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Induction of Regulatory T Cells by a Murine β-Defensin

Fateme Navid,* Michele Boniotto,†,1 Catherine Walker,† Kerstin Ahrens,* Ehrhardt Proksch,* Tim Sparwasser,‡ Werner Müller,† Thomas Schwarz,* and Agatha Schwarz*

β-Defensins are antimicrobial peptides of the innate immune system produced in the skin by various stimuli, including proinflammatory cytokines, bacterial infection, and exposure to UV radiation (UVR). In this study we demonstrate that the UVR-inducible antimicrobial peptide murine β-defensin-14 (mBD-14) switches CD4+CD25+ T cells into a regulatory phenotype by inducing the expression of specific markers like Foxp3 and CTLA-4. This is functionally relevant because mBD-14-treated T cells inhibit sensitization upon adoptive transfer into naive C57BL/6 mice. Accordingly, injection of mBD-14, comparable to UVR, suppresses the induction of contact hypersensitivity and induces Ag-specific regulatory T cells (Tregs). Further evidence for the ability of mBD-14 to induce Foxp3+ T cells is provided using DEREGR (depletion of Tregs) mice in which Foxp3-expressing cells can be depleted by injecting diphtheria toxin. mBD-14 does not suppress sensitization in IL-10 knockout mice, suggesting involvement of IL-10 in mBD-14-mediated immunosuppression. However, unlike UVR, mBD-14 does not appear to mediate its immunosuppressive effects by affecting dendritic cells. Accordingly, UVR-induced immunosuppression is not abrogated in mBD-14 knockout mice. Together, these data suggest that mBD-14, like UVR, has the capacity to induce Tregs but does not appear to play a major role in UVR-induced immunosuppression. Through this capacity, mBD-14 may protect the host from microbial attacks on the one hand, but tame T cell-driven reactions on the other hand, thereby enabling an antimicrobial defense without collateral damage by the adaptive immune system. The Journal of Immunology, 2012, 188: 000–000.

AMPs are either constitutively expressed or induced by various stimuli, including cytokines and bacteria (5). Recently, solar UV radiation (UVR)—in particular, the midwave range (UVB, 290–320 nm)—was shown to induce AMPs in the skin (6). Because UVR disturbs the epidermal barrier, the induction of AMPs by UVR may be regarded as a counterregulatory protective mechanism (7). In contrast, UVR suppresses adaptive immune reactions, primarily T cell-mediated immune responses. One of the major features of UVR-induced immunosuppression is its Ag specificity and the induction of regulatory T cells (Tregs) (8).

It was a constant matter of debate why bacterial superinfections are not observed upon solar/artificial UVR exposure, despite its well-known immunosuppressive properties, until the recent discovery of UVR’s capacity to induce AMPs (6). Because immunosuppression is caused by UVR doses in the same range as those producing beneficial antimicrobial effects, it is natural to speculate about the biological relevance of the immunosuppressive effects exerted by physiologic UVR doses (7). The vast majority of inflammatory dermatoses are T cell mediated (9), and thus T cell reactions in the skin may be more harmful than protective. In addition, the skin is constantly exposed to contact allergens, the reaction to which is T cell driven, and it is an organ prone to autoimmunity (10). This information gives rise to the speculation that a constant low level of immunosuppression by daily physiologic UVR doses may be beneficial rather than harmful (7).

Hence, these diverse effects of UVR may contribute to a protective mechanism after solar exposure, by protecting the skin from microbial attacks on the one hand, but taming T cell-driven pathologic reactions on the other hand. Today it is known that AMPs produce biological activities beyond antimicrobial defense. β-Defensins were shown to induce pigmentation via interaction with the melanocortin receptor (11). Cathelicidin affects cell proliferation and migration, as well as cyto- and chemokine release, thereby participating in wound healing (12). Thus, we postulated that AMPs might represent a master switch in these
protective mechanisms and studied whether AMPs can also contribute to suppression of the adaptive immune response in skin.

Materials and Methods

Animals

Female C57BL/6 mice 7 to 8 wk old were purchased from Harlan Laboratories (Roddorf, Germany). Female IL-10 knockout (KO) mice (C57BL/6 background) were purchased from The Jackson Laboratory (Bar Harbor, ME). DEREG (depletion of Tregs) mice were kindly provided by T. Sparwasser (13) and bred also in the central animal facilities of the University Clinics Schleswig-Holstein, Campus Kiel. Animal care was undertaken by expert personnel under specific pathogen-free conditions, in compliance with relevant laws and institutional guidelines.

mBD-14-deficient mice were generated by oligotargeting in msh3-deficient embryonic stem cells, provided by Hein te Riele of Amsterdam, The Netherlands. With this method (14), 4 bp (AATA) were inserted into the mBD-14 gene at position 21–50 of exon 2, resulting in the insertion of a stop codon and a frameshift mutation. The presence of the stop codon in the mBD14 gene was verified at the level of DNA and mRNA by sequencing. The mutation was backcrossed onto the C57BL/6 background for nine generations before generating homozygous mutants.

