MHC Dextramer® – Detect with Confidence
Get the full picture of **CD8+** and **CD4+** T-cell responses
Even the low-affinity ones
Available also in GMP

IMMUDEX
PRECISION IMMUNE MONITORING

The Journal of Immunology

RESEARCH ARTICLE | SEPTEMBER 15 2003

Cytokine Milieu of Atopic Dermatitis, as Compared to Psoriasis, Skin Prevents Induction of Innate Immune Response Genes [1] FREE

Ichiro Nomura; ... et. al

J Immunol (2003) 171 (6): 3262–3269.
https://doi.org/10.4049/jimmunol.171.6.3262

Related Content

8-Methoxypsoralen Plus Ultraviolet A Therapy Acts via Inhibition of the IL-23/Th17 Axis and Induction of Foxp3$^+$ Regulatory T Cells Involving CTLA4 Signaling in a Psoriasis-Like Skin Disorder

J Immunol (June,2010)

Transcriptomic meta-analysis reveals signatures of chronic inflammation in the classical monocyte population

J Immunol (May,2018)

Self-lipid specific T cells, restricted by CD1b, contribute to the development of hyperlipidemia-associated skin inflammation (BA11P.133)

J Immunol (May,2015)

Downloaded from http://journals.aai.org/jimmunol/article-pdf/171/6/3262/1170112/3262.pdf by guest on 24 May 2024

Cytokine Milieu of Atopic Dermatitis, as Compared to Psoriasis, Skin Prevents Induction of Innate Immune Response Genes[1]

Ichiro Nomura,* Elena Goleva,* Michael D. Howell,* Quatyba A. Hamid,† Peck Y. Ong,* Clifton F. Hall,* Marc A. Darst,‡ Bifeng Gao,§ Mark Boguniewicz,* Jeffrey B. Travers,‡ and Donald Y. M. Leung[2]*

Atopic dermatitis (AD) and psoriasis are the two most common chronic skin diseases. However patients with AD, but not psoriasis, suffer from frequent skin infections. To understand the molecular basis for this phenomenon, skin biopsies from AD and psoriasis patients were analyzed using GeneChip microarrays. The expression of innate immune response genes, human β defensin (HBD)-2, IL-8, and inducible NO synthetase (iNOS) was found to be decreased in AD, as compared with psoriasis, skin (HBD-2, $p = 0.00021$; IL-8, $p = 0.044$; iNOS, $p = 0.016$). Decreased expression of the novel antimicrobial peptide, HBD-3, was demonstrated at the mRNA level by real-time PCR ($p = 0.0002$) and at the protein level by immunohistochemistry ($p = 0.0005$). By real-time PCR, our data confirmed that AD, as compared with psoriasis, is associated with elevated skin production of Th2 cytokines and low levels of proinflammatory cytokines such as TNF-α, IFN-γ, and IL-1β. Because HBD-2, IL-8, and iNOS are known to be inhibited by Th2 cytokines, we examined the effects of IL-4 and IL-13 on HBD-3 expression in keratinocyte culture in vitro. We found that IL-13 and IL-4 inhibited TNF-α- and IFN-γ-induced HBD-3 production. These studies indicate that decreased expression of a constellation of antimicrobial genes occurs as the result of local up-regulation of Th2 cytokines and the lack of elevated amounts of TNF-α and IFN-γ under inflammatory conditions in AD skin. These observations could explain the increased susceptibility of AD skin to microorganisms, and suggest a new fundamental rule that may explain the mechanism for frequent infection in other Th2 cytokine-mediated diseases. *The Journal of Immunology,* **2003, 171: 3262–3269.**

Atopic dermatitis (AD)[3] and psoriasis are the two most common chronic inflammatory skin diseases found in the general population (1, 2). However, their mechanisms for skin inflammation and propensity for skin infection are quite different. The immune response in AD is Th2-mediated contributing to the high IgE levels and eosinophilia characteristic of this condition (3). In contrast, the immune response in psoriasis is Th1-mediated and associated with local neutrophil infiltration (4). Importantly, ~30% of AD patients suffer from frequent serious skin infections, and over 90% of AD patients are colonized with *Staphylococcus aureus* (5). In contrast, only 6.7% of psoriasis patients suffer from skin infections despite the fact that both skin diseases are characterized by defective skin barriers (6). This has recently been highlighted as a major public health problem because patients with AD are prone to eczema vaccinatum (7). Because nearly 15% of children have AD and close contacts are excluded from smallpox vaccination, it is estimated that nearly 50% of the general population cannot be vaccinated with the smallpox vaccine in any voluntary mass vaccination campaign to blunt the effects of a potential bioterrorist attack. Therefore, it is of great interest to better understand the mechanisms that make patients with AD prone to microbial skin infections.

Antimicrobial peptides have been shown to be key elements in the innate immune system providing the first line of defense in the skin against invading microbes (8). In normal skin, these peptides are present at only negligible levels. However, in response to injury or inflammation, keratinocytes produce high levels of these antimicrobial peptides, e.g., in the skin of patients with psoriasis. Animal models have shown that the induction of antimicrobial peptide expression is essential for skin to combat infection (9, 10). We have reported recently that both human β defensin (HBD)-2 and LL-37, a cathelicidin, are produced at low levels in the skin of AD patients compared with psoriasis patients (11). HBD-2 was able to kill *S. aureus* in the presence of LL-37 but not by itself.

Recently, a new antimicrobial peptide, HBD-3, has been identified which has potent killing activity against *S. aureus* by itself and can kill a broad spectrum of microbes (12, 13). Therefore, a deficiency of HBD-3 could contribute to the increased susceptibility of AD skin to infection. To capture a more complete picture of the innate immune response in atopic skin, we conducted the current study using a GeneChip microarray of multiple innate immune response genes using psoriasis skin samples as a control. The current study included a number of potentially important innate

*Department of Pediatrics, National Jewish Medical and Research Center, Denver, CO 80206, and Department of Pediatrics, University of Colorado Health Sciences Center, Denver, CO 80262; †Meakins-Christie Laboratories, McGill University, Montreal, Quebec, Canada; ‡Departments of Dermatology and Pediatrics and the H. B. Wells Center for Pediatric Research, Pharmacology, and Toxicology, Indiana University School of Medicine, Indianapolis, IN 46202; and §Microarray Core Laboratory, University of Colorado Health Sciences Center, Denver, CO 80262

Received for publication April 1, 2003. Accepted for publication July 9, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] D.Y.M.L. was supported in part by National Institutes of Health Grants HL36577, AR41256, HL37260; General Clinical Research Center Grant MO1 RR00051 from the Division of Research Resources; and the University of Colorado Cancer Center. P.Y.O. was the recipient of a National Institutes of Health National Research Service Award (T32 AI 07365) and an American Academy of Allergy Asthma and Immunology Fujisawa Health Care Allergic Skin Diseases Award.

[2] Address correspondence and reprint requests to Dr. Donald Y. M. Leung, National Jewish Medical and Research Center, 1400 Jackson Street, Room K926i, Denver, CO 80206. E-mail address: leungd@njc.org

[3] Abbreviations used in this paper: AD, atopic dermatitis; HBD, human β defensin; iNOS, inducible nitric oxide synthetase.

