutralizing antibodies to IL-10 and IL-13 and assayed for AMP expression. Addition of anti-IL-10 to skin explants resulted in a significant increase in HBD-2 expression (mean: 1346.2 ± 190.3 ; $p=0.007$) and LL-37 expression (mean: 61.2 ± 9.2 ; $p<0.001$) (Fig 7). Interestingly, the addition of anti-IL-13 to EAD skin explants augmented HBD-2 expression by 6-fold (mean: 3341.3 ± 691.2 ; $p=0.004$) and LL-37 expression by over 10-fold (mean: 113.5 ± 12.3 , $p<0.001$).

Discussion

The epidermis is an immunologically rich layer of skin comprised largely of keratinocytes that differentiate into anucleated cells to form the stratum corneum. The stratum corneum is the skin's first line of defense against invading microbial pathogens. When this physical barrier is compro-

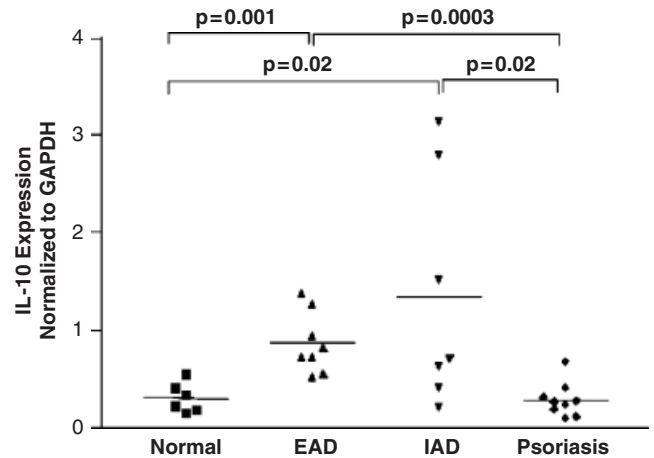


Figure 4
Increased interleukin (IL)-10 gene expression in extrinsic atopic dermatitis (EAD) and intrinsic atopic dermatitis (IAD) patients. RNA was isolated from the skin of normal subjects (\blacksquare , $n=7$), EAD (\blacktriangle , $n=9$), IAD (\blacktriangledown , $n=7$), and psoriasis (\blacklozenge , $n=8$) patients and the levels of IL-10 evaluated by real-time PCR. Significant differences between patient groups were determined by a non-parametric Mann-Whitney test.

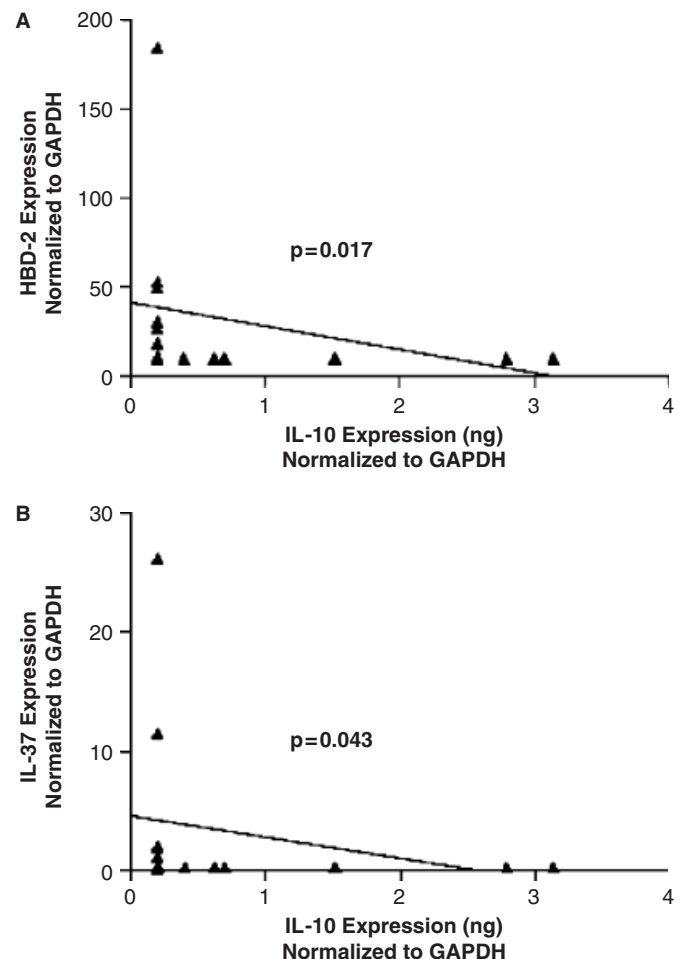


Figure 5
Inverse relationship between interleukin (IL)-10 and anti-microbial peptide (AMP) expression in intrinsic atopic dermatitis (IAD) patients. RNA was isolated from the skin of IAD ($n=7$) patients. The correlation between IL-10 and either human β -defensin (HBD)-2 (A) or LL-37 (B) gene expression was evaluated using linear regression.