Immunohistochemistry analysis

C57BL/6 mice were irradiated with 1.5 kJ/m² UVR on the shaved back. UV irradiation was performed using a bank of six fluorescent bulbs (TL12; Philips, Eindhoven, The Netherlands), which emit most of their energy in the UVB range (290–320 nm; emission peak, 313 nm). At 24 h later, skin samples were collected, fixed in 10% formaldehyde, and embedded in paraffin. Skin samples collected from unirradiated mice were used as controls. Next, 5-μm-thick sections were deparaffinized and stained with a specific affinity-purified polyclonal goat anti-mBD-14 Ab, as described previously (15). Negative control staining was performed by omitting the primary Ab. Expression of mBD-14 was evaluated by Axiostep 40 (Zeiss, Göttingen, Germany).

Quantitative real-time PCR

Total RNA was isolated with TRIzol Reagent (Invitrogen, Karlsruhe, Germany) from mouse skin samples. Then 1 μg total RNA was converted by reverse transcription into cDNA, using 250 ng random primers and 200 U SuperScript II (both Invitrogen). Quantitative real-time PCR was performed using a fluorescence temperature cycler (LightCycler; Roche, Mannheim, Germany) and corresponding sets of specific primers. For the evaluation of relative gene expression, levels of mBD-14 mRNA were normalized against housekeeping mRNA via the 2-ΔΔCt method (16).

The following sets of specific primers were used: Housekeeping 36B4, sense: 5'-GAAAAGTCAAAGCGCTTCT-3' and antisense: 5'-GGAGAAGACCGAATCCCAT-3'; mBD-14, sense: 5'-GCTCCTCCTAGGGTTTGT-3' and antisense: 5'-GAGACCCAGGGTCCCAATT-3'.

Flow cytometric analysis

To analyze the expression of mouse Foxp3, intracellular staining was performed using the Allophycocyanin Anti-Mouse/Rat Foxp3 Staining Set (eBioscience, San Diego, CA) according to the manufacturer’s instructions. For the expression of CTLA-4, CD44, and CD62L, the following Abs were used: FITC-labeled rat anti-mouse CD62L (Becton Dickinson, Franklin Lakes, NJ), rabbit anti-mouse intraperitoneal (Santa Cruz Biotechnology, Santa Cruz, CA), and hamster anti-mouse CTLA-4 (clone UC10-4F10). As secondary Abs, PE-labeled anti-rabbit IgG (Invitrogen) and PE-labeled anti-hamster IgG (BD Biosciences, Heidelberg, Germany) were used. For the expression of MHC class II, a PE-labeled rat anti-mouse I-A/B (BD) was used. Protein expression was analyzed by flow cytometry (FC500; Beckman Coulter, Krefeld, Germany).

Contact hypersensitivity

Mice were sensitized by painting 50 μl 2,4-dinitro-fluorobenzene (DNFB; Sigma-Aldrich, Taufkirchen, Germany) solution (0.5% in acetone:olive oil, 4:1) on their shaved back. At 5 d, an ear challenge was performed with 20 μl 0.3% DNFB on the left ear, and the ear-swelling response was measured with a spring-loaded micrometer 24 h later. For sensitization against oxazolone (Sigma-Aldrich) 100 μl 2% solution was used, and for challenge, 20 μl 1% solution. The ear-swelling response was determined as the amount of the haptener-treated ear compared with vehicle-treated ear and expressed in cm³ (mean ± SD). To neutralize IL-10 in vivo, a rat anti-mouse IL-10 Ab (R&D Systems, Minneapolis, MN) and the respective isotype control rat IgG (Beckman Coulter) were used.

Generation of bone marrow-derived dendritic cells

Dendritic cells (DCs) were generated from bone marrow cells and cultured as described before (17). For sensitization with hapten-labeled APCs, bone marrow-derived DCs (BMDCs) were labeled with 1 mM 2,4-dinitrobenzene sulfonic acid sodium salt (DNBS), the water-soluble derivative of DNFB, for 30 min. After washing, 7 × 10⁶ cells were injected s.c. into naive mice. At 5 d, ear challenge with DNFB was performed, and ear swelling was measured 24 h later.

