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Hapten Application to the Skin Induces an Inflammatory Program Directing Hapten-Primed Effector CD8 T Cell Interaction with Hapten-Presenting Endothelial Cells

Danielle D. Kish,* Nina Volokh,* William M. Baldwin, III,*‡δ and Robert L. Fairchild*‡δ

Contact hypersensitivity is a CD8 T cell-mediated response to hapten sensitization and challenge of the skin. Effector CD8 T cell recruitment into the skin parenchyma to elicit the response to hapten challenge requires prior CXCL1/KC-directed neutrophil infiltration within 3–6 h after challenge and is dependent on IFN-γ and IL-17 produced by the hapten-primed CD8 T cells. Mechanisms directing hapten-primed CD8 T cell localization and activation in the Ag challenge site to induce this early CXCL1 production in response to 2,4-dinitrofluorobenzene were investigated. Both TNF-α and IL-17, but not IFN-γ, mRNA was detectable within 1 h of hapten challenge of sensitized mice and increased thereafter. Expression of ICAM-1 was observed by 1 h after challenge of sensitized and nonsensitized mice and was dependent on TNF-α. The induction of IL-17, IFN-γ, and CXCL1 in the challenge site was not observed when ICAM-1 was absent or neutralized by specific Ab. During the elicitation of the contact hypersensitivity response, endothelial cells expressed ICAM-1 and produced CXCL1 suggesting this as the site of CD8 T cell localization and activation. Endothelial cells isolated from challenged skin of naive and sensitized mice had acquired the hapten and the ability to activate hapten-primed CD8 T cell cytokine production. These results indicate that hapten application to the skin of sensitized animals initiates an inflammatory response promoting hapten-primed CD8 T cell localization to the challenge site through TNF-α–induced ICAM-1 expression and CD8 T cell activation to produce IFN-γ and IL-17 through endothelial cell presentation of hapten. The Journal of Immunology, 2011, 186: 2117–2126.
Mechanisms directing the hapten-primed CD8 T cell populations to the challenge site shortly after challenge and activating the IL-17 and IFN-γ production mediating the initial CXCL1-dependent neutrophil infiltration remain undefined. The goal of the current study was to identify mechanisms mediating this initial CD8 T cell localization and activation in the challenge site. The results indicate that hapten application to the skin of immune animals initiates an inflammatory response that promotes the localization of hapten-primed CD8 T cells to the challenge site and that endothelial cell acquisition and presentation of the hapten activates the localized T cells to produce the cytokines initiating the innate immune response.

Materials and Methods

Mice

BALB/c (H-2b) and C57BL/6 (H-2b) mice were obtained through Dr. Clarence Reeder (National Cancer Institute, Frederick, MD). C3H (H-2b) mice were obtained from Taconic (Hudson, NY). ICAM-1−/− mice on a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). Female mice, 8-10 wk of age, were used throughout these studies.

Hapten sensitization and elicitation of CHS

Mice were sensitized to DNFB or OX by painting the shaved abdomen with 25 μl 0.25% DNFB (Sigma Aldrich, St. Louis, MO) or 25 μl 1% OX (Sigma Aldrich) and 10 μl to each paw on days 0 and +1 (7, 13). On day +5, hapten sensitized and control, nonsensitized mice were challenged on each side of each ear with 10 μl DNFB or OX to elicit the CHS response. Ear thickness was measured using an engineer's micrometer (Mitutoyo, Elk Grove Village, IL) and expressed in units of 10−0.5 in. The ear swelling response is given as the mean increase of each group of four individual animals ± SEM.

Abs and cytokines

Purified anti-CD4 mAb YTS 191.1.2 and GK1.5; anti-CD8 mAb YTS 169; anti-CD8 mAb LFA-1 mAb D441-8; and anti-mouse ICAM-1 mAb YN1-1.7.4 used for in vivo treatment were purchased from BioXCell (West Lebanon, NH). Anti-mouse IL-17 mAb was purchased from Southern Biotech (Birmingham, AL). Culture supernatant of the anti-mouse anti- TNF-α mAb producing hybridoma XT3 was used to purify IgG by protein G chromatography. Recombinant CXCL1/KC, IL-17, and IFN-γ were purchased from R&D Systems (Minneapolis, MN).

For in vivo depletion of CD4 T cells, mice were injected with 100 μg of each anti-CD4 mAb YTS 191 and GK1.5, i.p., on 3 consecutive days before hapten sensitization on days 0 and +1 as previously described (13, 18). CD8 T cells were depleted by injecting mice with 100 μg of each anti-CD8 mAb YTS 191, YTS 169, and TIB-150. In each experiment, treated sentinel mice were used to evaluate the efficiency of CD4 or CD8 T cell depletion by Ab staining and flow cytometry analysis of spleen and lymph node cells (LNCs) and was always >95% compared with that of cells from control, rat IgG-treated mice.

