Epithelium-innate immune cell axis in mucosal responses to SIV

L Shang¹, L Duan¹, KE Perkey¹, S Wietgrefe¹, M Zupancic¹, AJ Smith¹,³, PJ Southern¹, RP Johnson² and AT Haase¹

In the SIV (simian immunodeficiency virus)-rhesus macaque model of HIV-1 (human immunodeficiency virus type I) transmission to women, one hallmark of the mucosal response to exposure to high doses of SIV is CD4 T-cell recruitment that fuels local virus expansion in early infection. In this study, we systematically analyzed the cellular events and chemoattractant profiles in cervical tissues that precede CD4 T-cell recruitment. We show that vaginal exposure to the SIV inoculum rapidly induces chemokine expression in cervical epithelium including CCL3, CCL20, and CXCL8. The chemokine expression is associated with early recruitment of macrophages and plasmacytoid dendritic cells that are co-clustered underneath the cervical epithelium. Production of chemokines CCL3 and CXCL8 by these cells in turn generates a chemokine gradient that is spatially correlated with the recruitment of CD4 T cells. We further show that the protection of SIVmac239Δnef vaccination against vaginal challenge is correlated with the absence of this epithelium- innate immune cell-CD4 T-cell axis response in the cervical mucosa. Our results reveal a critical role for cervical epithelium in initiating early mucosal responses to vaginal infection, highlight an important role for macrophages in target cell recruitment, and provide further evidence of a paradoxical dampening effect of a protective vaccine on these early mucosal responses.

INTRODUCTION

Antiretroviral therapy has greatly reduced the morbidity and mortality from HIV-1 (human immunodeficiency virus type I) infection, and there has been continued progress as well in strategies and methods to prevent HIV transmission, e.g., the ALVAC-AIDSVAX vaccine,¹ topical and systemic pre-exposure prophylaxis,²–⁶ treatment as prevention,⁷ and male circumcision.⁸–¹¹ Nonetheless, even more effective prevention strategies are required to ultimately end the HIV/AIDS pandemic.

To that end, we have been seeking novel concepts and strategies for prevention through a deeper understanding of HIV-1 infection and transmission at mucosal surfaces, where the great majority of new HIV-1 infections are acquired.¹² We have specifically focused on the very early events in cervical vaginal mucosa in the studies carried out in the SIV (simian immunodeficiency virus)-rhesus macaque model of HIV-1 sexual transmission to women.¹³,¹⁴ In this non-human primate animal model, we have shown that small founder populations of infected cells are established and expand in the cervico-vaginal mucosa prior to systemic dissemination and infection in a time frame equivalent to the eclipse phase of HIV-1 transmission ~10 days after exposure.¹⁵ We have also shown that cervical epithelium has an important role in facilitating local expansion of the founder populations of infected cells that precedes virus dissemination and a robust systemic infection.

We initially discovered that vaginal inoculation of high doses of SIV elicited increased expression of MIP-3α/CCL20 in cervical epithelium,¹⁶,¹⁷ which was associated with recruitment of CCR6⁺ plasmacytoid dendritic cells (pDCs) beneath the epithelium. The pDCs in turn produced the beta-chemokines, MIP-1α/CCL3 and MIP-1β/CCL4, to recruit CD4 T cells to fuel local virus expansion.¹⁶,¹⁷ More recently, in studies of the natural killer cell response in the female reproductive tract (FRT) to vaginal inoculation of SIV,¹⁸ we were struck by the...
extent of macrophage recruitment at 7 days after inoculation (7 d.p.i.) in addition to the previously reported recruitment of pDCs.17 We therefore undertook a larger systematic investigation of the innate immune cells and chemokine signaling that precede and mediate CD4 T-cell recruitment into the FRT. We report further evidence that the cervical epithelium initiates the response to vaginal inoculation of SIV, but also now show that focal accumulations of pDCs and macrophages themselves comprise an environment for concentrating chemokines and their receptors to efficiently recruit CD4 T cells at sites of infection. Remarkably, SIVΔ nef vaccination disrupts this circuitry, thus serving as an example of novel strategies for prevention aimed at the mucosal epithelial-immune system axis to block the transmission-facilitating recruitment of CD4 T cells.