Generation of UVR-induced CD4⁺CD25⁺ Tregs and CD4⁺CD25⁻ T cells

Mice were exposed to UVB (1.5 kJ/m²) on their shaved back for 4 consecutive days. Animals were sensitized with DNFB 24 h after the last exposure. Lymph nodes and spleens were collected 5 d later, and single-cell suspensions were prepared. CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells were isolated via magnet-activated cell sorter (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany), using the CD4⁺CD25⁺ Regulatory T Cell Isolation Kit (Miltenyi Biotec) according to the manufacturer’s protocol. For adoptive transfer, cells obtained from lymph nodes and spleens (5 × 10⁵) were injected i.v. into naive mice. For the adoptive transfer of CD4⁺CD25⁺ and CD4⁺CD25⁻ T cells, 5 × 10⁵ cells were used. Recipients were sensitized against DNFB 24 h after injection, and ear challenge with the same hapten was performed 5 d later.

Statistical analysis

Statistical analysis was performed using the Student t test. Differences between the means and the corresponding control value, with p < 0.05, were considered statistically significant. Unless otherwise stated, the figures show one representative of three independent experiments, with six mice per group.

Results

The UVR-inducible AMP mBD-14 induces the expression of Foxp3 in murine CD4⁺CD25⁻ T cells

To study what impact AMPs might have on the adaptive immune response, we selected mBD-14, the murine ortholog of hBD-3, sharing 69% identity (18). Human AMPs were recently found to be induced by UVR (6). Similarly, mBD-14 was induced by UVR in murine skin in vivo both at the protein level, as demonstrated by immunohistochemistry (Fig. 1A), and at the mRNA level, as demonstrated by quantitative PCR (Fig. 1B).

![FIGURE 1. mBD-14 is induced by UVR. A, C57BL/6 mice were exposed to 1.5 kJ/m² UVR or left unirradiated (Co). Skin samples taken 24 h later were stained using an anti–mBD-14 Ab. Data show one representative of three independent experiments. Scale bar, 200 μm. B, In parallel, total RNA was isolated from the skin samples and mRNA transcribed to cDNA. Quantitative real-time PCR was performed with specific primers against mBD-14 and evaluated in comparison with the 36B4 housekeeping gene. Bars show mean ± SD of five different experiments. *p < 0.05 versus Co.](http://www.jimmunol.org/content/2/1/75)
The most frequently used model for studying UVR-induced immunosuppression is inhibition of the induction of contact hypersensitivity (CHS). Application of contact allergens like DNFB onto UVR-exposed skin does not result in sensitization, but in Ag-specific immunotolerance; the very same mice cannot be resensitized against DNFB at a later time, although other immune reactions are not suppressed (19). This immunotolerance is mediated via Ag-specific Treg, as demonstrated by adoptive transfer experiments (20). UVR-induced Tregs (UVR-Tregs) appear to represent a unique subtype of Tregs that express CD4, CD25, Foxp3, CTLA-4, neuropilin, and the lymph node homing receptor CD62L (21). Upon Ag-specific stimulation, UVR-Tregs release IL-10, which, at least in part, is responsible for the suppression.

Lymph node cells and splenocytes were obtained from mice that were tolerized against DNFB by having it painted onto their UVR-exposed skin. T cells were isolated by magnetobead separation into CD4\(^+\)CD25\(^-\) and CD4\(^+\)CD25\(^+\), the latter containing the UVR-Tregs. CD4\(^+\)CD25\(^-\) T cells were incubated overnight with mBD-14 and subjected to FACS analysis to determine the expression of the Treg marker Foxp3. Foxp3 has been described as a transcription factor expressed in naturally occurring CD4\(^+\)CD25\(^+\) Tregs (22). It is a key regulatory gene for the development of Tregs because retroviral gene transfer of Foxp3 converts naive T cells into a Treg phenotype similar to that of naturally occurring CD4\(^+\) Tregs. Upon incubation of CD4\(^+\)CD25\(^-\) cells with mBD-14, a clear induction of Foxp3 was noticed; this induction could be abolished by adding an anti-mBD-14 Ab (Fig. 2A).

UVR-Tregs express CTLA-4, CD62L, and neuropilin. CTLA-4, a negative regulator of T cell immune responses, is expressed on Tregs and is essentially involved in the suppressor mechanism by affecting the potency of APCs to activate T cells (23). CD62L is a lymph node homing receptor, primarily responsible for the migration of UVR-Tregs into the lymph nodes. The functional role of neuropilin expressed on UVR-Tregs remains to be determined. FACS analysis revealed that mBD-14 induced CTLA-4, CD62L, and neuropilin in CD4\(^+\)CD25\(^+\) T cells obtained from UVR-tolerized mice (Fig. 2B).