Copyright © 2003 by The American Association of Immunologists, Inc. 0022-1767/03/$02.00

Downloaded from http://journals.aai.org/jimmunol/article-pdf/171/6/3262/1170112/3262.pdf by guest on 24 May 2024

immunity genes such as induced NO synthetase (iNOS) which can kill viruses, bacteria, and fungi through production of NO (14); IL-8 which attracts neutrophils into the skin for phagocytosis and killing of bacteria; as well as Toll-like receptors and their signal transduction system needed to recognize and respond against microbial organisms. Our data indicate that the cytokine milieu in AD, as compared with psoriasis, prevents the induction of multiple innate immune response genes due to lower levels of proinflammatory cytokines such as TNF-α or IFN-γ needed for induction of inducible antimicrobial peptides, and the increased Th2 cytokines (IL-4 and IL-13, known to be overexpressed in AD skin but suppress antimicrobial peptide expression).

Materials and Methods

Subjects

This study was approved by the Institutional Review Board at National Jewish Medical and Research Center (Denver, CO). Skin biopsies were obtained from the skin lesions of 15 different patients with moderate to severe AD (mean age = 33.0 years; skin involvement 20–60%, the samples of six different patients were used for GeneChip study, six different patients for immunohistochemistry, 10 different patients for real-time PCR; some patients' skin biopsies were used in multiple assays), 15 psoriasis patients (mean age = 39.7 years; skin involvement 15–40%, the samples of seven patients were used for GeneChip study, 12 were used for real-time PCR, six for immunohistochemistry; some samples were used in multiple experiments), and six healthy individuals (mean age = 44.2 years, four healthy control samples were examined by both real-time PCR and immunohistochemistry, another two were used only for real-time PCR) were included in this study. None of the patients had previously received any systemic immunosuppressive drugs such as corticosteroids or cyclosporin. All patients were off topical corticosteroids or calcineurin inhibitor for more than 1 wk before their skin biopsy. Informed consent was obtained from all subjects before the study. The skin samples were immediately submerged in 1 ml of RNA later (RNeasy protect mini kit; Qiagen, Valencia, CA) and frozen at −80°C for future RNA isolation or fixed for immunohistochemistry.

RNA extraction from skin biopsies

Skin biopsies, preserved in RNA later were put into 600 μl of buffer RLT (Qiagen RNeasy minikit) and homogenized using a Pyrex tissue grinder. Total RNA was then extracted according to the manufacturer's protocol in the presence of DNase I (Qiagen).

Cell culture

Primary neonatal human keratinocytes (Clonetics, East Rutherford, NJ) were grown and subcultured based on manufacturer's recommendations. To study the effects of cytokines on HBD-3 mRNA expression in keratinocytes, 5×10^5 cells were differentiated in the presence of 1.3 mM $CaCl_2$ (as described in Ref. 15) for 3 days and then stimulated in the presence and absence of 20 ng/ml TNF-α, 200 U/ml (20 ng/ml) IFN-γ, or their combination with or without IL-4 and IL-13 (50 ng/ml) for 24 h. All cytokines were purchased from R&D Systems (Minneapolis, MN). The cells were then washed once and homogenized in buffer RLT (Qiagen RNeasy minikit) by repeated pipetting. Total RNA was isolated according to the manufacturer's protocol (Qiagen) in the presence of DNase I (Qiagen).

DNA microarray analysis

Five micrograms of total RNA isolated from skin samples was individually converted to double-stranded cDNA (ds-cDNA) using Superscript Choice system (Life Technologies, Rockville, MD). An oligo-dT primer containing a T7 RNA polymerase promoter (Promega, Madison, WI) was used. After second strand synthesis, the reaction mixture was extracted with phenol-chloroform-isoamyl alcohol and the ds-cDNA was recovered by ethanol precipitation. In vitro transcription and biotin-labeling were then performed with a RNA transcript labeling kit (Enzo Biochem, New York, NY). A ds-cDNA template was transcribed in the presence of a mixture of unlabeled ATP, CTP, GTP, UTP, biotin-labeled CTP and UTP (Enzo Biochem). Synthesized cRNA was fragmented in a fragmentation buffer at 94°C, for 35 min. Then, samples were resuspended in a hybridization solution containing 100 mM MES, 1M NaCl, and 20 mM EDTA in the presence of 0.01% Tween 20. Each of six AD samples and seven psoriasis samples were loaded individually into respective (total 13) human U-95Av2 GeneChip probe arrays (Affymetrix, Santa Clara, CA) and hybridized at 45°C for 16 h. After washing and staining with streptavidin-PE, the probe arrays were scanned using a Gene-array scanner (Hewlett Packard, Palo Alto, CA). The data was analyzed with GeneChip Suite version 5.0 software (Affymetrix). The GeneChip array analysis yielded a hybridization intensity for each represented gene as the signal intensity. A difference between AD and psoriasis skin was defined as significant if 2-fold difference in average signal intensity with a p value under 0.05 using a two-tailed unpaired t test was observed. Detailed protocols (16) and data analysis (17, 18) have been described in former reports.

Immunohistochemistry for HBD-3

For these experiments, skin biopsies were fixed in 4% paraformaldehyde in PBS for 4 h and transferred into 10% PBS-buffered formalin (Fisher Scientific, Fair Lawn, NJ). They were then transferred to PBS before being processed and embedded in paraffin. Three micron sections were cut onto poly-L-lysine-coated slides, dried and immunostained using polyclonal rabbit anti-human Ab directed against HBD-3 (Orbigen, San Diego, CA). The Ab was used at 1/100 dilution (10 μg/ml) which gave optimal staining following a titration study. The Ag-Ab complex was detected with an avidin-biotin peroxidase complex method, as described before (19). The signal was visualized with diamino benzidine. As a negative control, isotype Ab (purified nonimmune rabbit IgG; Southern Biotechnology Associates, Birmingham, AL) was used. The immunoreactivity was presented as the percentage of keratinocytes which were positively stained with a score of 0 to 8, where 0 was defined as no staining, 1 = up to 12.5%, 2 = up to 25% with stepwise increase by 12.5% to 8 = up to 100%.

Quantitative real-time PCR

The primers and probes used in the real-time PCR of HBD-3 mRNA were selected with Primer Express software (PerkinElmer/Applied Biosystems, Norwalk, CT) as follows: forward, 5′-GCTGCCTTCCAAAGGAGGA-3′ and reverse 5′-TTCTTCGGCAGCATTTTCG-3′ and 6FAM-ACGCGTC GAGCACTTGCCGATCT-TAMRA. Primers and probes for HBD-2 were the same as in the former report (11). Primers and probes for human IL-4, IL-8, IL-13, TNF-α, IFN-γ, IL-1β, GAPDH, and 18s RNA were purchased from PerkinElmer/Applied Biosystems (Norwalk, CT). Total RNA was isolated as described above. One microgram of RNA was reverse-transcribed in a 20-μl reaction containing Random Primers (Invitrogen, Carlsbad, CA) and Superscript II enzyme (Invitrogen). Each PCR system (50 μl) contained 1 μl of cDNA, 1× gold buffer II, 4 mM $MgCl_2$, 500 μM dNTP, primers (200 nM), 100 nM probe (labeled with 6-carboxyfluorescein), and 1.25 U AmpliTaq Gold DNA polymerase (PerkinElmer/Applied Biosystems). Real-time PCR was performed and analyzed by the dual-labeled fluorigenic probe method using an ABI Prism 7700 sequence detector (PerkinElmer/Applied Biosystems). 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 the 10-fold serial dilutions of total RNA of the highest expression sample. This was then used to calculate the relative amounts of target mRNA in test samples. Quantities of all targets in test samples were normalized to the corresponding GAPDH RNA transcript in the skin samples and 18S RNA transcripts in cultured keratinocytes.

