Response to Comment on "The Vast Majority of CLA + T Cells Are Resident in Normal Skin"

Rachael A. Clark and Thomas S. Kupper

*J Immunol* 2006; 177:1376-1377;
doi: 10.4049/jimmunol.177.3.1376
http://www.jimmunol.org/content/177/3/1376

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Comment on “The Vast Majority of CLA⁺ T Cells Are Resident in Normal Skin”

In a recent article, Clark et al. (1) characterized T cells present in normal human skin. Some of the conclusions they have drawn challenge our own published (2–4) and unpublished findings.

We were unable to identify cell surface CCR8 with the rat anti-CCR8 Ab that they have used (Fig. 1A). This was precisely the reason why we have generated our own anti-CCR8 Ab reagent (Fig. 1A and supplemental Fig. 1 in Ref. 2). In our study, staining for CCR8 was carefully controlled by the use of peptide-blocked Ab as negative control, and moreover, the level of CCR8 cell surface staining we observed on cultured T cells fully matched their chemotactic responsiveness to the CCR8 ligand CCL1 (Fig. 2 in Ref. 2). In the absence of controls being shown, it is difficult to determine whether the CCR8 stainings documented by Clark et al. represent an artifact.

Also, using their reagent, Clark et al. concluded that CCR8⁺ T cells extracted from normal human skin uniformly expressed CCR4 and CCR6, two chemokine receptors with prominent expression on inflammatory T cells (references in Ref. 5). In contrast, we found that CCR4 is present at low levels (Fig. 1B), and CCR6 is expressed on a small cell fraction (Fig. 3 in Ref. 4), which is in agreement with the reduced chemotactic migration of freshly isolated skin T cells in response to the corresponding chemokines (2). Clark et al. explained this obvious discrepancy by the treatment we have selected for extracting lymphocytes out of fresh skin tissue, which involved a brief collagenase D digestion step (2–4). However, in control experiments with cutaneous and peripheral blood T cells, this treatment did not reduce the stainings for cell surface CCR4 (Fig. 1B) or CCR6 (data not shown). We only observed an effect in chemokine receptor stainings after prolonged (>90 min) protease digestion (data not shown). Finally, minor differences between the study of Clark et al. (1) and our own findings (2–4) relate to the ratio of CD4⁺ vs CD8⁺ T cells in healthy human skin, which in our hands is not biased toward CD4⁺ T cells, and their effector status, as measured by the percentage of T cells able to produce cytokines in response to in vitro stimulation. These differences may be due to the distinct T cell isolation procedures used (recovery of migratory cells after >7 days of in vitro culture of full thickness skin tissue, representing 20% of total skin T cells (1), vs rapid cell extraction out of dermatoxed split skin (2–4)).

In our view, CCR8 marks a population of skin-resident lymphocytes, including αβ T cells, γδ T cells, and NK cells that appear to be largely underrepresented in inflamed skin tissue (2–4). CCL1, the ligand for CCR8, is present in normal human skin and, importantly, is not up-regulated under inflammatory conditions, suggesting to us that this chemokine system controls cutaneous T cell traffic under steady-state conditions.

Figure 1. Expression of chemokine receptors on T cells from healthy human skin. A. Difficulty in studying cell surface CCR8 using commercial Abs. Flow cytometric analysis of surface CCR8 expression on 300-19 pre-B cells stably transfected with human CCR8, freshly isolated CD3⁺ T cells from normal skin, or CD4⁺ skin T cells cultured for 7 days in 200 U/ml IL-2. No staining was observed with the PE-conjugated rat anti-CCR8 Ab from R&D Systems (191704) (filled histograms) compared with the IgG2b isotype control (141945) (bold lines), both at 6.25 μg/ml (upper panel). In contrast, clear CCR8 positivity was detected with our affinity-purified rabbit anti-CCR8 Ab (percent positive cells indicated by numbers) (lower panel). Anti-CCR8 Ab was used at 15 μg/ml, and negative control included 1 μg/ml blocking CCR8 peptide (2). B. Detection of CCR4 is unaffected by collagenase D treatment. CCR4 expression on CD3⁺ T cells isolated from minced, EDTA-treated (5 h/4°C) normal skin (dotted line) or recovered from normal skin after collagenase D treatment (1 mg/ml, 30 min/37°C) (filled histograms), using mouse anti-CCR4 (1G1) obtained from BD Biosciences. Also, the fraction of CCR4⁺ cells in peripheral blood CD4⁺ memory T cells was not reduced upon collagenase D treatment (filled histograms) as compared with untreated cells (dotted line). Normal lines show isotype control stainings.
In contrast, numerous reports describe the presence of CCR4 and CCR6 on T cells from inflammatory sites (references in Ref. 5). The report by Clark et al. argues against a distinct role for CCR8 in the control of steady-state T cell traffic within human skin because these cells would also respond to the CCR4 and CCR6 ligands that are greatly up-regulated during inflammation in diverse peripheral tissues.