FIGURE 2. mBD-14 switches CD4\(^+\)CD25\(^-\) T cells into a regulatory phenotype. A, Mice were sensitized with DNFB through UVR-exposed back skin. Lymph node cells and splenocytes were obtained 5 d later from lymph nodes and spleens and separated into CD4\(^+\)CD25\(^+\) and CD4\(^+\)CD25\(^-\) T cells. CD4\(^+\)CD25\(^-\) T cells were incubated with 10 \(\mu\)g/ml mBD-14 or left unstimulated (Co). Another group of cells was stimulated with mBD-14 in combination with an anti–mBD-14 Ab (300 \(\mu\)g/ml; mBD-14+Ab). At 24 h later, cells were permeabilized and stained with an allophycocyanin-labeled rat anti-mouse Ab directed against mouse Foxp3 and subjected to FACS analysis. Histograms show cell number (y-axis) versus fluorescence intensity (x-axis). Data show one representative of three independent experiments. B, Cells isolated as in A were stained with Abs against CTLA-4, neuropilin, or CD62L and subjected to FACS analysis. Data show one representative of three independent experiments. C, Cells isolated and treated as in A were injected i.v. into naive mice (5 \(\times\) 10\(^5\)/mouse). Recipients were sensitized 24 h later with DNFB through the shaved back skin, and 5 d later, ear challenge was performed. Ear swelling was measured 24 h after that. Positive control (Pos Co) mice were sensitized and challenged; negative control (Neg Co) mice were only challenged. Bars show mean \(\pm\) SD of increase in ear thickness expressed as the difference between the thicknesses of the challenged ear compared with the vehicle-treated ear. *\(p < 0.05\) versus Pos Co, **\(p < 0.05\) versus CD4\(^+\)CD25\(^-\) T cells. D, CD4\(^+\)CD25\(^+\) T cells were obtained from naive mice or from mice that were only UV irradiated or only sensitized against DNFB. After incubation with mBD-14, expression of Foxp3, CTLA-4, and neuropilin was evaluated by FACS analysis. Data show one representative of two independent experiments.
This finding indicates that mBD-14 shifts the surface marker expression of CD4+CD25− T cells toward a regulatory phenotype.

The functional relevance of this switch was studied by adoptive transfer experiments. CD4+CD25− T cells were incubated with mBD-14, as described above. Cells were washed and injected i.v. into naive mice. Recipients were sensitized against DNFB 24 h later. Mice receiving untreated CD4+CD25− T cells were not suppressed in their sensitization response, indicated by the pronounced ear swelling (Fig. 2C). In contrast, recipients of CD4+CD25− cells that were preincubated with mBD-14 were significantly suppressed in their CHS response. The suppression was similar to that achieved by the injection of CD4+CD25+ UVR-Tregs.

To study whether the switch into the regulatory phenotype by mBD-14 is specific to CD4+CD25− T cells derived from UVR-tolerized mice or a general phenomenon, CD4+CD25− T cells were obtained from naive mice or from mice that were only UV irradiated or only sensitized against DNFB. Cells were incubated with mBD-14, as described above, and subjected to FACS analysis. The in vitro incubation of these cells with mBD-14 resulted in an increased expression of these markers, except for neuropilin in the UVR-only group (Fig. 2D). However, gradual differences were obvious, indicating the strongest induction in T cells obtained from UVR-tolerized mice.

Injection of mBD-14 inhibits the induction of CHS and induces Tregs

To investigate the in vivo immunosuppressive effects of mBD-14, mice were injected i.v. with mBD-14 24 h before sensitization with DNFB. Mice were injected i.v. with 10 μg mBD-14 (250 μg) 1 h before and 24 h after mBD-14 injection (mBD-14+Ab). Another group of mice were injected with the Ab alone. Mice were sensitized with DNFB 24 h later. Ears were challenged with DNFB 5 d later, and ear swelling was measured 24 h beyond that. *p < 0.05 versus Pos Co, **p < 0.0005 versus mBD-14+Ab.

B, Lymph node cells and splenocytes were obtained from the mBD-14 and mBD-14+Ab groups, respectively, and injected i.v. (5 × 10^7/mouse) into naive mice (Transfer) 24 h before sensitization with DNFB. Ears were challenged 5 d later with DNFB, and ear swelling was measured after 24 h. *p < 0.0005 versus Pos Co, **p < 0.05 versus mBD-14+Ab.