Statistical methods

Data were presented as the means + SEM. Data were analyzed using unpaired two-tailed t test or one-way ANOVA followed by the Scheffe's F test or Bonferroni-Dunn test for multiple comparisons. An associated probability (p value) of <0.05 was considered significant.

Results

GeneChip microarray studies

GeneChip microarray analyses were conducted on biopsies of skin lesions from six different AD and seven different psoriasis patients studying all known innate immunity genes available in the human U-95Av2 GeneChip Affymetrix probe array. The GeneChip array analysis yielded a hybridization intensity for each represented gene as the signal intensity. The difference between AD and psoriasis skin was defined as significant if 2-fold or greater difference in average hybridization signal intensity with a p value under 0.05 using a two-tailed unpaired t test was observed.

Table I compares expression of various groups of those genes

Downloaded from http://journals.aai.org/jimmunol/article-pdf/171/6/3262/1170112/3262.pdf by guest on 24 May 2024

Table I. *Surveyed innate immunity genes in GeneChip microarray*

Classification	U-95 Av2	Gene Accession No.	Mean AD ± SE[a]	Mean PS ± SE[a]	t Test[b]
Cytokines and chemokines					
TNF-α	1852_at	X02910	156 ± 32	162 ± 14	
IL-1β	39402_at	M15330	195 ± 28	692 ± 229	
IL-6	38299_at	X04430	38 ± 14	45 ± 11	
IL-12	563_at	M65290	18 ± 6	16 ± 3[c]	
IFN-γ	40702_at	X13274	32 ± 11	54 ± 15	
IL-8	35372_r_at	M17017	63 ± 12	600 ± 210	$p = 0.044$
IL-8Rβ	664_at	L19593	62 ± 9	122 ± 12	$p = 0.0023$
IL-4	1574_s_at	M13982	72 ± 17	68 ± 14	
IL-13	494_at	U31120	135 ± 35	84 ± 20	
Toll-like receptors					
TLR1	36243_at	AL050262	97 ± 21	81 ± 17	
TLR2	40310_at	AF051152	108 ± 10	161 ± 26	
TLR3	33488_at	U88879	52 ± 16	25 ± 5	
TLR5	34473_at	AF051151	62 ± 12	69 ± 15	
TLR6	34144_at	AB020807	86 ± 18	79 ± 16	
Phagocytosis-promoting receptors					
MARCO[d]	40331_at	AF035819	126 ± 41	94 ± 41	
β_1 integrins	32808_at	X07979	2489 ± 132	2305 ± 228	
E-cadherin	2082_s_at	L08599	378 ± 69	379 ± 32	
CD36	36656_at	M98399	49 ± 7	74 ± 11	
CD11b	38533_s_at	J03925	52 ± 26	35 ± 11	
CD35	35892_at	Y00816	38 ± 7	34 ± 10	
Mannose receptor	36908_at	M93221	312 ± 74	217 ± 38	
CD14	36661_s_at	X06882	108 ± 10	161 ± 26	
Complement					
C1r	39409_at	M14058	2869 ± 326	2467 ± 109	
C1q B chain	38796_at	X03084	620 ± 141	815 ± 249	
C1r	40496_at	J04080	2485 ± 464	2310 ± 408	
C4	40766_at	U24578	253 ± 44	200 ± 48	
C5	37816_at	M57729	44 ± 9	32 ± 10	
Anti-microbial peptides, enzymes					
			(from neutrophils)		
Defensin, α1	31793_at	AL036554	61 ± 12	73 ± 24	
Defensin, α4	34546_at	AI250799	66 ± 9	49 ± 14	
Defensin, α6	34622_at	U33317	19 ± 9	18 ± 3	
Defensin, α5	34623_at	M97925	49 ± 23	51 ± 14	
Azurocidin	33963_at	M96326	55 ± 9	34 ± 3	
Cathepsin C	133_at	X87212	2875 ± 476	2063 ± 287	
Cathepsin D	239_at	M63138	4496 ± 287	6488 ± 444	$p = 0.0037$
Cathepsin H	37021_at	X16832	1630 ± 148	2604 ± 312	$p = 0.021$
			(from keratinocytes)		
iNOS	1948_f_at	U31511	65 ± 9	299 ± 71	$p = 0.016$
HBD-1	39660_at	AI309115	3255 ± 148	4279 ± 348	$p = 0.027$
HBD-2	32464_at	AF071216	13312 ± 4264	45863 ± 4177	$p = 0.00021$
Signal transduction					
MyD88[d]	38369_at	U70451	1063 ± 120	1235 ± 118	
TRAF6[d]	1209_at	U78798	72 ± 11	81 ± 12	
NF-κB1	1377_at	M58603	743 ± 70	808 ± 61	
NF-κB2	40363_r_at	U09609	78 ± 10	64 ± 8	

[a] Mean signal intensity ± SE of six AD skin biopsies and seven psoriasis (PS) skin biopsies.
[b] Two-tailed unpaired t test comparing AD vs psoriasis.
[c] Signal intensity under 100 is considered as under the detection limit by software used.
[d] MARCO, macrophage scavenger receptor with collagenous structure; MyD88, myeloid differentiation factor 88; TRAF, TNFR-associated factor.

that belong to the innate immunity system in AD and psoriasis skin. Interestingly, most of them revealed similar levels of expression in atopic and psoriatic skin lesions. Using the GeneChip microarrays lower levels of IFN-γ, IL-1β, and TNF-α were observed in AD, as compared with psoriasis, skin. However these differences did not achieve statistically significant differences. In contrast, expression of HBD-2 ($p = 0.00021$), iNOS ($p = 0.016$), and IL-8 ($p = 0.044$) was found to be significantly lower in AD skin lesions as compared with psoriasis skin lesions. Expression of HBD-1, Toll-like receptor-2, CD14, cathepsin D and H were elevated in psoriatic skin samples but was not greater than a 2-fold difference.

Immunohistochemistry of HBD-3 in AD, psoriasis, and normal skin

The U-95Av2 GeneChip Affymetrix probe array that was used in this study did not include a probe for HBD-3 which is a newly identified antimicrobial peptide that has been found to be active against a broad spectrum of microbes (12, 13). In particular, HBD-3 has strong killing activity at physiologic concentrations

Downloaded from http://journals.aai.org/jimmunol/article-pdf/171/6/3262/1170112/3262.pdf by guest on 24 May 2024

against *S. aureus* by itself while HBD-2 requires presence of LL-37 to kill *S. aureus* (11). Therefore, a defect in HBD-3 could be an important deficit in the pathogenesis of AD skin infections.

To determine whether HBD-3 expression in AD was lower than psoriasis skin at the protein level, immunohistochemistry studies were conducted (Fig. 1). Strong HBD-3 staining in keratinocytes from psoriasis skin lesions (Fig. 1*a*) was observed while AD skin samples revealed absent staining within keratinocytes (Fig. 1*b*). The specificity of staining was confirmed with the absence of immunostaining using an isotype control in psoriasis skin sample (Fig. 1*c*) and the lack of staining in normal skin (Fig. 1*d*). The staining for HBD-3 of six different psoriasis skin biopsies was significantly stronger ($p = 0.0005$) than six different AD skin biopsies (Fig. 1*e*).