In vivo neutralization of TNF-α was performed by injecting mice with 250 μg anti-mouse TNF-α mAb i.v. at the time of hapten challenge. In vivo antagonism of ICAM-1 and/or LFA-1 was performed by injecting mice with 300 μg anti-mouse ICAM-1 mAb, 300 μg anti-mouse LFA-1 mAb, or 150 μg of each on the day of hapten challenge.

Quantitation of CXCL1 production by immunomassay

Hapten-challenged or normal abdominal skin was excised and homogenized in 500 μl proteinase inhibitor mixture (Sigma Aldrich) with gentle shaking for 30 min. After centrifugation at 12,000 × g for 10 min, the supernatants were collected and the total protein concentration quantitated using a Coomassie Plus Protein Assay Reagent Kit (Pierce, Rockford, IL). All samples were diluted to an equivalent total protein concentration and tested for concentrations of CXCL1 by ELISA as previously reported (7). Supernatants from endothelial cell line cultures were also tested for concentrations of CXCL1 using the ELISA.

Analysis of gene expression by quantitative RT-PCR

Hapten-challenged or normal abdominal skin was excised and homogenized in TRIzol (Invitrogen Life Technologies, Carlsbad, CA) with subsequent chloroform extraction to isolate the whole-cell RNA. cDNA was synthesized from 2 μg RNA using the TaqMan Reverse Transcription Reagent Kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. PCR was performed using custom primers and FAM dye-labeled probes (Applied Biosystems) for mouse IFN-γ, IL-17, CXCL1, TNF-α, ICAM-1, and Mrpl 32 (gene assay ID nos.: Mm0081778-m1, Mm00439619-m1, Mm0043859-m1, Mm00443258-m1, Mm00516023-m1, and Mm00777741-sH, respectively).

The comparative Ct method for relative quantitation of cytokine gene expression was used where log measurements for each sample are made during amplification and the expression level of the Mrpl 32 housekeeping gene is subtracted from the expression level for each test cytokine gene. For each test cytokine, the expression level of a single RNA sample prepared from the unchallenged skin of nonsensitized wild-type mice was used as the calibrator and was arbitrarily set at 1.0, and the expression levels of all other samples were then normalized to the calibrator. Duplicate runs of each individual RNA sample prepared from a single mouse of three to four mice per group were tested, and the data from three to four RNA samples for each group are expressed as mean test cytokine expression level ± SEM.

T cell transfer

C57BL/6 mice were depleted of CD4+ T cells using specific mAb prior to sensitization to DNFB. On day +4 after sensitization, LNC suspensions were prepared from the sensitized mice, and aliquots of 4 × 107 LNcs were transferred i.v. into naive C57BL/6 or ICAM-1−/− recipients that were immediately challenged on the shaved abdomen with 25 μl 0.2% DNFB.

Sensitization with tetracyclidamidine isothiocyanate and analysis of hapten-expressing cells

Mice were sensitized to tetracyclidamidine isothiocyanate (TRITC) hapten by application of 10 μl 6.67 mg/ml TRITC (Sigma Aldrich) to each side of each ear on days 0 and +1. On day +5, TRITC-sensitized mice were challenged on the shaved abdomen with 25 μl TRITC. After 6 h, the challenged skin, as well as skin from nonsensitized, nonchallenged mice, was excised and incubated in 0.5% dispase (Invitrogen) for 18 h at 4˚C. The next day, the epidermis was separated from the dermis and incubated in 0.5% trypsin (Sigma Aldrich) for 60 min at 37˚C, 5% CO2. The epidermis was pressed through renal dialysis tubing to isolate individual cells. The cells were washed twice in HBSS, incubated in 0.2% DNase (Roche, Indianapolis, IN) for 10 min at room temperature, and washed again. Aliquots of 1 × 105 cells were washed in staining buffer (Dulbecco’s PBS with 2% FCS/0.2% NaN3) and incubated in FC block (BD Pharmingen, San Jose, CA) diluted 1:250,000 in the staining buffer for 20 min on ice. The cells were washed and resuspended in staining buffer, and analyzed by two-color flow cytometry using a FACSCalibur and CellQuest software (Becton-Dickinson, San Jose, CA). The cells were gated to exclude residual tissue debris, and nonsciable cells and sample data were collected on 20,000 cells.

Hapten-labeled endothelial cells isolated from the skin were tested for the ability to stimulate hapten-primed T cell populations. Cell suspensions were prepared from the skin of mice sensitized and challenged with a solution of TRITC plus DNFB. TRITC-expressing cell populations from the skin cell suspensions were stained with Pacific blue-labeled anti-CD31 mAb M-20 (Invitrogen) and sorted from the remaining cells using a FACSArria (Becton-Dickinson). The positively selected hapten-expressing endothelial cells were subsequently cultured with naïve CD8 or purified DNFB- or oxazolone-immune CD4 or CD8 T cells isolated from the lymph nodes of sensitized mice on day +4.