C, DEREG mice were injected with mBD-14 24 h before sensitization with DNFB. At 48 h after sensitization, one group of animals received 1 μg DT on 3 consecutive days. At 5 d after sensitization, lymph node cells and splenocytes were obtained from lymph nodes and spleens of mBD-14 and DT-treated (mBD-14+DT) or only mBD-14-treated (mBD-14) DEREG mice and were injected i.v. into naïve C57BL/6 mice (Transfer). At 24 h later, recipient animals were sensitized with DNFB, and at 5 d, ears were challenged with DNFB. Ear swelling was measured after another 24 h. *p < 0.05 versus Pos Co, **p < 0.0005 versus mBD-14. D, Lymph node cells and splenocytes obtained from mice sensitized against DNFB after injection of mBD-14 were injected into naïve recipients (Transfer) that were sensitized against DNFB or oxazolone (OXA). Ear challenge was performed 5 d later with DNFB and oxazolone, respectively. *p < 0.05 versus Pos Co. E, DNFB-sensitized C57BL/6 mice were injected i.v. with 10 μg mBD-14 24 h before challenge with DNFB. At 24 h later, ear swelling was measured. Positive control (Pos Co) mice were sensitized and challenged; negative control (Neg Co) mice were only challenged. Bars show mean ± SD of increase in ear thickness expressed as the difference between the thickness of the challenged ear and that of the vehicle-treated ear. Data show one representative of two independent experiments, with six mice per group.
DNFB. Ears were challenged 5 d later. The ear-swelling response was significantly reduced in mice that received mBD-14 (Fig. 3A). This effect could be inhibited by simultaneous injection of an Ab directed against mBD-14. To address whether Tregs were induced by mBD-14, lymph node cells and splenocytes were obtained from these mice and injected i.v. into naive syngeneic mice. Recipients were sensitized against DNFB 24 h thereafter. Recipients of cells obtained from mBD-14–treated donors were significantly suppressed in their CHS response (Fig. 3B). Transfer of cells obtained from mice that were additionally treated with the Ab did not generate suppressive activity. This finding indicates that injection of mBD-14 1) inhibits the induction of CHS and 2) induces cells with regulatory properties and thus acts in a fashion similar to UVR.

UVR-induced cells with regulatory properties belong to the T cell subset and express Foxp3. To prove whether the transferred suppression by bulk cells of mBD-14–treated donors is mediated via Foxp3-expressing Tregs, DEREG mice were used. These mice express a diphtheria toxin (DT) receptor-enhanced GFP under control of the foxp3 gene locus (13). Injection of 1 μg DT for 3 consecutive days results in the depletion of all Foxp3+ T cells. mBD-14 was injected into DEREG mice, followed by sensitization with DNFB 24 h later. After a further 48 h, DT was injected for 3 consecutive days. At 5 d after sensitization, lymph node cells and splenocytes were obtained and injected into naive wild-type (WT) recipients. Recipients were sensitized against DNFB 24 h later, and ear challenge was performed 5 d thereafter. Cells obtained from DEREG mice treated with mBD-14 significantly suppressed the CHS response in the recipient animals (Fig. 3C). In contrast, the sensitization response was not reduced upon transfer of cells obtained from DEREG mice treated with DT. This finding indicates that injection of mBD-14 induces Foxp3+ Tregs in the donors. Because depletion of Foxp3-expressing Tregs blocked mBD-14–induced suppression completely, one can conclude that Foxp3-expressing Tregs are the only cell fraction involved in mediating mBD-14–induced suppression. Hence, for practical reasons the majority of the subsequent transfer experiments were performed with bulk lymph node cells and splenocytes obtained from mBD-14–treated donors.

To study whether Tregs induced by mBD-14 suppress CHS in an Ag-specific fashion, lymph node cells and splenocytes were obtained from mice treated with mBD-14 and DNFB. Bulk cells were injected i.v. into naive recipients, which subsequently were sensitized against either DNFB or oxazolone. At 5 d later, mice were challenged with DNFB and oxazolone, respectively. Transferred cells suppressed in the recipients the sensitization response only against DNFB, but not against the unrelated contact allergen oxazolone (Fig. 3D), indicating that mBD-14 induces Ag-specific Tregs.

Despite its pronounced inhibitory effect on sensitization, mBD-14 did not suppress the elicitation of CHS. Injection of mBD-14 into DNFB-sensitized mice 24 h before challenge did not reduce the ear-swelling response (Fig. 3E).

To prove whether immunosuppressive activities are unique to mBD-14, similar experiments were performed with mBD-3. Like mBD-14, mBD-3 increased the expression of Foxp3 in CD4+CD25− cells obtained from UVR-tolerized mice (Fig. 4A). Intravenous injection of mBD-3 inhibited the induction of CHS (Fig. 4B). Adoptively transferred lymph node cells and splenocytes obtained from these mice inhibited sensitization in the recipients (Fig. 4C), suggesting that injection of mBD-3 induces Tregs in the donors. Together, these findings indicate that mBD-3 might exert immunosuppressive effects similar to those of mBD-14.