Table I. Augmented TNF- α and IFN- γ production in AD PBMC treated with anti-IL-10

	TNF- α (pg per mL)	IFN- γ (pg per mL)
Media	<15.6	<15.6
Media + anti-IL-10	<15.6	<15.6
Anti-CD3	150.0 \pm 19.2	574.2 \pm 171.5
Anti-CD3 + anti-IL-10	209.0 \pm 18.5*	1376.7 \pm 270.1*

*Significance of $p \leq 0.05$ as compared with stimulation with anti-CD3 alone.

TNF- α , tumor necrosis factor- α ; IFN- γ , interferon- γ ; AD, atopic dermatitis; PBMC, peripheral blood mononuclear cell; IL, interleukin.

mised, further invasion by the pathogen is prevented by the innate immune response. AMP are an integral part of the innate immune response as they have been shown to be effective in killing bacterial (Ong *et al*, 2002) and viral (Howell *et al*, 2004) pathogens.

This study demonstrates that the skin of both IAD and EAD skin is deficient of AMP. Combined with a previous study which demonstrated that the levels of LL-37 and

HBD-2 in psoriasis skin, but not AD skin, can kill *S. aureus*, our observations may explain why both IAD and EAD exhibit similar propensity to skin infections. But the primary mechanism(s) underlying the AMP deficiency may be different between the two subsets of AD. Previous studies have demonstrated that the Th2 cytokines, IL-4 and IL-13, inhibit the expression of HBD-2 and HBD-3 in human keratinocytes (Ong *et al*, 2002; Nomura *et al*, 2003). Additional studies have demonstrated that patients with EAD express higher levels of IL-13 in the lesional skin (Jeong *et al*, 2003), skin T cells (Akdis *et al*, 1999), and PBMC (Kagi *et al*, 1994) than IAD. Therefore, we investigated other mechanisms to explain the decreased AMP expression in patients with IAD.

Pro-inflammatory cytokines such as TNF- α , IL-6, IL-1, and IFN- γ have been shown to induce the expression of AMP (Erdag and Morgan, 2002; Liu *et al*, 2002; Ong *et al*, 2002; Nomura *et al*, 2003). These cytokines are highly expressed in psoriasis skin (Grossman *et al*, 1989; Nomura *et al*, 2003), whereas negligible levels of TNF- α and IFN- γ have been shown in AD skin (Nomura *et al*, 2003). Despite higher levels of IFN- γ in chronic as compared with acute AD, skin explants from patients with chronic AD expressed minimal levels of AMP. This is likely because of the fact that pre-incubation of keratinocytes with IL-4/IL-13, as would be the case in acute AD that has evolved into chronic AD, inhibits the induction of AMP expression by TNF- α and IFN- γ (Nomura *et al*, 2003). This does not, however, explain the deficiency of AMP in IAD. Previous studies have demonstrated that IL-10 is expressed by mononuclear cells in AD and that the overexpression of IL-10 acts to inhibit or downregulate the pro-inflammatory and Th1 immune responses (Lester *et al*, 1995; Ohmen *et al*, 1995; Asadullah *et al*, 1998; Borish, 1998; Laouini *et al*, 2003).

Using real-time RT-PCR, we demonstrate that IL-10 is elevated in both EAD and IAD lesional skin whereas negligible levels are expressed in psoriasis skin. Additionally, we demonstrate an inverse relationship between the expression of IL-10 and AMP in lesional skin from IAD patients. In EAD skin, the expression of IL-10 did not correlate with AMP expression; however, the expression of both IL-10 and IL-13 correlated with decreased AMP expression. This further supports the immunoregulatory role of IL-10 in IAD and added role of IL-13 plus IL-10 in EAD.