RESULTS

In the studies reported here, we focused on the potential relationships between epithelial signaling and innate immune responses that could recruit CD4 T cells into the transition zone of the ectocervix and endocervix and adjoining endocervix, because this is the site where small founder populations of infected cells (viral (v)RNA + ) have been most consistently detected, and the site where the influx of CD4 T-cell targets has been associated with the local expansion of infection at 7 d.p.i. (co-clustering of CD4 T cells and vRNA + CD4 T cells is illustrated in Figure 1 and described in refs. 13, 14, 16, and 17). It is also a site where increasing evidence supports an active role of the epithelium in orchestrating the response to SIV.19 Thus, this endocervical region is a promising site to extend our understanding of the relationships between the cervical epithelium and innate immune responses to SIV. We also used a high dose of virus in order to increase the likelihood of sampling key events in FRT tissues.

Kinetics of cell recruitment and co-localization of vRNA + cells, CD4 T cells, pDCs, and macrophages beneath cervical epithelium

Following high dose vaginal inoculation, local expansion of small founder populations of infected cells is driven by the influx of CD4 T cells in the cervical transition zone.14,17 Here, we show a detailed kinetics of CD4 T-cell recruitment in cervical tissues (Figure 2a). Consistent with previous observations,14,17 cervical CD4 T cells are rare in uninfected animals, but their numbers were significantly increased by 7 d.p.i. (Figure 2a). The massive CD4 influx into cervical tissues peaked at 14 d.p.i., and then declined, but to levels still considerably higher than baseline levels by 28 d.p.i., the last time point at which archived tissues were obtained in these studies of acute SIV infection following vaginal inoculation.14 We found that the recruited CD4 T cells were clustered into foci in close proximity to the larger number of epithelial cells in the palmate folds of the endocervix near the transition zone at 7 d.p.i. (Figure 2b). Further examination of earlier tissues demonstrated that even though the increase in CD4 T cells in cervical tissue by 4–6 d.p.i. did not reach statistical significance at the population level, in three out of seven animals, accumulation of recruited CD4 T cells in local foci was already evident as early as 3 d.p.i. (Figure 2b). By 7 d.p.i., local expansion in vRNA + cells predominantly mapped to these foci (Figure 2c) where the infected cells co-cluster with CD4 T cells.14,17

Figure 1 Co-clustering of CD4 T cells (white) and SIV vRNA + infected cells (red) in cervical mucosa close to the endocervical epithelium after vaginal challenge. The montage was constructed with images (original magnification > 200) of the cervical tissue from a rhesus macaque 7 days after vaginal infection with SIVmac251. Insert shows co-clustering in the marked region at higher magnification. The vRNA + cells are predominantly CD4 + T cells (co-localized red and white). SIV, simian immunodeficiency virus.
One such cluster is encircled and enlarged in Figure 1. The majority of vRNA+ cells were CD4 T cells; fewer than 5% vRNA+ cells were macrophages, as we had shown previously.\textsuperscript{14-17,20} We later discuss the small proportion of infected macrophages despite their high frequency in these foci.

In addition to increased numbers of CD4 T cells underlying cervical epithelium, we also observed increases in numbers of macrophages (CD68\textsuperscript{+} and CD163\textsuperscript{+}) and pDCs (CD123\textsuperscript{+} and BDCA-2\textsuperscript{+}) (Figure 3). The number of sub-epithelial macrophages increased rapidly within 1–3 d.p.i. and peaked at 4–6 d.p.i., followed by a decline to levels of about twice higher than baseline levels (Figure 3a, b). Similarly, the number of sub-epithelial pDCs also increased significantly by 3 d.p.i., peaked at 7 d.p.i., and then remained at the higher levels through 14 d.p.i. (Figure 3c, d).