Analysis of tissue-infiltrating cells by flow cytometry

On day +5, the shaved trunk skin of sensitized and nonsensitized mice was challenged with DNFB, and 6 h later, the challenged skin was removed and digested to prepare cell suspensions as previously described (7). The isolated cells were washed, stained with fluorochrome-labeled FITC-labeled rat anti-mouse CD45 mAb 50-F11 (BD Pharmingen) and PE-labeled rat anti-mouse Gr-1 mAb RB6.8C5 (eBioscience, San Diego, CA) and analyzed by two-color flow cytometry.

Histological analyses

Hapten-challenged skin was excised from naive and sensitized mice 6 h after challenge and fixed with acid methanol (60% methanol, 10% acetic acid). Paraffin-embedded sections (8 μm) were cut on edge and mounted onto slides. The slides were deparaffinized, rehydrated, and boiled in Ag retrieval solution (Biogenex, San Ramon, CA). Overnight staining was
done with 5 μg/ml polyclonal goat anti-mouse CXCL1 Ab (R&D Systems) diluted in PBS/1% BSA solution at 4°C. Control slides were incubated with normal goat serum as the primary Ab (Vector Laboratories, Burlingame, CA). Primary Ab binding was detected using biotinylated rabbit anti-goat IgG followed by streptavidin HRP and developed using the substrate chromagen 3,3’-diaminobenzidine.

For immunohistochemistry to detect ICAM-1, Ag retrieval was performed on fixed sections by immersion of slides in two changes of Trilogy-EDTA, pH 8 (Cell Marque, Hot Springs, AR) in a steamer for 1 h. Endogenous peroxidase activity was blocked by incubation with 0.3% H2O2 in methanol. Nonspecific protein activity was blocked by incubation with a serum-free protein block (DAKO Corp, Carpinteria, CA). Staining was performed with a 1:20 dilution of purified polyclonal goat anti-mouse ICAM-1 IgG (R&D Systems) for 60 min at room temperature. Primary Ab was detected using biotinylated anti-goat Ab. Staining was performed with a Vectastain ABC Elite kit (Vector) and developed using 3,3’-diaminobenzidine.

For immunofluorescent analyses, excised skin was fixed in Histochoice, and frozen sections (8 μm) were cut on edge and mounted onto slides. The slides were stained with 5 μg/ml polyclonal goat anti-mouse CXCL1 Ab (R&D Systems) and 4 μg/ml rat anti-CD31 mAb M-20 (Santa Cruz Biotechnology, Santa Cruz, CA) diluted in PBS/1% BSA overnight at 4°C. The slides were washed and stained sequentially for 1 h at room temperature with 2 μg/ml rabbit anti-goat IgG Alexa Fluor 488 and then 2 μg/ml goat anti-rat IgG Alexa Fluor 568 (Molecular Probes, Eugene, OR) diluted in HBSS. After washing in HBSS, slides were mounted with Vectashield/ DAPI (Vector Laboratories), were viewed at 488 and 568 nm, and images captured using Image ProPlus 5.0.

Statistical analysis
Statistical analysis to assess differences between experimental groups was performed using Student t test. Differences were considered significant when p < 0.05.

Results

TNF-α is expressed in the skin challenge site as early as 1 hour after challenge of sensitized mice

To begin to identify factors that direct the recruitment of the hapten-primed CD8 T cells producing IL-17 and IFN-γ to the skin challenge site to initiate the CHS response, the temporal expression of inflammatory mediators was tested in the skin of naive and sensitized mice within the first 4 h after challenge with hapten. The prediction was that candidate factors would be expressed equivalently at early times in response to hapten application in both naive and immune animals but may increase further in the immune animals as the Ag-specific CD8 T cell response is initiated and progresses. DNFB-challenged skin from naive and sensitized mice was excised 1, 2, 3, and 4 h after the challenge, and whole-cell RNA was isolated and tested for expression levels of TNF-α, ICAM-1, IL-17, and IFN-γ by quantitative RT-PCR (qRT-PCR). Expression of the test genes was not detected in skin that had not been challenged with hapten (Fig. 1). TNF-α expression was detected within 1 h after hapten application to the skin of naive and hapten-sensitized mice although the expression levels were 2-fold higher in hapten-challenged skin from sensitized versus naive mice as early as 1 h after challenge and increased thereafter to the 4-h time point. ICAM-1 expression was also detected as early as 1 h after hapten challenge but was expressed at similar levels in the challenged skin of both naive and sensitized mice at 1 and 3 h after challenge. At 4 h postchallenge, ICAM-1 expression increased in the challenged skin of sensitized but not naive mice. Similar to the expression of TNF-α, the expression of IL-17A was also evident within an hour of skin challenge of sensitized but not naive mice and increased with time after challenge. In contrast to the rapid expression of IL-17, the expression of IFN-γ was at low levels in the challenged skin of naive and sensitized mice until 3 h after challenge and then only increased in the challenged skin of the sensitized mice.