In this study and in previous studies (Ong *et al*, 2002; Nomura *et al*, 2003), IL-13 has been shown to act directly on the keratinocytes to downregulate the expression of the β -defensin family. We found, however, that IL-10 does not act on human keratinocytes to directly inhibit AMP expression. This is likely due, in part, to the absence of IL-10R1 on keratinocytes and the requirement for both IL-10 receptors for signaling (Seifert *et al*, 2003). Previous studies have demonstrated that IL-10 acts on mononuclear cells and natural killer cells to inhibit the expression of pro-inflammatory cytokines and IFN- γ (de Waal Malefyt *et al*, 1991; Fiorentino *et al*, 1991; D'Andrea *et al*, 1993; Chernoff *et al*, 1995). Therefore, we explored the possibility that IL-10 indirectly inhibits AMP expression by downregulating the expression of the pro-inflammatory cytokines in mononuclear cells, thereby preventing the induction of the AMP. This concept is supported by the following findings in our study.

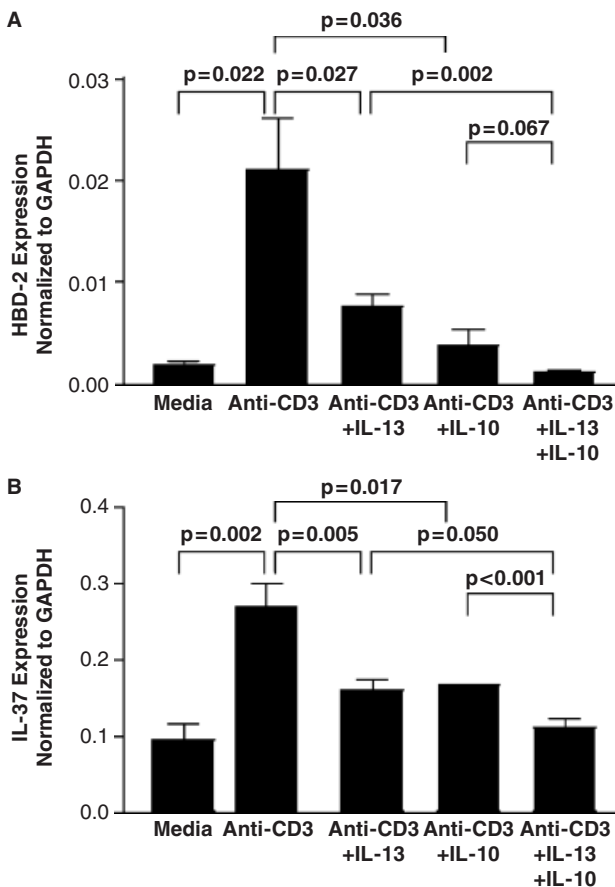


Figure 6
Anti-microbial expression in HaCaT keratinocytes is downregulated by interleukin (IL)-10 and IL-13. RNA was isolated from keratinocytes stimulated with the supernatants from normal peripheral blood mononuclear cell ($n = 6$) cultured with media or anti-CD3 (100 ng per mL) in the presence or absence of IL-10 and/or IL-13 and the levels of human β -defensin (HBD)-2 (A) and LL-37 (B) were evaluated by real-time RT-PCR. Significant differences between exposure groups were determined by a two-tailed t test.

First, we have found that stimulation of normal PBMC with anti-CD3 induces the expression of TNF- α , IL-6, IL-1, and IFN- γ . These cytokines have been previously reported to increase AMP expression in keratinocytes (Erdag and Morgan, 2002; Liu *et al*, 2002; Ong *et al*, 2002; Nomura *et al*, 2003). The addition of IL-10 to this system inhibited TNF- α and IFN- γ expression at both the mRNA and protein level. Second, supernatant from normal PBMC stimulated with anti-CD3, in the presence of IL-10, failed to induce the expression of AMP in human keratinocytes. This is likely to be because of the lack of the pro-inflammatory cytokines. In contrast, supernatants from PBMC stimulated with anti-CD3 in the absence of IL-10 induced significantly greater expression of HBD-2 and LL-37 than media alone. Our study additionally proposes the novel idea that IL-10 and IL-13 work through different mechanisms to synergistically downregulate AMP expression in AD skin. By stimulating PBMC in the presence of IL-10 and adding exogenous IL-13 to keratinocyte cultures, AMP expression was inhibited to a greater degree than observed for each cytokine alone.

The mechanism by which IL-10 inhibits cytokine production in macrophages and natural killer cells is still unclear. IL-10 has, however, been shown to inhibit the expression of B7.1 (CD80), B7.2 (CD86), and major histocompatibility complex II (MHC class II) expression in monocytes and Langerhan's cells (Ding *et al*, 1993). B7 and MHC class II are required for T cell activation and proliferation. By inhibiting the expression of these molecules in monocytes, IL-10 is able to prevent T cell activation and the subsequent release of pro-inflammatory cytokines (Ding *et al*, 1993). Since IL-10 is highly expressed in both EAD and IAD skin, this may explain the reduced levels of pro-inflammatory cytokines and negligible expression of the AMP.