The immunosuppressive effect of mBD-14 is not mediated via APCs

UVR induces Treg via targeting APCs. It was shown that cutaneous APCs damaged by UVR migrate into the regional lymph nodes, present the Ag in a nonprofessional fashion, and thereby induce Tregs (24). To clarify whether APCs are as crucially involved in the induction of Tregs by mBD-14, BMDCs were stimulated overnight with mBD-14 and subjected to FACS analysis to determine the expression of MHC class II, which is downregulated by UVR (25). MHC class II expression was not affected by mBD-14, in comparison with untreated DCs (Fig. 5A).

To further analyze whether mBD-14 influences the capacity of DCs to present Ag, BMDCs were coupled with DNBS, the water-soluble analog of DNFB. One group of BMDCs was incubated with the addition of mBD-14. After an incubation period of 24 h, BMDCs were injected s.c. into naive mice, which were challenged with DNFB on the left ear 5 d later. Mice injected with DNBS-coupled BMDCs responded with significant ear swelling, indicating successful sensitization (Fig. 5B). A similar CHS response was observed in mice injected with DNBS-coupled BMDCs that were preincubated with mBD-14. This observa-
tion indicates that mBD-14 does not impair DCs in their Ag-presenting capacity and implies that mBD-14 might produce its immunosuppressive activities via different mechanisms than UVR.

Induction of Tregs by mBD-14 requires IL-10

The immunosuppressive cytokine IL-10 plays an important role both in the induction of UVR-Tregs and in the exertion of their suppressive activity (26). To determine whether the same might apply for mBD-14–induced Tregs, we used IL-10 KO mice. Injection of mBD-14 into IL-10 KO mice, in contrast to WT mice, did not inhibit the induction of sensitization against DNFB (Fig. 6A) and did not induce Tregs, as demonstrated by a negative adoptive transfer (Fig. 6B).

To determine whether IL-10 is involved in the suppressive activity of mBD-14–induced Tregs, lymph node cells and splenocytes were obtained from mBD-14–injected mice and adoptively transferred into naive recipients. One group of recipients received an anti–IL-10 Ab or the respective isotype 1 h before and 3 h after sensitization with DNFB. Ear challenge was performed 5 d later. Suppression of the CHS response by mBD-14–induced Tregs was abolished by injection of the anti–IL-10 Ab (Fig. 6C), indicating that the suppressive activity of mBD-14–induced Tregs, is mediated, at least partially, by IL-10.

**FIGURE 5.** mBD-14 does not affect APCs. A, DCs were obtained from the bone marrow of C57BL/6 mice (BMDC) and incubated with 10 μg/ml mBD-14 or left unstimulated (Co). Cells were stained 24 h later with an Ab against MHC class II and subjected to FACS analysis. Histograms show cell number (y-axis) versus fluorescence intensity (x-axis). Data show one experiment. B, BMDCs were coupled with DNBS; one group was additionally exposed to mBD-14. At 24 h later, cells were harvested and injected s.c. into naive mice (7 × 10^7/mouse). Ear challenge with DNFB was performed 5 d later. Ear swelling was measured 24 h beyond that. Positive controls (Pos Co) were sensitized by s.c. injection of DNBS-coupled BMDCs followed by DNFB challenge. Negative controls (Neg Co) were challenged only. Bars show mean ± SD of increase in ear thickness expressed as the difference between the thickness of the challenged ear and that of the vehicle-treated ear. Data show one representative of two independent experiments, with six mice per group.

**FIGURE 6.** Induction of Tregs by mBD-14 is IL-10 dependent. A, C57BL/6 (WT) and IL-10 KO mice were injected with mBD-14 and sensitized with DNFB 24 h later. Ears were challenged 5 d thereafter with DNFB, and ear swelling was measured 24 h later. *p < 0.005 versus Pos Co. B, Lymph node cells and splenocytes obtained from mBD-14–treated WT and IL-10 KO mice were injected i.v. into naive WT mice (Transfer). Recipients were sensitized against DNFB 24 h after injection. Ears were challenged 5 d later, and ear swelling was measured. Data show one representative of three independent experiments, with five mice per group. *p < 0.05 versus Pos Co. C, Lymph node cells and splenocytes were obtained from lymph nodes and spleens of mBD-14–treated donors and were injected i.v. (5 × 10^7/mouse) into naive mice 24 h before sensitization with DNFB (Transfer). One group of recipients was injected i.p. with an anti–IL-10 Ab (25 μg; IL-10 Ab) or the respective isotype control (IgG) 1 h before and 3 h after sensitization. Ears were challenged 5 d later with DNFB, and ear swelling was measured after 24 h. Positive control (Pos Co) mice were sensitized and challenged; negative control (Neg Co) mice were only challenged. Bars show mean ± SD of increase in ear thickness expressed as the difference between the thickness of the challenged ear and that of the vehicle-treated ear. Data show one experiment, with five mice per group. *p < 0.05 versus Pos Co, **p < 0.05 versus bulk (–).
**UVR-induced immunosuppression is not impaired in mBD-14 KO mice**

The data so far suggest that mBD-14 has the capacity to inhibit the induction of CHS and to induce Tregs and thus acts in a fashion similar to UVR. Nevertheless, different pathways appear to be involved because UVR exerts these effects primarily by affecting APCs, whereas mBD-14 seems to directly affect T cells. Hence, we were interested to discover whether mBD-14 is involved in UVR-induced immunosuppression. For that purpose, mBD-14 KO mice were used.