Early expression of IL-17 and IFN-γ is dependent upon TNF-α

The impact of TNF-α production on the expression of ICAM-1, IL-17, and IFN-γ in the hapten challenge site was then tested. Groups of naive and sensitized mice were treated with control rat IgG or with anti–TNF-α mAb, challenged with DNFB, and the challenged skin excised 2, 4, and 6 h later to assess expression levels of the target genes (Fig. 2A–C). Expression of ICAM-1 observed in challenged skin of both naive and sensitized mice was significantly decreased by TNF-α neutralization. Similarly, expression of IL-17 observed in the challenged skin of sensitized but not naive mice as early as 1 h after challenge was decreased by neutralization of TNF-α, and the later expression of IFN-γ in the challenged skin of sensitized mice was also decreased by treatment with the anti–TNF-α mAb. The downregulation of these mediators was reflected by the marked inhibition of the CHS response when anti–TNF-α mAb was given to sensitized mice at the time of hapten challenge (Fig. 2D).

TNF-α, ICAM-1, and LFA-1 are required to induce CXCL1 production and CHS response in vivo

Because neutralization of TNF-α down-modulated both the expression of ICAM-1 and CD8 T cell cytokines required for elicitation of CHS, the role of ICAM-1 in the recruitment of the IL-17– and IFN-γ–producing CD8 T cells to the challenge site was assessed. This was first approached by testing the expression of

![FIGURE 1. Induction of TNF-α, ICAM-1, IL-17, and IFN-γ expression during elicitation of CHS. Groups of four C57BL/6 mice were sensitized with 0.25% DNFB on days 0 and +1. On day +5, the sensitized and groups of control naive mice were challenged with 0.2% DNFB. Challenged skin was excised at 1, 2, 3, and 4 h after challenge and snap-frozen. Skin from naive nonchallenged (NNC) mice was excised as a control. Whole-cell RNA was prepared and was used to assess mRNA expression of TNF-α, ICAM-1, IL-17, and IFN-γ in the skin samples by qRT-PCR. The mean expression level for each of four samples per group ± SEM is shown. All results are representative of two individual experiments. *p ≤ 0.05.](http://www.jimmunol.org/doi/abs/10.4049/jimmunol.82.6.2119)
sensitization and challenge of ICAM-1 type mice 6 h after challenge, levels were barely detectable after CXCL1 were observed in the challenged skin of sensitized wild-type mice were challenged with hapten. Whereas high levels of the cytokines were tested by qRT-PCR. The mean expression level for each of four samples per group ± SEM is shown. D, Ear thickness was monitored prechallenge and 24 h after challenge. The mean increase in ear thickness after hapten challenge is shown in units of 10⁻⁴ in. ± SEM for groups of four mice. All results are representative of two individual experiments each. *p ≤ 0.05.

de the IL-17- and IFN-γ-induced CXCL1 in the challenge site when groups of DNFB-sensitized wild-type mice were treated with control Ig, anti–ICAM-1 mAb, anti–LFA-1 mAb, or with both anti–ICAM-1 plus anti–LFA-1 mAb at the time of challenge. The hapten-challenged skin was excised 6 h later, and prepared homogenates were tested for the levels of CXCL1 as an indication of IL-17 and IFN-γ production at the site. Whereas skin from naive mice challenged with hapten did not produce CXCL1, challenged skin from sensitized mice produced CXCL1 by 6 h after challenge, and this production was inhibited by treatment with either anti–ICAM-1 mAb or anti–LFA-1 mAb at the time of DNFB challenge (Fig. 3A). To test directly the effect of ICAM-1 and LFA-1 neutralization on the production of IL-17 and IFN-γ at the challenge site, groups of DNFB-sensitized mice were treated with control rat IgG or with anti–ICAM-1 and/or anti–LFA-1 mAb at the time of hapten challenge, and 6 h later, the challenged skin was harvested, whole-cell RNA was isolated, and the mRNA levels of the cytokines were tested by qRT-PCR. Treatment with anti–ICAM-1 and/or anti–LFA-1 mAb at the time of challenge reduced the mRNA expression of CXCL1, IFN-γ, and IL-17 to the background levels observed in the skin of naive mice challenged with the hapten (Fig. 3B). Consistent with the decreased CXCL1 production was the attenuated or absent neutrophil infiltration into the skin challenge site at 6 h postchallenge in mice treated with ICAM-1- and/or LFA-1–specific Abs (Fig. 3C) as well as the absence of CHS responses read at 24 h after the hapten challenge (data not shown).

These studies were extended by testing the expression of CXCL1 in the skin challenge site when sensitized ICAM-1⁻/⁻ and wild-type mice were challenged with hapten. Whereas high levels of CXCL1 were observed in the challenged skin of sensitized wild-type mice 6 h after challenge, levels were barely detectable after sensitization and challenge of ICAM-1⁻/⁻ mice (Fig. 4A). However, T cell priming is severely compromised in ICAM-1⁻/⁻ deficient mice (19) and is likely to account at least in part for the absence of the CD8 T cell-induced CXCL1 at the site. Therefore, we tested the ability of transferred hapten-primed CD8 T cells from sensitized wild-type donors to induce CXCL1 in challenged naive wild-type and ICAM-1⁻/⁻ mice. Transfer of hapten-primed wild-type CD8 T cells to naive wild-type recipients induced high levels of CXCL1 production in response to hapten challenge of the skin. In contrast, transfer of the wild-type CD8 T cells to naive ICAM-1⁻/⁻ mice did not induce this production.