There was thus both temporal and spatial evidence consistent with the hypothesis that macrophages as well as pDCs could have an important role in recruiting CD4 T cells into cervical foci to facilitate local expansion of infection. First, the kinetic analysis showed that both macrophages and pDCs were recruited into the cervical tissues slightly before CD4 T cells, and were thus temporally antecedent to CD4 T-cell recruitment (Figure 2a and Figure 3). Second, macrophages and pDCs were often accumulated in clusters beneath the epithelium lining the endocervix (Figure 4a, b), consistently observed prior to CD4 T-cell recruitment (Figure 4). By 7 d.p.i., these clusters were comprised of CD4 T cells co-localizing with vRNA+ cells (Figure 4c-f) and subepithelial pDCs and macrophages (immune cells were only rarely found between epithelial cells) (Figure 4g, h).
We also determined the temporal and spatial profiles of recruitment of other types of leukocytes in the cervical tissues besides CD4 T cells, macrophages, and pDCs at the same time points (Table 1). First, eosinophils, basophils, plasma cells, and myeloid dendritic cells were absent in all examined cervical tissues before and after vaginal infection. Second, B cells, neutrophils, and CD8 T cells were only occasionally detected in cervical tissues close to the transition zone regardless of the status of infection, and were not co-localized with CD4 T-cell foci. Third, consistent with previous reports, Langerhans cells were only located in the ectocervical epithelium, thereby spatially separated from the CD4 T-cell foci. Fourth, that even though a small number of natural killer cells infiltrated cervical tissues after vaginal infection, they were also spatially separated from CD4 T-cell foci and vRNA+ cells. Finally, we observed a slight increase in mast cells in the cervical tissues at 7 d.p.i. (Figure 5). However, the spatial distribution of mast cells showed no evidence of co-clustering with CD4 T cells and vRNA+ cells (data not shown). On the basis of these results, we conclude that other cell types of leukocytes do not show a consistent and commensurate increase in size of the population as a potential source of chemokines that precedes CD4 T-cell recruitment, nor do they co-localize with local CD4 T-cell clusters and vRNA+ cells. Thus, they were not quantitatively, temporally, or spatially associated with CD4 T-cell recruitment and local expansion of infection.

Cervical epithelium-innate immune cell axis and CD4 T-cell recruitment

On the basis of the spatiotemporal profiles of macrophage, pDC, and CD4 T-cell accumulation in cervical tissues, we hypothesized the following mechanism for CD4 T-cell recruitment: cervical epithelium produces chemokines after vaginal inoculation to initiate the recruitment of macrophages and pDCs beneath the epithelium; the recruited macrophages and pDCs accumulate in clusters to serve as a concentrated source of chemokines, and this chemokine gradient drives the initial recruitment of CD4 T cells into these clusters. This mechanism extends the previous model of cell recruitment, in which pDCs were recruited as a source of beta-chemokines by epithelium-expressed CCL20, by attributing an important new role for macrophages in this process. In addition, this mechanism enlarges our view of hypothesized mechanisms for CD4 T-cell recruitment, where macrophages and pDCs in
clusters become a source of chemokine ligands that produce a gradient to recruit more macrophages, pDCs, and CD4 T cells.

We tested predictions of this feed-forward mechanism by determining the profiles of chemokine expression in the epithelium and macrophages. We first confirmed the expression of CCL20 in the epithelium (Figure 6a), and newly found that CCL3 and CXCL8 expression rapidly increased in epithelium compared with uninfected animals not exposed vaginally to SIV, both at the mRNA level from microarray analysis (Figure 6b) and at the protein level by immunohistochemical staining and quantitative image analysis (Figure 6c). Expression of CCL3, CCL20, and CXCL8 on the cervical epithelium increased by 1 d.p.i., and thereafter remained elevated in naive animals, but, remarkably, in the vaccinated animals, expression did not increase following vaginal exposure (Figure 6a–c), an observation we discuss below.

This profile of chemokine ligand expression is consistent with the observed early recruitment of CCR5+, CCR6+, CXCR1+, and CXCR2+ macrophages (Figure 7a); CCR6+, CXCR1+, and CXCR3+ pDCs (Figure 7b); and subsequent recruitment of CCR5+, CCR6+, CXCR1+, and CXCR2+ CD4 T cells (Figure 7c). Note that the macrophages in these foci were also CCL3+, CCL5+, and CXCL8+ (Figure 7a); and the pDCs were CCL5+ as well as CCL3+ and CCL4+, as previously reported (Figure 7b). Moreover, the macrophages, but not the pDCs in these foci, were CXCL10+, consistent with a primary role of macrophages in recruiting CXCR3+ pDCs and CD4 T cells (Figure 7). We further discovered in this chemokine profiling that the recruited CD4