Mice were irradiated on 4 consecutive days with UVR before sensitization with DNFB. Ear challenge was performed 5 d later, and ear swelling was measured. UVR also significantly reduced the CHS response in mBD-14 KO mice (Fig. 7A). Finally, lymph node cells and splenocytes were obtained from these mice and injected i.v. into naive WT mice. Recipients were sensitized 24 h after injection. The CHS response in these mice was found to be reduced in comparison with that in positive control mice that were sensitized and challenged, indicating mBD-14 KO mice generate Tregs upon UVR exposure (Fig. 7B). This finding suggests that mBD-14 is not a crucial component of UVR-induced immunosuppression.

**Discussion**

Because UVR is known to inhibit sensitization against haptens and to induce Tregs, and because we recently showed that similar UVR doses induce the release of AMPs, it was our original aim to determine whether AMPs have immunosuppressive properties and whether they are involved in UVR-induced immunosuppression. To address this issue, we used the immunologic model of CHS and the murine AMP mBD-14, which was shown to be induced by UVR.

The present data indicate that mBD-14, like UVR, inhibits the induction of CHS and induces Ag-specific Tregs, but does not appear to be involved in mediating UVR-induced immunosuppression. The latter conclusion is also supported by the fact that mBD-14 may induce Tregs via mechanisms other than UVR.

**β-Defensins initially were considered peptides with direct antimicrobial activity and thus were regarded as the relevant antimicrobial component of the innate immune system. Biragyn et al. (27) were the first to suggest that defensins may bridge the innate and adaptive immune response by demonstrating that mBD-2 acts directly on immature DCs via TLR-4, thereby upregulating costimulatory molecules and inducing DC maturation. In accordance with this line of thought, LL37 was shown to mediate activation of plasmacytoid DCs in psoriasis by converting inert self-DNA into a potent trigger of IFN-α production (28). In addition, LL37 as well as hBD-3 is chemotactic for T cells, immature DCs, neutrophils, and monocytes (29–31). To exert these effects, hBD-3 appears to interact with the chemokine receptor CCR6, LL37 with the formyl peptide receptor-like 1 (30). Although these studies imply that AMPs trigger an adaptive immune response, our findings suggest that AMPs can act the other way around as well and turn down adaptive immune responses.

Because the generation of Tregs is a hallmark feature of UVR-induced immunosuppression, we first checked whether mBD-14 is able to induce Tregs. This appears to be the case because in vitro incubation of CD4⁺CD25⁻ T cells obtained from UVR-tolerized mice induced the expression of Foxp3, a specific marker for Tregs (22). In addition, CTLA-4, CD62L, and neuropilin, which are expressed on UVR-Tregs as well (21), were induced by mBD-14. This phenotypic alteration appears to be functionally relevant because injection of these cells into naive mice inhibited the induction of CHS in the recipients.

The induction of Foxp3, CTLA-4, and neuropilin by mBD-14, however, does not appear to be restricted to T cells from UVR-tolerized mice but was also observed when CD4⁺CD25⁻ T cells were obtained from naive mice or mice that were only UVR exposed or only sensitized. Although these alterations were observed in all groups, with the exception of neuropilin in the UVR-only group, the differences were gradual, revealing the strongest induction in cells obtained from UVR-tolerized mice. Currently, we can only speculate that the effect of mBD-14 may be more pronounced in activated T cells. Furthermore, it remains to be determined whether these phenotypic alterations are associated with a regulatory function. If this is the case, we would expect that mBD-14-induced regulatory cells obtained from naive mice should act in a nonspecific fashion. Whether this is the case remains to be determined.

The induction of Tregs does not appear to be restricted to mBD-14, because a similar in vivo phenomenon was observed with mBD-3. We also have preliminary in vitro data that human CD4⁺CD25⁻ T cells after incubation with hBD-3 acquire a regulatory phenotype as well (data not shown).