Gene regulation of HBD-3 by cytokine treatment of cultured keratinocytes

To examine the mechanism of suppression of HBD-3 in AD skin, mRNA expression of HBD-3 was studied in differentiated primary human keratinocyte cell cultures. Differentiated cells were stimulated with TNF-α, IFN-γ, or their combination for 24 h. Stimulated cells revealed increased HBD-2 and HBD-3 mRNA expression as compared with the medium control (Fig. 2), confirming that both antimicrobial peptides can be induced by proinflammatory cytokines. The combination of TNF-α/IFN-γ was the most potent stimuli for induction of HBD-3 transcripts in keratinocyte cultures. The proinflammatory cytokine IL-1β was also tested based on differences observed between skin from AD and psoriasis patients (Table I). However, stimulation of keratinocyte cultures with IL-1β did not augment the induction of HBD-2 or HBD-3 observed following stimulation with the TNF-α/IFN-γ combination (data not shown).

Because induction of HBD-2, iNOS and IL-8 are known to be inhibited by Th2 cytokines, Th2 cytokines might play an active role also in the inhibition of HBD-3 gene expression. To explore this possibility, we studied the effects of IL-4 and IL-13 on the gene expression of HBD-3 in primary human keratinocytes. Our current data was consistent with previous observations that Th2 cytokines suppress HBD-2 induction in transformed HaCAT cell lines (11). Fig. 2 shows that IL-4 in combination with IL-13 significantly inhibited the up-regulation of HBD-2 and HBD-3 mRNA by TNF-α and IFN-γ combination. Because AD lesions are characterized by a predominance of IL-4 and IL-13 expression (20, 21), the suppression of HBD-3 mRNA by these cytokines may represent an important mechanism for the observed low expression of HBD-3 in AD lesions.

Real-time PCR of HBD-3 and cytokines in AD and psoriasis skin samples

To confirm the finding in immunohistochemistry and to determine the clinical relevancy of cultured keratinocyte experiments in Fig. 2, real-time PCR to evaluate HBD-3 and expression of various cytokines (TNF-α, IFN-γ, IL-4, IL-8, and IL-13) in skin lesions from psoriasis and AD patients was performed. Although the gene array is a good method to determine initial differences between populations, evaluation of individual gene targets using real-time PCR is a much more sensitive and quantitative method. Because TNF-α and IFN-γ were the main inducers for HBDs as shown in Fig. 2, we initially evaluated levels of TNF-α and IFN-γ mRNA in skin samples of all study groups by real-time PCR. We found that TNF-α and IFN-γ gene expression were significantly increased (TNF-α, $p = 0.0365$; IFN-γ, $p = 0.0192$) in psoriasis as compared with AD skin samples (Fig. 3).

As shown in Fig. 4*a*, we found that HBD-3 mRNA expression was significantly lower ($p = 0.0002$) in AD skin lesions than in psoriasis skin lesions. Although IL-4 mRNA expression showed increased levels in AD (3.07 $\pm$ 1.38), as compared with psoriasis skin samples (0.70 $\pm$ 0.18), the gene expression level in AD was quite variable and did not reach statistically significant difference between these two skin conditions ($p = 0.06$). Expression of IL-8 showed a statistically significant up-regulation in psoriasis skin compared with AD, confirming the GeneChip results (data not shown). The most interesting relationship was observed for IL-13 (Fig. 4*b*). IL-13 mRNA expression was significantly higher in AD,

Downloaded from http://journals.aai.org/jimmunol/article-pdf/171/6/3262/1170112/3262.pdf by guest on 24 May 2024

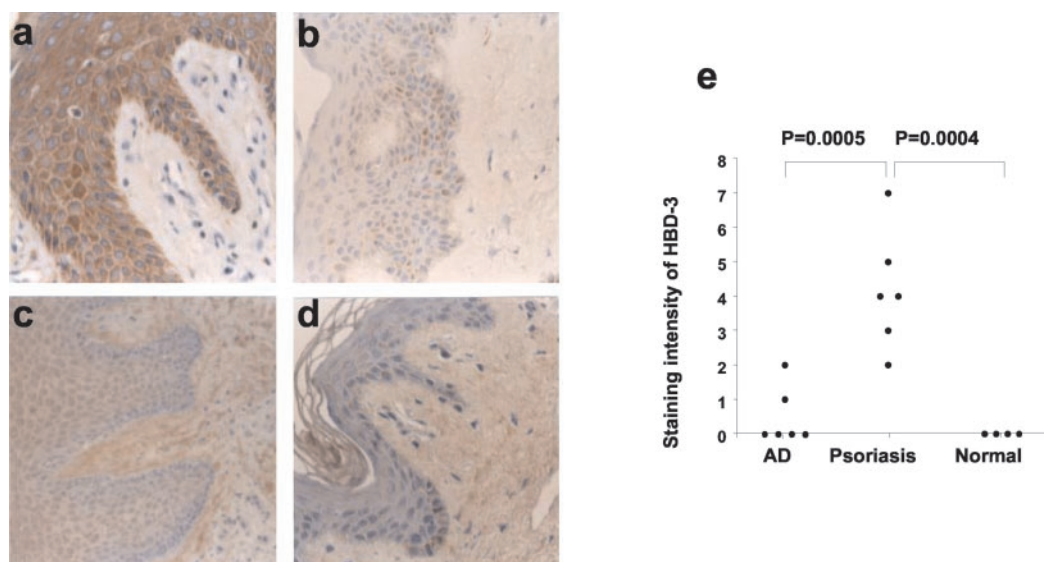

FIGURE 1. Immunohistochemistry demonstrates increased HBD-3 staining in the skin of psoriasis, as compared with AD, patients. *a*, Psoriasis skin lesions show strong staining for HBD-3 in their keratinocytes; *b*, AD skin lesions show weak staining within keratinocytes. *c*, Absence of immunostaining in isotype control in psoriasis skin sample. *d*, Minimal HBD-3 staining is observed in normal skin. *e*, Summary of HBD-3 staining intensity in six different AD patients, six different psoriasis patients, and four different normal control skin biopsies. The intensity of the immunostaining was graded as the percentage of keratinocytes which were positively stained with a score of 0 to 8, where 0 is no staining, 1 = up to 12.5%, 2 = up to 25% with stepwise increase in scoring by 12.5% until 8 = up to 100%.