Using antibodies against IL-10, we have directly demonstrated that the downregulatory role of IL-10 on pro-inflammatory cytokines could be reversed in PBMC from AD patients. Extending this observation, the addition of anti-IL-10 to lesional skin explants from AD patients augmented HBD-2 and LL-37 expression. The importance of this observation is further delineated by a previous study that demonstrated reduced bacterial growth and lethality in mice treated with anti-IL-10 prior to challenge with *Streptococcus pneumoniae* (van der Sluijs *et al*, 2004). Based on this study, we propose that neutralization of IL-10 in AD skin may decrease the susceptibility of these individuals to recurrent bacterial and viral skin infections.

This study demonstrates that reduced expression of AMP in AD is not constitutive but acquired as a result of increased Th2 cytokine expression. Moreover, this study demonstrates a novel mechanism by which AMP production can be deficient in AD, i.e. because of increased IL-10 expression. We propose that IL-10 indirectly inhibits AMP expression in keratinocytes by suppressing the production of pro-inflammatory cytokines, known to induce AMP, by mononuclear cells. This explains why, despite different clinical characteristics, both EAD and IAD are deficient of AMP and suffer from recurrent skin infections. Importantly, it identifies a new therapeutic target for augmentation of host defense, i.e. neutralization of IL-10 secretion in atopic skin (Fig 7).

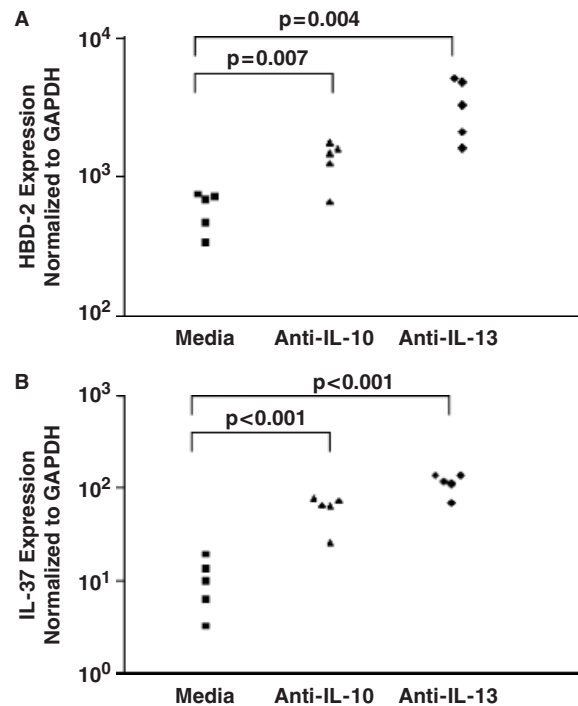


Figure 7

Neutralization of interleukin (IL)-10 and IL-13 in atopic dermatitis (AD) skin augments anti-microbial peptide expression. Lesional skin from extrinsic AD patients ($n=5$) was cultured in the presence of anti-IL-10 (1 μ g per mL) or anti-IL-13 (1 μ g per mL) for 24 h and the levels of human β -defensin (HBD)-2 (A) and LL-37 (B) evaluated by real-time RT-PCR. Significant differences between exposure groups were determined by a two-tailed *t* test.

Materials and Methods

Subjects Study participants included six healthy controls (mean age: 44.2 y), seven IAD patients (mean age: 29.1 y; SCORAD: 22.2 ± 5.6 ; serum IgE: 34–146 kU per liter), 14 EAD patients (mean age: 38.7 y; SCORAD: 45.0 ± 11.5 ; serum IgE: 288–5000 kU per liter), eight psoriasis patients (mean age: 39.7 y; 15%–40% skin involvement), and five ACD patients (mean age: 60.6 y). Sera from AD patients were tested against house dust mite, *Aspergillus*, *Candida albicans*, birch pollen, timothy grass pollen, ragweed, apple, milk, egg, peanut, hazelnut, *Staphylococcus enterotoxin A*, and *Staphylococcus enterotoxin B* for specific IgE with the Immulite 2000 system (DPC Biermann, Bad Nauheim, Germany). The skin prick test was conducted with the following allergens: house dust mite, grass, birch pollen, *Aspergillus*, *C. albicans*, milk, egg, soy, and peanut. Patients with IAD had no allergen-specific IgE and were skin prick test negative to inhalant and food allergens. In contrast, patients with EAD had allergen-specific IgE and were skin prick test positive to inhalant and food allergens.