Patrick Schae ri, Lisa M. E bert, and Bernhard Moser

†Ludwig Institute for Cancer Research (Melbourne Branch)
Heidelberg, Victoria, Australia

Department of Pathology
The CBR Institute for Biomedical Research
Boston, MA 02115

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We and others have access only to commercially available CCR8 Abs, one of which we used in our recently published studies (1). The letter authors apparently did not see reactivity using this Ab and have generated a polyclonal Ab that they believe is superior. Certainly, we agree that Ab staining is only as good as the Ab used. Polyclonal Abs can have the disadvantage of decreased specificity; for example, polyclonal Abs to CCR8 may also react with CCR5 or other chemokine receptors. Additional specificity controls, beyond the peptide immunogen blocking control reported, should be performed before we can verify that the authors’ polyclonal Ab is superior. However, it should be noted that both the letter authors and we agree that ~50% of skin resident T cells express CCR8 (2). In their original article, the authors found CCR8 expression on 46% of CD4 T cells and 61% of CD8 T cells. In the current letter, the percentage of positive cells is significantly higher, a variance from the previously published data that is not explained. Although the intensity of staining we report is lower, we believe that it is real. Our negative control was an isotype-matched Ab used at the same concentration as the CCR8 Ab. Gates for positive staining were based on this control, with 99% of isotype control-stained cells gated in the negative. Thus, we are comfortable that our CCR8 staining is not an artifact. Indeed, the fact that both our groups found similar expression of CCR8 on skin resident T cells should be considered independent confirmation of this observation, rather than a bone of contention. Of course, if we were provided with this group’s CCR8 polyclonal Ab, we could directly replicate their findings.

In contrast, we do take serious issue with the contention that skin T cells resident in normal skin do not express CCR4. We have clear, convincing, and abundant data that the vast majority of skin T cells isolated freshly from skin—whether using EDTA treatment, isolated freshly from skin using collagenase treatment, or isolated from skin using explant cultures—express high levels of CCR4 (Fig. 1a in Ref. 1). Moreover, we find high expression of CCR4 on virtually all T cells isolated from both sun-exposed (face) and sun-protected skin (breast and abdomen). The authors provide some data above in histogram form showing that collagenase treatment does not affect CCR4 levels. However, dot plots showing CCR8 staining vs CCR4 staining would be much more convincing. We have now tested upward of 30 normal skin donors and have found high expression of CCR4 on T cells from all donors. To settle this issue, we stained frozen sections of normal skin for CD3 and CCR4. Virtually all CD3+ T cells found in normal skin stained for CCR4 (Fig. 1), confirming that T cells in normal skin do express this homing receptor. Using frozen sections, we have likewise confirmed our finding regarding the percentage of CD4 vs CD8 T cells in normal skin and the expression of CLA and CCR6 by T cells resident in normal skin. These data are not shown but can be provided upon request.

As for the “reduced chemotactic migration” of T cells that the authors describe, with due respect, we do not find the chemotaxis data they describe particularly compelling. The letter authors claim that CCR8 is present on at least 50% of skin T cells, but migration to CCL1 is only 12% of input T cells (compared with a random migration of 6%). This low percentage of migration is concerning, and calls into question the results using CCR4 ligands.

![Figure 1](http://www.jimmunol.org/) T cells in normal human skin express CCR4. Sections of normal human skin were stained with directly conjugated CD3 and CCR4 (1G1) Abs and analyzed by fluorescence microscopy. In the sections shown, CD3 T cells are present within the epidermis and the dermis of normal skin (two epidermal T cells are specifically indicated by arrows). Virtually all CD3+ T cells (left panels) present in normal skin costained for CCR4 expression (right panels). Control sections stained with identical concentrations of isotype-matched directly conjugated Abs showed no staining aside from nonspecific staining of the stratum corneum.
Lastly, we would stress the point that a homing receptor present on only 50% of skin resident T cells, according to the authors’ own data, is unlikely to be the major addressin guiding T cells into skin under conditions of normal immunosurveillance. In contrast, CLA and CCR4 are present on the vast majority of T cells from normal skin regardless of the method of T cell isolation, a result we have confirmed by staining frozen sections of normal skin. Moreover, the ligands for CLA and CCR4, E-selectin and thymus and activation-regulated chemokine, are expressed at low but detectable levels in resting cutaneous endothelium (3). This suggests that homing to normal skin uses the same receptors as trafficking to inflamed skin, and thus, it is our belief that homeostatic trafficking to skin relies on quantitative, not qualitative, differences in vascular homing receptor expression. While we do not mean to minimize a potential contributory role for CCR8 in skin homing, we think that the evidence is overwhelming that CLA and CCR4 are both better candidates for homing receptors that support migration of T cells into normal skin. We thank the letter authors for their comments but are confident that our results will stand the test of time.