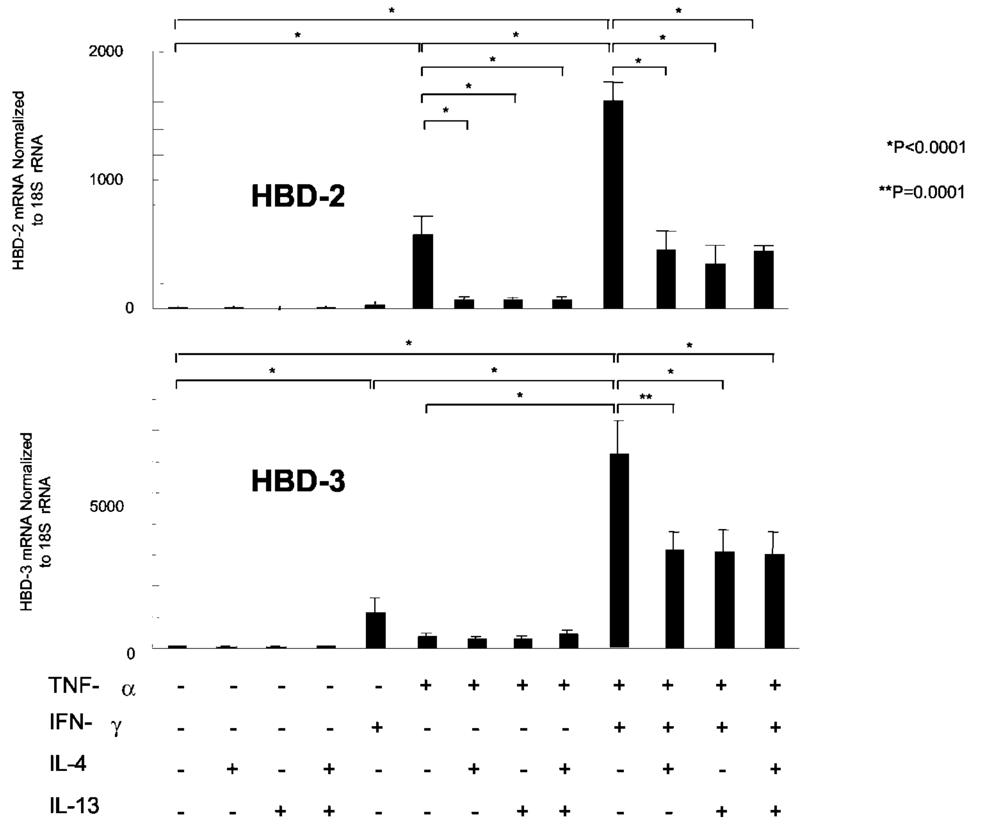

FIGURE 2. Suppression of TNF-α- and IFN-γ-induced HBD-2 and HBD-3 RNA induction by Th2 cytokines in primary human keratinocytes. Human primary differentiated keratinocytes were cultured and stimulated with or without TNF-α, IFN-γ, and IL-4/IL-13 for 24 h. After incubation, RNA was extracted, converted into cDNA, and real-time PCR was performed for HBD-2 and HBD-3. Each bar represents mean + SEM from five independent experiments.

as compared with psoriasis skin. Importantly, as shown in Fig. 4*c*, a statistically significant inverse relationship was found between in vitro mRNA expression of IL-13 and HBD-3 ($p = 0.032$, $r = 0.456$). This data supports our observation in cultured keratinocytes that Th2 cytokines suppress HBD-3 expression.

Discussion

Our current study demonstrates the novel observation that a recently identified antimicrobial peptide, HBD-3, which independently has antimicrobial activity against a broad spectrum of microbes including *S. aureus* (12) is extremely low at both the mRNA and protein level in AD skin and may therefore contribute to the increased susceptibility of such patients to skin infections especially to *S. aureus*. We have also confirmed and extended previous reports of separately examined expression of individual antimicrobial molecules in AD. Using GeneChip microarray technology we found the orchestrated suppression of a constellation of innate immune response genes in AD skin lesions as compared with psoriasis skin lesions. In addition to HBD-3, we found that AD skin lesions contain significantly lower levels of iNOS, IL-8, and HBD-2 transcripts. Combined with a previous report that the levels of LL-37 and HBD-2 in psoriasis skin, but not AD skin, can kill *S. aureus* (11), and our current observation that the majority of AD patients have undetectable or negligible levels of other antimicrobial molecules, deficient production of iNOS and IL-8, the lack of an adequate inducible innate immune response likely accounts for the failure of patients with AD to mount an adequate host response against a variety of microbes. Due to the limited amounts of RNA that were isolated from normal skin samples we were unable to compare levels of innate immune response genes in normal skin vs AD skin using the GeneChip Microarray technology. However, a sufficient amount of RNA was isolated to evaluate individual target genes by real-time PCR.

We propose that the reason antimicrobial molecules such as HBD-3 are suppressed in AD skin may be due to the overexpression of Th2 cytokines (20, 21) and low levels of TNF-α, and IFN-γ

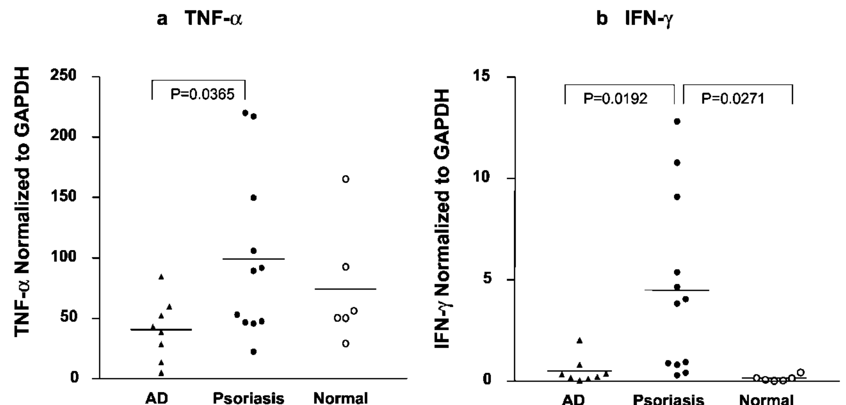

FIGURE 3. Decreased production of proinflammatory cytokines in AD skin. Real-time PCR was performed to evaluate TNF-α and IFN-γ gene expression in RNA samples isolated from 10 different AD patients (▲), 12 different psoriasis patients (●), and six different normal donor (○) skin biopsies. TNF-α and IFN-γ gene expression were significantly lower ($p = 0.0365$, $p = 0.0192$, correspondently) in AD skin as compared with psoriasis.

Downloaded from http://journals.aai.org/jimmunol/article-pdf/171/6/3262/1170112/3262.pdf by guest on 24 May 2024

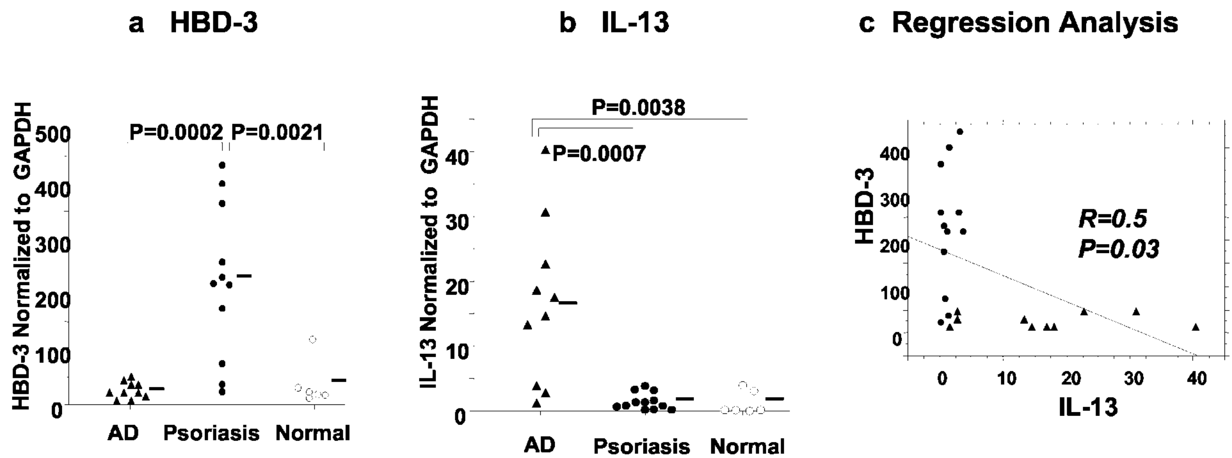

FIGURE 4. Significant inverse correlation exists between mRNA expression of IL-13 and HBD-3 mRNA. In these experiments, IL-13 and HBD-3 expression were compared by real-time PCR in RNA samples isolated from 10 different AD patients (▲), 12 different psoriasis patients (●), and six different normal donor (○) skin biopsies. *a*, HBD-3 gene expression was significantly lower ($p = 0.0002$) in AD skin as compared with psoriasis; *b*, IL-13 showed significantly higher ($p = 0.0007$) expression in AD skin; *c*, regression analysis of HBD-3 vs IL-13 ($p = 0.03$; $R = 0.5$).