None of the patients had received oral corticosteroids within 1 mo of skin explant, and topical corticosteroids were not allowed for a period of at least 1 wk prior to enrollment. These studies were conducted according to the Declaration of the Helsinki Guidelines and were approved by the institutional review board at National Jewish Medical and Research Center in Denver, the University of Bonn Medical Center in Germany, and the Istituto Dermatologico dell'Immacolata in Italy. All subjects gave written informed consent prior to enrollment.

PBMC culture PBMC were isolated from heparinized blood using Ficoll-Hypaque (Pharmacia Biotech, Piscataway, New Jersey) centrifugation and suspended in RPMI 1640 (Bio-Whittaker, Walkersville, Maryland) as previously described (Hauk *et al*, 2000).

PBMC were isolated from subjects and seeded at 1×10^6 cells per mL in 25 cm² flasks in the presence or absence of 100 ng per mL of anti-CD3. To investigate the effects of IL-10 on anti-CD3-stimulated normal PBMC, up to 50 ng per mL of IL-10 (R&D Systems, Minneapolis, Minnesota) was added to corresponding flasks and cultured for 24 h. Conversely, 1 µg per mL of anti-IL-10 (R&D Systems) was added to anti-CD3-stimulated PBMC from AD patients and cultured for 24 h. Following the incubation period, the supernatant was collected from stimulated PBMC and either stored at -80°C until further analysis or added to cultured HaCaT keratinocyte cells (2×10^5 cells per well) in 24-well plates. RNA was isolated from PBMC according to the manufacturer's guidelines (Qiagen, Valencia, California).

Cytokine protein analysis Cytokine quantification was performed on supernatants from stimulated PBMC. The Bio-Plex human cytokine 17-plex panel was purchased from Bio-Rad Laboratories and analyzed using the Bio-Plex array reader (Bio-Rad Laboratories) according to the manufacturer's guidelines. TNF-α and IFN-γ were further evaluated in supernatants using Quantikine ELISA kits according to the manufacturer's guidelines (R&D Systems). Data were calculated as mean picograms (pg) per mL ± standard error.

Keratinocyte and skin explant cultures Primary keratinocytes were isolated from six patients with moderate-to-severe chronic IAD or EAD (age range: 19–45 y), six patients with moderate-to-severe chronic plaque psoriasis (age range: 32–49 y), and six healthy control subjects (age range: 25–50 y). In these experiments, epidermal sheets were procured from the roof of suction blisters raised on normal-appearing skin of the forearms of patients with IAD or EAD, patients with psoriasis or healthy subjects, and primary keratinocyte cultures were obtained as described (Pastore *et al*, 1997). Keratinocytes were routinely grown in serum-free keratinocyte growth medium (Clonetics, San Diego, California), prepared from the essential nutrient solution keratinocyte basal medium supplemented with 10 ng per mL epidermal growth factor (Clonetics), 0.4 µg per mL hydrocortisone (Clonetics), bovine pituitary extract (Clonetics), and antibiotics. To study AMP gene expression, keratinocyte cultures were differentiated in the presence of 1.3 mM CaCl₂ for 3 d and then stimulated for 24 h with a combination of 20 ng per mL TNF-α (R&D Systems, Abingdon, UK) and 200 U per mL IFN-γ (R&D Systems), or with 1 per 10 dilution in culture medium of heat-inactivated GAS (strain NZ131) extract. The cells were then washed once and homogenized in buffer RLT (Qiagen). Total RNA was isolated according to the manufacturer's protocol in the presence of DNase I (Qiagen) for real-time RT-PCR.

The HaCaT human keratinocyte cell line was grown in Dulbecco's modified Eagle's medium (Cellgro, Herndon, Virginia), supplemented with 10% fetal calf serum (Gemini Bio Products, Calabasas, California) and 1% of the following: penicillin/streptomycin, L-glutamine, minimal essential medium (MEM) with non-essential amino acids (GIBCO, Grand Island, New York), MEM vitamins solution (GIBCO) until confluent. Once confluent, cells were split and seeded in 24-well plates at 2×10^5 cells per well and cultured for 24 h to allow adherence. To examine the direct effect of IL-10 on AMP production, HaCaT cells were stimulated with 20 ng per mL TNF-α (R&D Systems) and 200 U per mL IFN-γ (R&D Systems) in the presence and absence of either 50 ng per mL IL-10 and/or 50 ng per mL IL-13 (R&D Systems). Following a 24 h incubation, media were removed and RNA isolated from keratinocytes according to the manufacturer's guidelines (Qiagen). The indirect effect of IL-10 on AMP expression was assessed by adding culture supernatants from PBMC experiments to HaCaT cells. In some cases, IL-13 was added to the cultures in order to determine the combinatory effect of IL-13 on the keratinocytes and IL-10 on the pro-inflammatory cytokines and AMP expression. Cells were cultured for 24 h and RNA isolated from HaCaT cells according to the manufacturer's guidelines (Qiagen) for real-time RT-PCR.