To test directly the presence or absence of hapten-primed CD8 T cell activation in the skin challenge site, skin was excised 6 h after challenge either of sensitized wild-type and ICAM-1⁻/⁻ mice or of naive wild-type and ICAM-1⁻/⁻ mice that had received hapten-primed wild-type CD8 T cells (Fig. 4B). After 6 h, the challenged skin from DNFB-sensitized wild-type mice expressed high levels of CXCL1, IFN-γ, and IL-17. In contrast, the challenged skin from sensitized ICAM-1⁻/⁻ mice expressed very low to undetectable levels of these genes, and this was not corrected by transferring hapten-primed wild-type CD8 T cells to the ICAM-1⁻/⁻ mice. Thus, ICAM-1 expression is required in the skin challenge site for the activation of the IL-17- and IFN-γ-producing CD8 T cells and the induction of CXCL1 during the initiation of CHS.

Because the expression of ICAM-1 was required for the activation of hapten-primed CD8 T cell expression of IL-17 and IFN-γ within the skin challenge site, the cells expressing ICAM-1 in the site were examined by staining prepared sections of hapten-challenged skin from naive and sensitized mice by immunohistochemistry. ICAM-1 staining was not visible in skin from nonsensitized mice not challenged with hapten (Fig. 5A). ICAM-1 staining was also not detected in hapten-challenged skin from naive and sensitized challenged mice at 2 h postchallenge. However, ICAM-1 staining of vascular endothelial cells was observed within 4 h postchallenge in hapten-challenged skin from both nonsensitized and sensitized mice. ICAM-1⁻/⁻ vessels were more abundant in the hapten-challenged skin from sensitized versus naive mice, and this increase was observed in both groups at 6 h postchallenge. Consistent with the mRNA levels detected in the challenge site (Fig. 2), treatment of sensitized mice with anti–TNF-α mAb at the time of hapten challenge markedly decreased ICAM-1 staining (Fig. 5B).
Endothelial cells in the skin challenge site produce CXCL1 during elicitation of CHS

To identify CXCL1-producing cells in the hapten-challenge site of sensitized mice at 6 h postchallenge, immunohistochemical staining was performed. Challenged skin was excised from both DNFB-sensitized and nonsensitized mice at 6 h after DNFB challenge and sections stained with a CXCL1-specific antiserum. In the hapten-challenged skin from naive mice, staining was restricted...
we examined the relationship between endothelial cells and hapten-primed CD8 T cells to elicit the CHS response. Therefore, the endothelial cells as the hapten-presenting cells activating this chemokine by endothelial cells in the challenge site suggested that IL-17 produced by hapten-primed CD8 T cells, the expression of CXCL1. Because CXCL1 is induced by the IFN-γ and IL-17 produced by hapten-primed CD8 T cells, the expression of this chemokine by endothelial cells in the challenge site suggested the endothelial cells as the hapten-presenting cells activating the hapten-primed CD8 T cells to elicit the CHS response. Therefore, we examined the relationship between endothelial cells and hapten acquisition and presentation. To identify hapten-expressing endothelial cells in the skin challenge site, challenged skin was removed 6 h after TRITC challenge of sensitized and nonsensitized mice. As a negative control, skin was also excised from nonsensitized/nonchallenged mice. The excised skin samples were digested, and isolated cell aliquots were stained with fluorescent-labeled anti-CD31 mAb and analyzed by flow cytometry analysis. TRITC-labeled endothelial cells were clearly observed from the skin of both sensitized and naive mice challenged with the hapten (Fig. 7A).

To test the ability of these hapten-expressing endothelial cells to stimulate hapten-primed CD8 T cells, fluorescent-labeled endothelial cells from TRITC + DNFB-sensitized mice were isolated by flow cell sorting from cell suspensions prepared from digested hapten-challenged skin. Aliquots of $2 \times 10^4$ sorted cells were cultured alone or with $1 \times 10^6$ DNFB-immune CD4 or DNFB-immune or nonimmune CD8 T cells. After 6-h culture, supernatants were harvested and tested for CXCL1 production by ELISA. CXCL1 was not detected in the culture supernatants from

FIGURE 5. ICAM-1 is induced by hapten application to the skin. C57BL/6 mice were sensitized with 0.25% DNFB on days 0 and +1. On day +5, sensitized and nonsensitized mice were challenged on a shaved square area of trunk skin with 0.2% DNFB. A, Challenged areas of skin were removed at 2, 4, and 6 h postchallenge from challenged and nonchallenged mice and fixed in methanol. Paraffin-embedded sections were prepared and stained with anti–ICAM-1 mAb or with control rat IgG. Slides were examined by light microscopy and representative images captured. B, Groups of mice were treated with 250 μg anti–TNF-α mAb immediately prior to hapten challenge, and skin was prepared as above for analysis of ICAM-1 expression in the skin. Original magnification ×40.