production in AD skin as compared with psoriasis. This concept is supported by the following findings in our current study. First, using real-time PCR we have found that inflammation in AD skin is associated with negligible levels of TNF-α and IFN-γ which are considered to be the main inducers of antimicrobial peptides. Second, we have demonstrated a significant inverse correlation between in vivo expression of IL-13 mRNA transcripts and levels of HBD-3 in AD and psoriasis skin lesions. High IL-13 expression in skin lesions was always associated with decreased expression of HBD-3 in AD skin. In contrast, psoriatic lesions showed high levels of HBD-3 transcripts and low IL-13 expression. IL-4 mRNA expression showed increased levels in AD, as compared with psoriasis skin samples, but the difference observed was not statistically significant. This may be accounted for by the transient nature of IL-4 mRNA expression in the skin because our skin biopsies were not timed for peak expression of IL-4 in AD and other studies have found significantly increased levels of IL-4 in AD, as compared with psoriasis (20). Third, IL-4 and IL-13 were found to suppress the generation of TNF-α- and IFN-γ-induced HBD-2 and HBD-3 in primary human keratinocytes. This is consistent with our recent report that IL-4 and IL-13 suppress the generation of TNF-α-induced HBD-2 in the HaCaT keratinocyte tumor cell line (11).

Our current study also confirms a previous report that iNOS is suppressed in AD skin compared with psoriasis skin (14). iNOS is known to be produced by keratinocytes, and can kill HSV (22–24), bacteria (25–27), and fungi through the generation of NO. It is induced by bacterial endotoxins, staphylococcal lipoteichoic acid,

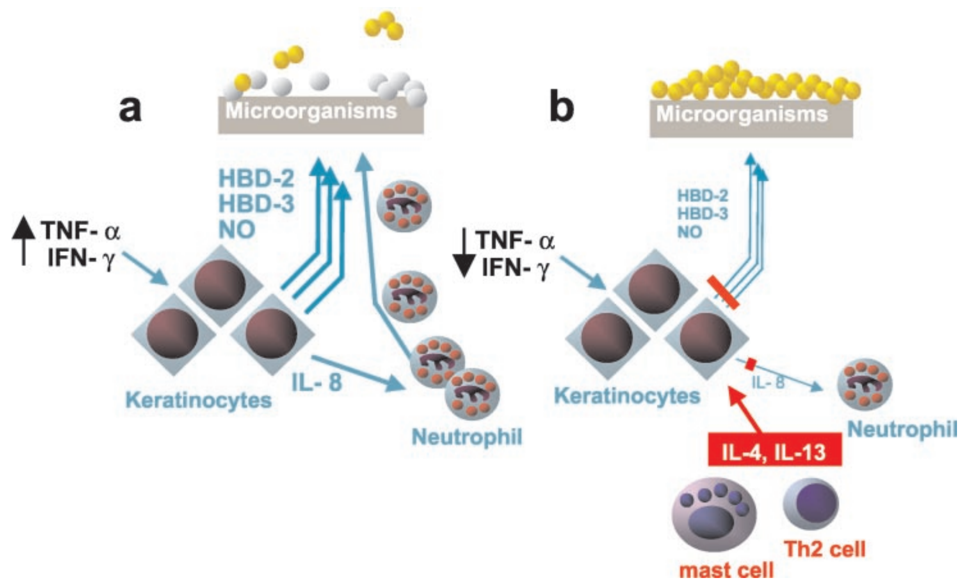

FIGURE 5. Proposed scheme depiction of interference by Th2 cytokines with innate immunity response of keratinocytes. *a*, In normal skin, microorganisms are recognized by innate immune receptors such as Toll-like receptors on the keratinocyte surface and keratinocytes produce antimicrobial molecules such as HBD-2, HBD-3, and NO. IL-8 (neutrophil chemokine) is also produced to drive neutrophils from the bone marrow pool into inflamed skin. *b*, In the Th2 environment, IL-4 and IL-13 induce phosphorylation of STAT6 which inhibits NF-κB and IFN-γ response elements mediated transcription by competition for their binding sites in the promoter regions of several antimicrobial agents. Decreased production of HBD-2, HBD-3, and NO leads to lower killing activity against microorganisms. Lower IL-8 production results in defective neutrophil accumulation in the skin. These events may allow microbes to grow in the Th2 environment of AD skin.

Downloaded from http://journals.aai.org/jimmunol/article-pdf/171/6/3262/1170112/3262.pdf by guest on 24 May 2024

TNF-α, IL-1β, and IL-8. Because AD skin is densely colonized with *S. aureus*, iNOS should be induced by staphylococcal lipoteichoic acid. Consistent with the inhibitory effects of Th2 cytokines on HBDs, induction of iNOS is known to be inhibited by IL-4 (28).

Interestingly, IL-8, a chemokine now referred to as CXC chemokine ligand 8, is one of the most potent neutrophil chemoattractants (29), and was found in our current study to be decreased at the mRNA level in the skin of AD compared with psoriasis. Neutrophils play an important role in bacterial killing cooperating with antimicrobial-specific Abs and complement. In contrast to psoriasis, absence of neutrophils is one of the most prominent histologic features of AD. Suppression of IL-8 expression may explain this phenomenon and could significantly impair host defense against various infectious agents in AD skin. It is well-established that IL-8 is induced by TNF-α and IL-1 through activation of the transcription factor, NF-κB. Several reports have indicated that Th2 cytokines can suppress IL-8 production in airway smooth muscle cells (30), bronchial epithelial cells (31), and monocytes (32). Therefore, increased expression of Th2 cytokines could cause IL-8 suppression as well in AD skin.

Taken together, the induction of various innate immune response genes has been found to be suppressed in AD skin, i.e., HBD-2, HBD-3, IL-8, and iNOS. These genes are known to be up-regulated by proinflammatory cytokines like TNF-α and IFN-γ. Interestingly, our data indicate that TNF-α and IFN-γ are decreased in AD skin, as compared with psoriasis, this by itself may result in a relative inability to induce production of the above mentioned innate immunity genes. Although psoriasis skin samples showed higher levels of IL-1β mRNA in psoriasis, as compared with AD skin, this does not account for the difference in antimicrobial peptide production in these two skin conditions because IL-1β does not induce HBDs in keratinocyte cell cultures (data not shown). This observation is consistent with a recent observation by Sorensen et al. (33) which also failed to demonstrate significant elevations in either HBD-2 or HBD-3 gene expression in primary keratinocytes following IL-1β stimulation.

TNF-α is now believed to mainly act through the activation of NF-κB transcription factor. To date, three NF-κB binding sites have been reported within the HBD-2 promoter region (34). Mutation analysis of HBD-2 promoter construct revealed that two of them were essential for full LPS responsiveness of HBD-2 gene (34).