Skin explants were cultured under various conditions in complete RPMI 1640 (Bio-Whittaker). To investigate whether the baseline levels of AMP expression were similar between normal and AD patients; punch biopsies were collected from non-lesional skin of four AD patients as well as four normal subjects and added to 96-well tissue culture plates. Culture supernatants from either media or anti-CD3-stimulated PBMC were added to skin explants and allowed to culture for 24 h. Following exposure, RNA was isolated from skin explants for real-time RT-PCR. In another experiment, lesional skin from five EAD patients was cultured *ex vivo* in complete RPMI 1640 (Bio-Whittaker). Anti-IL-10 (R&D Systems) or anti-IL-13 (R&D Systems) were added to skin explants for 24 h to investigate the effect of Th2 cytokine neutralization on AMP expression. Following exposure, RNA was isolated from skin explants for real-time RT-PCR.

RNA preparation and analysis Total RNA was isolated from skin explants by chloroform:phenol extraction and isopropanol precipitation according to the manufacturer's guidelines (Sigma Chemical Co., St Louis, Missouri). RNeasy Mini Kits (Qiagen) were used according to the manufacturer's protocol to isolate RNA from cell cultures and to further purify RNA from skin explants. One microgram of RNA was reverse transcribed in a 20 µL reaction containing Random Primers (Invitrogen, Carlsbad, California), RNase Inhibitor (Invitrogen), and Superscript II enzyme (Invitrogen). Real-time PCR was performed and analyzed by the dual-labeled fluorogenic probe method using an ABI Prism 7000 sequence detector (Applied Biosystems, Foster City, California). Primers and probes for human IL-10, TNF-α, IFN-γ, and GAPDH were purchased from Applied Biosystems. HBD-2 and LL-37 primer and probes were prepared as previously described (Ong *et al*, 2003). The TaqMan probes were purchased from Applied Biosystems and were 5' labeled with 6-carboxyfluorescein and 3' labeled with 6-carboxy-tetramethylrhodamine. Amplification reactions were performed in MicroAmp optical tubes (Applied Biosystems) in a 25 µL volume containing 2 × TaqMan Master Mix (Applied Biosystems), 900 nM forward primer, 900 nM reverse primer, 200 nM probe, and the template RNA. Thermal cycling conditions were: 50°C for 2 min, 95°C for 10 min for one cycle. Subsequently, 40 cycles of amplification were performed at 94°C for 15 s and 60°C for 1 min. Relative expression levels were calculated by the relative standard curve method as outlined in the manufacturer's technical bulletin. A standard curve was generated using the fluorescent data from 10-fold serial dilutions of total RNA of the highest expression sample. To allow for comparisons between samples and groups, quantities of all targets in test samples were normalized to the corresponding GAPDH levels in the skin explants, cultured keratinocytes, and PBMC, and expressed as Target Gene normalized to GAPDH.

Immunohistochemistry for LL-37 Skin explants were submerged in 10% buffered formalin for immunostaining. Paraffin-embedded tissues were cut at 5 µm and placed on frosted microscope slides. Using toluene and a series of ethanol washes, slides were deparaffinized and then re-hydrated. Tissue sections were then immersed in antigen retrieval solution (0.01 M citric acid, 0.05 M NaOH, pH 6.0) and microwaved for 4 min. Endogenous peroxidase was blocked by incubating slides for 30 min in a 0.3% H₂O₂ solution. Skin sections were then blocked with 2% goat serum and 3% bovine serum albumin in PBS for 30 min and then stained with rabbit anti-LL-37 (5 µg per mL) as previously described (Dorschner *et al*, 2001). Sections were washed in PBS, stained with goat anti-rabbit horseradish peroxidase (Vectastain Elite ABC Rabbit kit, Vector Laboratories, Burlingame, California), and detected with diaminobenzidine substrate (Sigma Chemical Co.) according to the manufacturer's instructions. The skin sections were counterstained with hematoxylin. Primary antibody specificity was confirmed in separate experiments by adsorption of anti-LL-37 antibody with excess amounts of the synthetic peptide. Specificity of the goat anti-rabbit antibody and immunostaining reagents was confirmed by routine use of the rabbit non-immune sera.