Accordingly, i.v. injection of mBD-14 into naive mice inhibited the induction of CHS. Elicitation, however, was not affected by mBD-14 because the ear-swelling response upon challenge was not inhibited or reduced by mBD-14 when injected into sensitized mice. This finding excludes an anti-inflammatory effect by mBD-14 as the cause of CHS suppression. Semple et al. (32) attributed anti-inflammatory effects to defensins, reporting that hBD-3 and mBD-14 inhibited LPS-induced release of TNF-α and IFN-γ by macrophages and blocked IFN-γ- and CD40-triggered stimulation.
of macrophages. In addition, we could demonstrate that mBD-14, when injected into naive mice that were subsequently sensitized, induced Tregs that acted in an Ag-specific fashion, as demonstrated by adoptive transfer experiments. In this respect, mBD-14 acted in a manner similar to UVR.

Adaptive transfer experiments with lymph node cells and splenocytes obtained from mBD-14–injected and sensitized mice resulted in Ag-specific suppression of sensitization in the recipients. To determine whether the suppressive activity of these bulk cells is due to Tregs expressing Foxp3, DEREGL mice were used. Depletion of Foxp3 Tregs by DT resulted in complete inhibition of suppression, which indicates that suppressive activity in the bulk cells is exclusively mediated by Foxp3–expressing Tregs and not by other cell populations. Hence, for practical reasons the majority of subsequent adoptive transfer experiments were done with bulk cells.

UVR-Tregs mediate their suppressive activity, at least in part, by IL-10, as demonstrated by Ab blocking experiments (33). Furthermore, UVR-Tregs cannot be detected in IL-10 KO mice. However, because UVR-Tregs can be identified only by functional assays (adoptive transfer) owing to lack of a highly specific marker, it is not possible to discern whether Tregs do not develop in IL-10–deficient mice or whether they develop but cannot exert their suppressive activity as a result of an inability to secrete IL-10 (34). The same appears to apply to mBD-14–induced Tregs. Adoptive transfer experiments with cells obtained from IL-10 KO mice that were injected with mBD-14 before sensitization did not show suppression in the recipients. mBD-14–induced Tregs apparently require IL-10 to exert their suppressive activity because injection of an anti–IL-10 Ab into recipients prevented suppression by adoptively transferred mBD-14–induced Tregs obtained from WT mice.

Although we could detect a variety of similarities between UVR- and mBD-14–induced Tregs, as discussed above, differences also were noted. UVR induces Tregs by primarily affecting cutaneous APCs. Low-dose UVR induces specific DNA lesions (cyclobutane pyrimidine dimers, 6-4 photoproducts). This appears to be a critical molecular event because upon removal of these DNA lesions—either by DNA repair enzymes (35) or by IL-12, which induces DNA repair (36)—the development of Tregs can be prevented (24). The current hypothesis claims that UVR-damaged APCs carry the haptens to the lymph nodes, where they present the Ag in a nonprofessional fashion and thus do not induce T effector cells, but Tregs. The hypothesis explains that upon UVR exposure, sensitization is not induced and Tregs develop, which ultimately mediate Ag-specific immunotolerance. Hence, we were interested to study the effect of mBD-14 on APCs. In contrast to UVR exposure, incubation with mBD-14 did not downregulate MHC class II expression on BMDCs. In addition, hapten-coupled BMDCs treated with mBD-14 were not impaired in their sensitizing capacity but induced a vigorous CHS response when injected s.c. into naive mice. Together, these findings imply that mBD-14 does not induce Tregs via its effect on APCs but rather acts directly on naive mice. This idea is also in accordance with the observation that s.c. injection of mBD-14 before hapten application did not prevent sensitization and did not induce Tregs (data not shown). Tregs were induced only by mBD-14 upon i.v. injection. This observation favors the concept of a direct effect of mBD-14 on T cells, which we could demonstrate in vitro (Fig. 2).

The observation that UVR and mBD-14 appear to induce Tregs by different mechanisms contradicted our initial hypothesis that AMPs may be involved in mediating UVR-induced immunosuppression. In fact, this does not appear to be the case because mBD-14 KO mice were as suppressed as WT mice in their sensitization response following UVR exposure. In addition, cells obtained from UVR-exposed mBD-14 KO mice were able to transfer suppression into naive recipients.

Thus, the present data did not confirm one of our initial hypotheses, but demonstrated that mBD-14 has the capacity to induce Tregs and thereby identified a new activity for defensins. Preliminary findings indicate that mBD-3 as well as hBD-3 might exert similar effects. Whether this is the case, as well as whether defensin-induced Tregs are active in immunologic models other than that of CHS, must be demonstrated in the future.

In conclusion, our findings demonstrate that b-defensins can induce Tregs and thereby inhibit immune responses in an Ag-specific fashion. By having this dual role in antimicrobial activity and immunosuppression, b-defensins may enable an antimicrobial defense without collateral damage by the adaptive T cell–driven immune system. In addition, it is tempting to speculate on whether b-defensins are suitable tools for future immunosuppressive strategies in a therapeutic setting.

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Disclosures

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References