An increasing number of reports on defensins and other antimicrobial peptides provide strong evidence that they possess important roles in antimicrobial host defense. Molecular mechanisms of their transcriptional regulation are, however, only starting to be delineated (35). The role of several inflammatory stimuli in upregulation of HBD-3 expression has been studied in epithelial cells (12, 36). TNF-α was found to be able to induce HBD-3 gene expression in primary keratinocytes as well as in primary tracheal epithelial cells at physiologically relevant concentrations (10 ng/ml) within 6 h. In addition, contact of epithelial cells with heat inactivated Gram-negative and Gram-positive bacteria like *Pseudomonas aeruginosa* and *S. aureus*, respectively, induced HBD-3 mRNA (12). Interestingly, HBD-3 mRNA expression in fetal lung explants and gingival keratinocytes was shown to be markedly induced by IL-1β, despite the absence of NF-κB consensus sequences in the immediate 5′ flanking sequence or intron of the gene. It was suggested that HBD-3 expression can be regulated from NF-κB sites at the 5′ end of the HBD-2 gene (36). In our studies, TNF-α stimulation induced 4-fold increased production of HBD-3. IFN-γ stimulation as well appeared to be able to induce HBD-3 in our experiments. Previously genetic analysis reported the presence of IFN-γ response elements within the 5′ flanking region of HBD-3 gene (36). Based on our observations, the most potent transcriptional activation of HBD-3 was achieved by a combination of TNF-α and IFN-γ. Our data as well indicated that Th2 cytokines suppress HBD-3 induction.

Recently, it has been reported that Th2 cytokines can inhibit the combination of TNF-α/NF-κB system through activation of STAT6 which can act as a transcriptional inhibitor of NF-κB target genes (37–39). Because Th2 cytokines are at high levels in AD skin, they may act directly by inhibiting the TNF-α/NF-κB system and thus inhibit the expression of innate immunity genes in the AD skin. We suggest that STAT6 induced by IL-4 and IL-13 can interfere with NF-κB binding to the consensus target sequences for NF-κB. Similar observations have been reported in the experimental model of NF-κB induced osteoclastogenesis (37). An excess of unlabeled consensus sequence STAT6, but not its mutated form, inhibited NF-κB binding. The presence of exogenously added STAT6 protein as well inhibited NF-κB/DNA interaction. STAT6 acts as an NF-κB antagonist, perhaps allosterically or by binding to DNA sequences overlapping with that of NF-κB (37). An analogous mechanism was described by Bennett et al. (38)–in this study IL-4 was found to inhibit TNF-induced NF-κB DNA binding due to STAT6 antagonism in the E-selectin gene promoter of endothelial cells. It has been established that STAT6 as well can compete with IFN-γ-induced STAT1 for IFN-γ activation sequences in the promoters of sensitive genes. Interestingly, STAT1 binding to the IFN-γ activation sequence motif induces transactivation, while STAT6 binding to the same motive has no transactivation potential (39).

In conclusion, we have demonstrated that AD, as compared with psoriasis skin lesions are associated with the suppression of a constellation of innate immunity genes. We speculate that this orchestrated suppression of innate immune response genes appears to be a combination of two events, due to the low levels of TNF-α/IFN-γ production in AD skin and inhibition of TNF-α/IFN-γ-induced expression of these genes by Th2 cytokines, likely as the result of competition between STAT6 and NF-κB or IFN-γ response elements (STAT1) for the same consensus sequence elements in the promoter regions of these innate immune response genes (Fig. 5). Importantly, our current report suggests a new concept to potentially explain the mechanism for frequent infections that accompany other Th2-mediated disease conditions, e.g., hyper-IgE syndrome (40), Netherton syndrome, and superinfection of bacteria in the parasite-infected organ (41). Although further studies are needed to confirm this speculation, our current study suggests that the blockage of Th2 cytokine action may augment innate immune responses in such disease conditions.

Acknowledgments

We thank Maureen Sandoval for her assistance in the preparation of this manuscript. We thank Aaron Carmody and Todd Woessner for their assistance in performing GeneChip microarray, Umarani Pugazhenthi for her technical assistance in performance of real-time PCR, nursing staff in the General Clinical Research Center for their help in collecting specimens from patients and Dr. Kevin Kisich for his review of these data.

References

1. Williams, H., C. Robertson, A. Stewart, N. Ait-Khaled, G. Anabwani, R. Anderson, I. Asher, R. Beasley, B. Bjorksten, M. Burr, et al. 1999. Worldwide variations in the prevalence of symptoms of atopic eczema in the International Study of Asthma and Allergies in Childhood. *J. Allergy Clin. Immunol. 103:125*.
2. Christophers, E., and U. Mrowietz. 1999. Psoriasis. In *Dermatology in General Medicine*. I. M. Freedberg, A. Z. Eisen, K. Wolff, K. F. Austen, L. A. Goldsmith, S. I. Katz, and T. B. Fitzpatrick, eds. McGraw-Hill, New York, p. 495.
3. Leung, D. Y. 2000. Atopic dermatitis: new insights and opportunities for therapeutic intervention. *J. Allergy Clin. Immunol. 105:860*.

Downloaded from http://journals.aai.org/jimmunol/article-pdf/171/6/3262/1170112/3262.pdf by guest on 24 May 2024