All slides were coded before the samples were evaluated so that the identity of the patients would be blinded to the observer. Images were collected at $\times 40$ magnification and the intensity of the immunostaining was scored on a scale from 0 to 5, with 0 indicating no staining and 5 the most intense staining.

Statistical analyses Statistical analysis was conducted using Graph Pad Prism, version 3.01 (San Diego, California) and SAS (Research Triangle Park, North Carolina). Statistical differences in gene expression or protein staining between groups were determined using a non-parametric Mann–Whitney test, with significant differences conferred when $p \leq 0.05$. In cases where multiple exposure groups were compared with a control, data were analyzed by a one-way ANOVA and significant differences between exposure groups were determined by a Tukey–Kramer test. Regression analysis was performed using PROC GLM in SAS.

This work was supported in part by the NIH National Research Service Award (T32 AI 07365) and AAAAI Fujisawa Skin Diseases Award (M. D. H.); Deutsche Forschungsgemeinschaft DFG no. 454/1-1 and DFG no. 454/2-1 (N. N. and T. B.); Italian Ministry of Health (S. P. and G. G.); NIH grants AI052453, AR45676, and a VA Merit Award (R. L. G.); NIH grants HL36577, AR41256, HL37260, R21 AR051634-01, General Clinical Research Center grant MO1 RR00051 from the Division of Research Resources, the Edelstein Family Chair in Pediatric Allergy and Immunology, and the University of Colorado Cancer Center (D. Y. M. L.). The authors thank Ichiro Nomura, Lening Zhang, Jean-Pierre Allam, and Annegret Dunlap for their technical help with this project. The authors are additionally indebted to the nursing staff in the General Clinical Research Center for the recruitment of patients and collection of specimens. The authors also thank Maureen Sandoval for her help in preparing this manuscript.

DOI: 10.1111/j.0022-202X.2005.23776.x

Manuscript received November 18, 2004; revised February 25, 2005; accepted for publication March 1, 2005

Address correspondence to: Donald Y. M. Leung, MD, PhD, National Jewish Medical and Research Center, Department of Pediatrics, Room K926, 1400 Jackson Street, Denver, Colorado 80206, USA. Email: leungd@njc.org