Rachael A. Clark and Thomas S. Kupper

Department of Dermatology
Brigham and Women’s Hospital and Harvard Skin Disease Research Center
Boston, MA 02115

References


Comment on “Mast Cell-Mediated Remodeling and Fibrinolytic Activity Protect against Fatal Glomerulonephritis”

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ochegger et al. challenge our conclusion (1) that protection is mediated by the ability of mast cells (MCs) to engender repair mechanisms. Like Hochegger et al., we found in the glomeruli of wild-type mice decreased signs of inflammation and diminished numbers of inflammatory cells as compared with MC-deficient W/W mice. These findings were surprising to us, because in various inflammatory disease models, the presence of MC is rather known to promote an inflammatory response with leucocyte recruitment to the local site. We proposed a direct effector role of MC to explain these somewhat unexpected findings instead of an MC-mediated inflammatory response with leukocyte recruitment to the local site.

Kathrin Hochegger,* Alexander R. Rosenkranz,* Frank Siebenhaar,1 and Marcus Maurer1

†Department of Dermatology and Allergy
Charité—Universitätsmedizin Berlin
Berlin, Germany

References


Response to Comment on “Mast Cell-Mediated Remodeling and Fibrinolytic Activity Protect against Fatal Glomerulonephritis”

Hochegger et al. challenge our conclusion (1) that protection is mediated by the ability of mast cells (MCs) to engender repair mechanisms. Like Hochegger et al., we found in the glomeruli of wild-type mice decreased signs of inflammation and diminished numbers of inflammatory cells as compared with MC-deficient W/W mice. These findings were surprising to us, because in various inflammatory disease models, the presence of MC is rather known to promote an inflammatory response with leukocyte recruitment to the local site. We proposed a direct effector role of MC to explain these somewhat unexpected findings instead of an MC-mediated immunomodulatory action. This is supported by the kinetics of appearance of the thick fibrin- and collagen-containing subendothelial deposits that were increased in MC-deficient W/W mice. Favored by the high doses of anti-GBM Abs used in our study, these differences in deposits appeared already during the Ab-dependent heterologous phase of the disease (2), even though at day 8 urine tissue-type plasminogen activator and urokinase-type plasminogen activator activity were also affected. The net fibrinolytic activity was in
accord with the described multiple fibrinolytic effector functions of MCs. Our somewhat unexpected findings are also supported by the fact that fibrin deposits readily explain the enhancement of macrophages in the glomeruli of W/Wmice. A strong reduction in glomerular macrophage recruitment was demonstrated in fibrinogen-deficient mice subjected to anti-GBM-induced glomerulonephritis (3). Concerning the point that P-selectin-deficient mice also show increased fibrin deposits, we think that these studies are not directly comparable because fibrin deposits are a general marker of disease aggravation. Furthermore, the effect of P-selectin deficiency on MC activation, for example through interference with complement activation, has not been evaluated.

As the role of MCs in inflammation is complex, we do not rule out an additional immunomodulatory role of MCs, especially during the late phase of the disease. However, in contrast to our results obtained in the early phase of the disease, MC-mediated enhanced infiltration with T cells, macrophages, and, accordingly, TGF-β production appear relatively late in the study of Hochegger et al., which agrees with previous findings in the literature that indicated a role of T cells rather during the autologous phase of the disease in the accelerated model of anti-GBM-induced glomerulonephritis (4).

In conclusion, although several mechanisms may be involved, we believe that the available evidence points to a direct effect of MC activation in anti-GBM-induced glomerulonephritis. It now becomes important to dissect which of the various mediators are involved.

Yutaka Kanamaru, Lisa Scandiuzzi, Marie Essig, Renato C. Monteiro, and Ulrich Blank

Institut National de la Santé et de la Recherche Médicale Unité 699
Bichat Medical School
Paris, France

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