Tissue localization of CXCL1 to endothelial cells during elicitation of CHS. BALB/c mice were sensitized with 0.25% DNFB on days 0 and +1. On day +5 after sensitization, mice were challenged on a shaved square area of trunk skin with 0.2% DNFB. Challenged areas of skin were removed at 6 h postchallenge from (A) nonsensitized and (B) DNFB-sensitized mice. Paraffin-embedded sections were prepared and stained with CXCL1-specific antisera. Slides were examined by light microscopy and representative images are shown. Original magnification ×40. C–H, Frozen sections were prepared and stained with both CXCL1-specific antisera and primary antibodies for CXCL1 (C, D), CXCL1 (E, F), or both CD31 and CXCL1 (G, H) staining in challenged skin from naive (C, E, G) and sensitized (D, F, H) mice are shown. Original magnification ×40.

Hapten-expressing endothelial cells isolated from the skin challenge site produce CXCL1 during culture with immune CD8 T cells

Endothelial cells in the skin challenge site were identified as expressing CXCL1. Because CXCL1 is induced by the IFN-γ and IL-17 produced by hapten-primed CD8 T cells, the expression of this chemokine by endothelial cells in the challenge site suggested the endothelial cells as the hapten-presenting cells activating the hapten-primed CD8 T cells to elicit the CHS response. Therefore, we examined the relationship between endothelial cells and hapten expression in the skin.
the isolated endothelial cells alone or from endothelial cells cultured with nonimmune CD8 T cells (Fig. 7B). Whereas DNFB-immune CD4 T cells induced low production of CXCL1 (2.26 ± 0.2 pg/ml), DNFB-immune CD8 T cells induced nearly 4-fold higher production (8.53 ± 0.7 pg/ml). Furthermore, purified CD8 T cells from the draining lymph nodes of Ox-sensitized mice did not stimulate isolated endothelial cells from DNFB-sensitized skin to produce CXCL1 indicating the hapten-specificity of the chemokine production by the endothelial cells (Fig. 7C).

**Hapten-presenting endothelial cell production of CXCL1 in vitro**

The expression of ICAM-1 on vascular structures and the requirement of ICAM-1 for the expression of the hapten-primed CD8 T cell-derived IL-17 and IFN-γ suggested that the CD8 T cells might interact with the endothelial cells in the challenge site. To investigate these potential interactions in more detail, an in vitro culture system was developed using the endothelial cell line 2F2B. Aliquots of DNBS-labeled or -unlabeled 2F2B cells were cultured with syngeneic CD4 or CD8 T cells prepared from the lymph nodes of Ox- or DNFB-sensitized mice on day +4 after sensitization. Culture supernatants were removed after 6 h and tested for CXCL1 production by ELISA (Fig. 8A). The DNFB-sensitized CD8 T cells induced DNBS-labeled, but not unlabeled, 2F2B cells to produce CXCL1. Culture of the sensitized CD8 T cells alone did not result in CXCL1 production, and stimulation of these CD8 T cells with Con A also did not stimulate this production, whereas LPS stimulation of unlabeled 2F2B cells did stimulate CXCL1 production (data not shown), indicating that the CXCL1 was produced by the 2F2B cells and not by the hapten-primed CD8 T cells. Culture of DNBS-labeled 2F2B cells with either Ox-sensitized CD8 or DNFB-sensitized CD4 T cells did not stimulate the production of CXCL1 (Fig. 8A).

To test directly the role of the CD8 T cell-derived cytokines IFN-γ and IL-17 in the production of CXCL1 by the hapten-labeled 2F2B cells, neutralizing Abs to these cytokines were added to cultures of the DNBS-labeled endothelial cells and DNFB-sensitized CD8 T cells (Fig. 8B). Culture supernatants were removed after 6 h and tested for CXCL1 production by ELISA. Addition of Ab to either IL-17 or IFN-γ significantly reduced the production of CXCL1 by the 2F2B cells. Addition of Ab to either ICAM-1 or LFA-1 also decreased CXCL1 production to levels observed by neutralization of either anti–IFN-γ or anti–IL-17 indicating the requirement for LFA-1/ICAM-1 interactions for the CD8 T cells to become activated by the hapten-labeled endothelial cells. However, addition of Ab to TNF-α did not affect the production of CXCL1 in the immune CD8 T cell–hapten-presenting endothelial cell cultures.