References

- Akdis CA, Akdis M, Simon D, *et al*: T cells and T cell-derived cytokines as pathogenic factors in the nonallergic form of atopic dermatitis. *J Invest Dermatol* 113:628–634, 1999
- Asadullah K, Sterry W, Stephanek K, *et al*: IL-10 is a key cytokine in psoriasis. Proof of principle by IL-10 therapy: A new therapeutic approach. *J Clin Invest* 101:783–794, 1998
- Borish L: IL-10: Evolving concepts. *J Allergy Clin Immunol* 101:293–297, 1998
- Chernoff AE, Granowitz EV, Shapiro L, *et al*: A randomized, controlled trial of IL-10 in humans. Inhibition of inflammatory cytokine production and immune responses. *J Immunol* 154:5492–5499, 1995
- D'Andrea A, Aste-Amezaga M, Valiante NM, Ma X, Kubin M, Trinchieri G: Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. *J Exp Med* 178:1041–1048, 1993
- de Waal Malefyt R, Abrams J, Bennett B, Figdor CG, de Vries JE: Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: An autoregulatory role of IL-10 produced by monocytes. *J Exp Med* 174:1209–1220, 1991
- Ding L, Linsley PS, Huang LY, Germain RN, Shevach EM: IL-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 expression. *J Immunol* 151:1224–1234, 1993
- Dorschner RA, Pestonjamas VK, Tamakuwala S, *et al*: Cutaneous injury induces the release of cathelicidin anti-microbial peptides active against group A *Streptococcus*. *J Invest Dermatol* 117:91–97, 2001
- Erdag G, Morgan JR: Interleukin-1alpha and interleukin-6 enhance the antibacterial properties of cultured composite keratinocyte grafts. *Ann Surg* 235:113–124, 2002
- Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A: IL-10 inhibits cytokine production by activated macrophages. *J Immunol* 147:3815–3822, 1991
- Gallo RL, Murakami M, Ohtake T, Zaiou M: Biology and clinical relevance of naturally occurring antimicrobial peptides. *J Allergy Clin Immunol* 110:823–831, 2002
- Grossman RM, Krueger J, Yourish D, *et al*: Interleukin 6 is expressed in high levels in psoriatic skin and stimulates proliferation of cultured human keratinocytes. *Proc Natl Acad Sci USA* 86:6367–6371, 1989
- Hanifin JM, Rajka G: Diagnostic features of atopic dermatitis. *Acta Derm Venereol (Stockh)* 92:44–47, 1980
- Harder J, Bartels J, Christophers E, Schroder JM: A peptide antibiotic from human skin. *Nature* 387:861, 1997
- Hauk PJ, Hamid QA, Chrousos GP, Leung DY: Induction of corticosteroid insensitivity in human PBMCs by microbial superantigens. *J Allergy Clin Immunol* 105:782–787, 2000
- Howell MD, Jones JF, Kisich KO, Streib JE, Gallo RL, Leung DY: Selective killing of vaccinia virus by LL-37: Implications for eczema vaccinatum. *J Immunol* 172:1763–1767, 2004
- Jeong CW, Ahn KS, Rho NK, *et al*: Differential *in vivo* cytokine mRNA expression in lesional skin of intrinsic vs. extrinsic atopic dermatitis patients using semiquantitative RT-PCR. *Clin Exp Allergy* 33:1717–1724, 2003
- Johansson SG, Bieber T, Dahl R, *et al*: Revised nomenclature for allergy for global use: Report of the Nomenclature Review Committee of the World Allergy Organization, October 2003. *J Allergy Clin Immunol* 113:832–836, 2004
- Kagi MK, Wuthrich B, Montano E, Barandun J, Blaser K, Walker C: Differential cytokine profiles in peripheral blood lymphocyte supernatants and skin biopsies from patients with different forms of atopic dermatitis, psoriasis and normal individuals. *Int Arch Allergy Immunol* 103:332–340, 1994
- Laouini D, Alenius H, Bryce P, Oettgen H, Tsitsikov E, Geha RS: IL-10 is critical for Th2 responses in a murine model of allergic dermatitis. *J Clin Invest* 112:1058–1066, 2003
- Lester MR, Hofer MF, Gately M, Trumble A, Leung DY: Down-regulating effects of IL-4 and IL-10 on the IFN-gamma response in atopic dermatitis. *J Immunol* 154:6174–6181, 1995
- Leung DY, Bieber T: Atopic dermatitis. *Lancet* 361:151–160, 2003
- Leung DY, Boguniewicz M, Howell MD, Nomura I, Hamid QA: New insights into atopic dermatitis. *J Clin Invest* 113:651–657, 2004
- Liu AY, Destoumieux D, Wong AV, Park CH, Valore EV, Liu L, Ganz T: Human beta-defensin-2 production in keratinocytes is regulated by interleukin-1, bacteria, and the state of differentiation. *J Invest Dermatol* 118:275–281, 2002
- Nizet V, Ohtake T, Lauth X, *et al*: Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature* 414:454–457, 2001
- Nomura I, Goleva E, Howell MD, *et al*: Cytokine milieu of atopic dermatitis, as compared to psoriasis, skin prevents induction of innate immune response genes. *J Immunol* 171:3262–3269, 2003
- Novak N, Bieber T: Allergic and nonallergic forms of atopic diseases. *J Allergy Clin Immunol* 112:252–262, 2003
- Ohmen JD, Hanifin JM, Nickoloff BJ, *et al*: Overexpression of IL-10 in atopic dermatitis. Contrasting cytokine patterns with delayed-type hypersensitivity reactions. *J Immunol* 154:1956–1963, 1995
- Ong PY, Ohtake T, Brandt C, *et al*: Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N Engl J Med* 347:1151–1160, 2002
- Oppel T, Schuller E, Gunther S, Moderer M, Haberstock J, Bieber T, Wollenberg A: Phenotyping of epidermal dendritic cells allows the differentiation between extrinsic and intrinsic forms of atopic dermatitis. *Br J Dermatol* 143:1193–1198, 2000
- Pastore S, Fanales-Belasio E, Albanesi C, Chinni LM, Giannetti A, Girolomoni G: Granulocyte macrophage colony-stimulating factor is overproduced by keratinocytes in atopic dermatitis. Implications for sustained dendritic cell activation in the skin. *J Clin Invest* 99:3009–3017, 1997
- Seifert M, Gruenberg BH, Sabat R, *et al*: Keratinocyte unresponsiveness towards interleukin-10: Lack of specific binding due to deficient IL-10 receptor 1 expression. *Exp Dermatol* 12:137–144, 2003
- van der Sluijs KF, van Elden LJ, Nijhuis M, *et al*: IL-10 is an important mediator of the enhanced susceptibility to pneumococcal pneumonia after influenza infection. *J Immunol* 172:7603–7609, 2004