4. Nickoloff, B. J. 2001. Creation of psoriatic plaques: the ultimate tumor suppressor pathway: a new model for an ancient T-cell-mediated skin disease: viewpoint. *J. Cutan. Pathol. 28:57*.
5. Christophers, E., and T. Henseler. 1987. Contrasting disease patterns in psoriasis and atopic dermatitis. *Arch. Dermatol. Res. 279(Suppl.):S48.*
6. Grice, K., H. Sattar, H. Baker, and M. Sharratt. 1975. The relationship of transepidermal water loss to skin temperature in psoriasis and eczema. *J. Invest. Dermatol. 64:313*.
7. Engler, R. J., J. Kenner, and D. Y. Leung. 2002. Smallpox vaccination: risk considerations for patients with atopic dermatitis. *J. Allergy Clin. Immunol. 110:357*.
8. Gallo, R. L., M. Murakami, and M. Zaiou. 2003. Biology and clinical relevance of naturally occurring antimicrobial peptides. *J. Allergy Clin. Immunol. 110:823.*
9. Nizet, V., T. Ohtake, X. Lauth, J. Trowbridge, J. Rudisill, R. A. Dorschner, V. Pestonjamasp, J. Piraino, K. Huttner, and R. L. Gallo. 2001. Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature 414:454*.
10. Panyutich, A., J. Shi, P. L. Boutz, C. Zhao, and T. Ganz. 1997. Porcine polymorphonuclear leukocytes generate extracellular microbicidal activity by elastase-mediated activation of secreted proprotegrins. *Infect. Immun. 65:978*.
11. Ong, P. Y., T. Ohtake, C. Brandt, I. Strickland, M. Boguniewicz, T. Ganz, R. L. Gallo, and D. Y. Leung. 2002. Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N. Engl. J. Med. 347:1151*.
12. Harder, J., J. Bartels, E. Christophers, and J. M. Schroder. 2001. Isolation and characterization of human β-defensin-3, a novel human inducible peptide antibiotic. *J. Biol. Chem. 276:5707*.
13. Schibli, D. J., H. N. Hunter, V. Aseyev, T. D. Starner, J. M. Wiencek, P. B. McCray, Jr., B. F. Tack, and H. J. Vogel. 2002. The solution structures of the human β-defensins lead to a better understanding of the potent bactericidal activity of HBD3 against *Staphylococcus aureus. J. Biol. Chem. 277:8279*.
14. Bruch-Gerharz, D., K. Fehsel, C. Suschek, G. Michel, T. Ruzicka, and V. Kolb-Bachofen. 1996. A proinflammatory activity of interleukin 8 in human skin: expression of the inducible nitric oxide synthase in psoriatic lesions and cultured keratinocytes. *J. Exp. Med. 184:2007*.
15. Liu, A. Y., D. Destoumieux, A. V. Wong, C. H. Park, E. V. Valore, L. Liu, and T. Ganz. 2002. Human β-defensin-2 production in keratinocytes is regulated by interleukin-1, bacteria, and the state of differentiation. *J. Invest. Dermatol. 118:275*.
16. Geraci, M. W., M. Moore, T. Gesell, M. E. Yeager, L. Alger, H. Golpon, B. Gao, J. E. Loyd, R. M. Tuder, and N. F. Voelkel. 2001. Gene expression patterns in the lungs of patients with primary pulmonary hypertension: a gene microarray analysis. *Circ. Res. 88:555*.
17. Lockhart, D. J., H. Dong, M. C. Byrne, M. T. Follettie, M. V. Gallo, M. S. Chee, M. Mittmann, C. Wang, M. Kobayashi, H. Horton, and E. L. Brown. 1996. Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nat. Biotechnol. 14:1675*.
18. Lee, C. K., R. G. Klopp, R. Weindruch, and T. A. Prolla. 1999. Gene expression profile of aging and its retardation by caloric restriction. *Science 285:1390*.
19. Hamid, Q. A., S. E. Wenzel, P. J. Hauk, A. Tsicopoulos, B. Wallaert, J. J. Lafitte, G. P. Chrousos, S. J. Szefler, and D. Y. Leung. 1999. Increased glucocorticoid receptor beta in airway cells of glucocorticoid-insensitive asthma. *Am. J. Respir. Crit. Care Med. 159:1600*.
20. Hamid, Q., M. Boguniewicz, and D. Y. Leung. 1994. Differential in situ cytokine gene expression in acute versus chronic atopic dermatitis. *J. Clin. Invest. 94:870*.
21. Hamid, Q., T. Naseer, E. M. Minshall, Y. L. Song, M. Boguniewicz, and D. Y. Leung. 1996. In vivo expression of IL-12 and IL-13 in atopic dermatitis. *J. Allergy Clin. Immunol. 98:225*.
22. Paludan, S. R., S. Ellermann-Eriksen, L. Malmgaard, and S. C. Mogensen. 2000. Inhibition of NO production in macrophages by IL-13 is counteracted by herpes simplex virus infection through tumor necrosis factor-α-induced activation of NK-κB. *Eur. Cytokine Network 11:275*.
23. Paludan, S. R., L. Malmgaard, S. Ellermann-Eriksen, L. Bosca, and S. C. Mogensen. 2001. Interferon (IFN)-γ and herpes simplex virus/tumor necrosis factor-α synergistically induce nitric oxide synthase 2 in macrophages through cooperative action of nuclear factor-κB and IFN regulatory factor-1. *Eur. Cytokine Network 12:297*.
24. Harris, N., R. M. Buller, and G. Karupiah. 1995. γ Interferon-induced, nitric oxide-mediated inhibition of vaccinia virus replication. *J. Virol. 69:910*.
25. Sakiniene, E., T. Bremell, and A. Tarkowski. 1997. Inhibition of nitric oxide synthase (NOS) aggravates *Staphylococcus aureus* septicaemia and septic arthritis. *Clin. Exp. Immunol. 110:370*.
26. Zhang, B., G. L. Cao, A. Cross, J. B. Domachowske, and G. M. Rosen. 2002. Differential antibacterial activity of nitric oxide from the immunological isozyme of nitric oxide synthase transduced into endothelial cells. *Nitric Oxide 7:42*.
27. McInnes, I. B., B. Leung, X. Q. Wei, C. C. Gemmell, and F. Y. Liew. 1998. Septic arthritis following *Staphylococcus aureus* infection in mice lacking inducible nitric oxide synthase. *J. Immunol. 160:308*.
28. Paludan, S. R., S. Ellermann-Eriksen, J. Lovmand, and S. C. Mogensen. 1999. Interleukin-4-mediated inhibition of nitric oxide production in interferon-γ-treated and virus-infected macrophages. *Scand. J. Immunol. 49:169*.
29. Strieter, R. M. 2002. Interleukin-8: a very important chemokine of the human airway epithelium. *Am. J. Physiol. 283:L688.*
30. John, M., B. T. Au, P. J. Jose, S. Lim, M. Saunders, P. J. Barnes, J. A. Mitchell, M. G. Belvisi, and K. F. Chung. 1998. Expression and release of interleukin-8 by human airway smooth muscle cells: inhibition by Th-2 cytokines and corticosteroids. *Am. J. Respir. Cell Mol. Biol. 18:84*.
31. Fujisawa, T., Y. Kato, J. Atsuta, A. Terada, K. Iguchi, H. Kamiya, H. Yamada, T. Nakajima, M. Miyamasu, and K. Hirai. 2000. Chemokine production by the BEAS-2B human bronchial epithelial cells: differential regulation of eotaxin, IL-8, and RANTES by TH2- and TH1-derived cytokines. *J. Allergy Clin. Immunol. 105:126*.
32. Ameixa, C., and J. S. Friedland. 2001. Down-regulation of interleukin-8 secretion from *Mycobacterium tuberculosis*-infected monocytes by interleukin-4 and -10 but not by interleukin-13. *Infect. Immun. 69:2470*.
33. Sorensen, O. E., J. B. Cowland, K. Theilgaard-Monch, L. Liu, T. Ganz, and N. Borregaard. 2003. Wound healing and expression of antimicrobial peptides/polypeptides in human keratinocytes, a consequence of common growth factors. *J. Immunol. 170:5583.*
34. Tsutsumi-Ishii, Y., and I. Nagaoka. 2002. NF-κB-mediated transcriptional regulation of human β-defensin-2 gene following lipopolysaccharide stimulation. *J. Leukocyte Biol. 71:154*.
35. Lehrer, R. I., and T. Ganz. 2002. Defensins of vertebrate animals. *Curr. Opin. Immunol. 14:96*.
36. Jia, H. P., B. C. Schutte, A. Schudy, R. Linzmeier, J. M. Guthmiller, G. K. Johnson, B. F. Tack, J. P. Mitros, A. Rosenthal, T. Ganz, and P. B. McCray, Jr. 2001. Discovery of new human β-defensins using a genomics-based approach. *Gene 263:211.*
37. Abu-Amer, Y. 2001. IL-4 abrogates osteoclastogenesis through STAT6-dependent inhibition of NF-κB. *J. Clin. Invest. 107:1375*.
38. Bennett, B. L., R. Cruz, R. G. Lacson, and A. M. Manning. 1997. Interleukin-4 suppression of tumor necrosis factor α-stimulated E-selectin gene transcription is mediated by STAT6 antagonism of NF-κB. *J. Biol. Chem. 272:10212*.
39. Ohmori, Y., and T. A. Hamilton. 2000. Interleukin-4/STAT6 represses STAT1 and NF-κB-dependent transcription through distinct mechanisms. *J. Biol. Chem. 275:38095*.
40. Erlewyn-Lajeunesse, M. D. 2000. Hyperimmunoglobulin-E syndrome with recurrent infection: a review of current opinion and treatment. *Pediatr. Allergy Immunol. 11:133*.
41. Lambertucci, J. R., A. A. Rayes, J. C. Serufo, and V. Nobre. 2001. Pyogenic abscesses and parasitic diseases. *Rev. Inst. Med. Trop. Sao Paulo 43:67*.

Downloaded from http://journals.aai.org/jimmunol/article-pdf/171/6/3262/1170112/3262.pdf by guest on 24 May 2024