Because neutralization of either IL-17 or IFN-γ inhibited hapten-presenting endothelial cell production of CXCL1 during culture with immune CD8 T cells, the ability of these cytokines to directly induce unlabeled 2F2B cells to produce CXCL1 was tested (Fig. 8C). Addition of either rIL-17 or rIFN-γ induced low amounts of CXCL1 production, whereas addition of equivalent amounts of these cytokines to cultures of DNBS-labeled endothelial cells did not result in CXCL1 production (data not shown), indicating that the CXCL1 was produced by the 2F2B cells and not by the hapten-primed CD8 T cells.
SEM for four individual cell culture samples is shown. After 6 h and tested for CXCL1 by ELISA. The mean concentration was added to each culture as indicated. Culture supernatants were collected after 6 h and assessed for levels of CXCL1 by ELISA. The mean concentration or both recombinant cytokines. Culture supernatants were collected after 6 h and assessed for levels of CXCL1 by ELISA. The mean concentration was added to each culture as indicated. Culture supernatants were collected after 6 h and assessed for levels of CXCL1 by ELISA.

Hapten-immune CD8 T cells induce endothelial cell production of CXCL1 in vitro. A, 2F2B endothelial cells were labeled with DNBS, washed four times, and 2 × 10⁶ cells were cultured with 1 × 10⁶ isolated CD4⁺ or CD8⁺ T cells from DNFB- or Ox-sensitized C3H mice. Culture supernatants were collected after 6 h and assessed for levels of CXCL1 by ELISA. The mean concentration ± SEM for four individual cell culture samples is shown. Results are representative of two individual experiments each. *p ≤ 0.05. B, DNBS-labeled or-unlabeled 2F2B cells were cultured with purified CD8 T cells from the lymph nodes of DNFB-sensitized mice, and 10 μg mAb to IFN-γ, IL-17, TNF-α, ICAM, or LFA-1 was added to each culture as indicated. Culture supernatants were collected after 6 h and tested for CXCL1 by ELISA. The mean concentration ± SEM for four individual cell culture samples is shown. *p ≤ 0.05. C, Unlabeled 2F2B cells were cultured with 4 ng aliquots of rIFN-γ, rIL-17, or both recombinant cytokines. Culture supernatants were collected after 6 h and assessed for levels of CXCL1 by ELISA. The mean concentration ± SEM for four individual cell culture samples is shown. Results are representative of two individual experiments each. *p ≤ 0.05 versus 2F2B only, **p ≤ 0.05 versus cultures with individual cytokines added.

Discussion

A key event during CD8 T cell-mediated immune responses is the recruitment of the Ag-primed CD8 T cells to the tissue site where the response will be elicited. Generally, this recruitment is thought to be mediated by the synergistic functions of chemokines and adhesion molecules, but how the local expression of these chemokines and adhesion molecules is coordinated is not well understood and is likely to be different for specific tissue sites as has been shown for the trafficking of CD4 T cells (20-22). Previous studies from this laboratory have documented that the infiltration of hapten-primed CD8 T cells into the skin to mediate CHS requires prior CXCL1-mediated recruitment and activation of neutrophils (16, 17). Based on work from many laboratories indicating the induction of CXCL1 and other neutrophil and macrophage chemoattractants as the result of an inflammatory insult (23-26), we proposed that application of hapten to the skin directly induced cells, such as keratinocytes, in the challenge site to produce the CXCL1 and direct this initial neutrophil infiltration. This proposal was proved wrong when we recently observed that CXCL1 and CXCL2 production 3–6 h after challenge required both IL-17 and IFN-γ produced by two separate populations of hapten-primed CD8 T cells (7). The goal of the current studies was to identify mechanisms directing the initial localization of the CD8 T cells to the challenge site and activating the T cells to produce the cytokines inducing the innate immune component during elicitation of CHS.

Many studies have identified TNF-α as an acute-phase cytokine produced early during tissue inflammation that induces the participation of additional components that amplify the intensity of inflammation (25, 27, 28). Such TNF-α-induced downstream events include the production of chemokines and other proinflammatory cytokines and the mobilization of selectins and integrin ligands to the luminal membrane of vascular endothelium (1-3, 29). Administration of anti–TNF-α Ab or recombinant TNF-α receptors has been shown to attenuate inflammation and Ag-specific immune responses in animal models as well as in patients with psoriasis and inflammatory bowel disease (30-35). Anti–TNF-α Abs inhibit the leukocyte infiltration and ear swelling of CHS responses when given to sensitized mice at the time of hapten challenge (36). CHS responses are also absent after sensitization and challenge of TNF-α−/− and TNF-α receptor p75−/− mice (37, 38). It is important to note that TNF-α is also a critical factor in the activation and mobilization of interstitial dendritic cells including Langerhans cells from the periphery to draining lymphoid organs, and TNF-α antagonism during sensitization certainly attenuates hapten-specific T cell priming through this mechanism (38, 39). However, the role of TNF-α during the early stages of CHS elicitation has remained poorly defined. The current studies provide further insights into the role TNF-α plays to initiate inflammatory events during the elicitation of CHS. TNF-α expression was observed in the skin as early as 1 h after hapten application to both nonsensitized and sensitized animals but was 2-fold-higher in the sensitized animals suggesting synergy with a hapten-primed component. The expression of IL-17 (and not IFN-γ) was also evident at this early time postchallenge but, as previously reported, only in the sensitized mice. It is likely that the IL-17 either directly or in synergy with TNF-α amplifies further TNF-α expression in the challenge site during the initial elicitation of the response as has been observed in other immune responses (40, 41).

One of the major consequences of this early TNF-α production in the site of hapten challenge is the upregulation of ICAM-1 expression. In contrast to TNF-α, expression of ICAM-1 was equivalent in the skin of both naive and sensitized mice challenged with hapten, and neutralization of TNF-α downregulated this expression implicating a role for TNF-α in ICAM-1 expression after hapten application to the skin of both naive and sensitized animals. Two experimental findings indicate that this TNF-α–induced ICAM-1 expression is required for the localization and activation of the IL-17- and IFN-γ-producing CD8 T cell populations in the challenge site. First, induction of IL-17 and IFN-γ were absent in the challenge site of sensitized ICAM-1−/− mice. Because ICAM-1 is also required for dendritic cell migration from the skin sensitization site to the draining lymph nodes for optimal Ag priming of T cells and could account for the absence of these cytokines in response to challenge of sensitized ICAM-1–deficient mice (19, 42), we transferred hapten-primed CD8 T cells from sensitized
wild-type donors to naive ICAM-1$^{-/-}$ recipients and observed the same absence of cytokine expression in response to hapten challenge. Second, CXCL1 mRNA expression and protein production required the expression of ICAM-1 in the challenge site, and we have previously demonstrated that this production is induced by the IL-17 and IFN-$\gamma$ produced by the hapten-primed CD8 T cells at the challenge site. Similarly, antagonism of ICAM-1, or its ligand LFA-1, during challenge of sensitized wild-type animals substantially decreased IL-17, IFN-$\gamma$, and CXCL1 production as well as neutrophil infiltration into the skin challenge site.

Anti–TNF-$\alpha$ Abs decreased the local expression of ICAM-1 on endothelial cells implicating the effects of TNF-$\alpha$ directly on the endothelial cells during the early stages of CHS elicitation. Several studies have documented TNF-$\alpha$ receptor expression and the TNF-$\alpha$–mediated induction of inflammatory events on endothelial cells, including ICAM-1 expression (43, 44). Endothelial cells in the hapten-challenge site of sensitized animals were the primary source of the CXCL1 observed 6 h after challenge suggesting that the ICAM-1–expressing endothelial cells were stimulated by the hapten-specific CD8 T cell–derived IL-17 and IFN-$\gamma$ to produce the neutrophil chemoattractant where it is accessible to circulating neutrophils.

Vascular endothelial cells are the first cells that Ag-primed T cells in the circulation encounter at an inflammatory site prior to infiltration into peripheral tissues. Although direct perfusion of hapten–protein complexes through the blood into the spleen and through the afferent lymph to the nodes after cutaneous hapten application has been previously documented (45), the presentation through the afferent lymph to the nodes after cutaneous hapten–protein complexes through the blood into the spleen and to infiltration into peripheral tissues. Although direct perfusion of neutrophils.

The results of these experiments demonstrate an intricate system of early events initiated by hapten application to the skin of sensitized animals that culminate in the elicitation of the CHS response. The results indicate two immediate consequences of hapten application to the skin. The first is the rapid induction of TNF-$\alpha$ that induces the expression of ICAM-1 on endothelial cells and facilitates the localization of hapten-primed CD8 T cells to the challenge site. The second is the acquisition of the hapten by the vascular endothelial cells in the challenge site and their presentation to the hapten-primed T cells resulting in their activation to produce IL-17 and IFN-$\gamma$. It is these cytokines that induce the CXCL1 and CXCL2 directing the neutrophils into the site to initiate the innate immune component of the response required for the subsequent infiltration of the CD8 T cells into the skin parenchyma.

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Disclosures
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References
10. Macatonia, S. E., S. C. Knight, A. J. Edwards, S. Griffiths, and P. Fryer. 1987. Localization of antigen on lymph node dendritic cells after exposure to the infiltrating CD8 T cells to express the functions resulting in the vascular leak and edema that are the hallmarks of the CHS response. The functions expressed by neutrophils that promote Ag-primed CD8 T cell infiltration through the endothelium and into the site are not yet identified. Depletion of neutrophils results in increased levels of CXCL1 and CXCL2 production 6 h after challenge of sensitized animals (results not shown), likely due to the absence of neutrophil-mediated digestion of the chemokines during transendothelial cell migration (49). Cytokine activation also induces neutrophils to produce T cell chemoattractants such as CXCL9/Mig and CXCL10/IP-10 during transendothelial migration and peripheral tissue infiltration and may promote the subsequent infiltration of Ag-primed CD8 T cells into the tissue (50–53). Neutrophil-dependent leukocyte infiltration into the murine liver during CMV infection is associated with neutrophil expression of specific matrix metalloproteinases, suggesting that digestion and possibly structural alteration of extracellular matrix may be required for Ag-primed T cell infiltration into peripheral tissues during certain immune responses (54).