Figure 4  Co-localization in subjacent tissue sections of CD4+ T cells, macrophages, pDCs, and vRNA+ cells (original magnification × 200) in the cervical transformation zone and adjoining endocervical tissues. Macrophages were defined as CD68+ and CD163+ cells; and pDCs as CD123+ and BDCA-2+ cells. (a, b) Macrophages and pDCs often co-cluster under the cervical epithelium, even prior to CD4 T-cell recruitment. Representative images in each panel were obtained from two different animals (4–5 d.p.i.), in which CD4 recruitment was not yet detectable in the cervical tissues. (c–f) By 7 d.p.i., macrophages and pDCs were consistently found to co-localize in cervical CD4 T-cell foci in all eight examined animals. Representative images in every panel were obtained from four different animals. Adjacent sections were used in panels (a–f). (g, h) vRNA+ cells (appear black in transmitted light) were primarily localized in regions with macrophages and pDCs. Representative images in each panel were obtained from two different animals (7 d.p.i.). d.p.i., day post infection; pDC, plasmacytoid dendritic cell.
T cells themselves produced β-chemokines (Figure 7c). Thus, the cells in these foci concentrate chemokines beneath the cervical epithelium close to the transformation zone (Figure 8) that by a positive feed-forward loop mechanism maximize the availability of CD4 T-cell targets in spatial proximity to infected cells to fuel expansion of the infected founder population in the endocervix.

**Quiescent cervical epithelium-innate immune cell axis as a correlation of protection in SIV\textsubscript{D}nef-vaccinated animals**

In our hypothesized model, early activation of the cervical epithelium has a critical role in initiating the cascade of innate cell infiltration leading to CD4 T-cell recruitment in naive animals. By contrast, SIVmac239\textsubscript{D}nef vaccination has recently been shown to inhibit the recruitment of CD4 T cells and subsequent local expansion of infected cells in the FRT mucosa, as one correlate of the protection against high dose vaginal challenge. Because the numbers of cervical macrophages and pDCs in vaccinated animals also remained at the same levels as naive animals after challenge, we examined the potential role of epithelial responses in the cervix of vaccinated animals in the inhibition of cell recruitment. In striking contrast to unvaccinated animals, expression of CCL3, CCL20, and CXCL8 by the cervical epithelium was not detected even 7 days after high dose vaginal challenge (Figure 6a–c). These results imply that the protection mediated by SIVmac239\textsubscript{D}nef vaccine is correlated in part with the absence of an early activation signaling triggered in the cervical epithelium, and the subsequent recruitment of CD4 T cells.

**DISCUSSION**

We have previously shown that: (i) vaginal exposure to high doses of SIV infection elicited MIP-3β/CCL20 expression

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**Table 1** Profiles of other cell types of leukocytes in the cervical transition zone after vaginal infection of SIV

| Cell types                  | Markers             | Abundance                       | Co-cluster with CD4 T cells and vRNA+ cells |
|-----------------------------|---------------------|---------------------------------|---------------------------------------------|
| Eosinophil                  | BMK13               | Not detected                    | –                                           |
| Basophil                    | 2D7                 | Not detected                    | –                                           |
| Plasma cell                 | CD138               | Not detected                    | –                                           |
| Myeloid dendritic cell (mDC) | DC-SIGN, S100B, Fascin, CD83 | Not detected                    | –                                           |
| Langerhans cell\textsuperscript{a} | CD1a, Langerin      | Not detected                    | No                                          |
| B cell                      | CD20                | Not consistently detected       | No                                          |
| CD8\textsuperscript{c} cell\textsuperscript{b} | CD8                | Not consistently detected       | No                                          |
| Neutrophil                  | Neutrophil elastase, Nuclei morphology | Not consistently detected       | No                                          |
| NK cell\textsuperscript{b}  | NKG2A               | Small increase                  | No                                          |
| Mast cell                   | Mast cell tryptase, Toluidine staining | See Figure 5                   | No                                          |

\textsuperscript{a}Consistent with previous reports.\textsuperscript{18,27,36,37}
Figure 6  For caption see page on 513.
Figure 7 For caption see page on 516.
in the endocervical epithelium; (ii) MIP-3α/CCL20 expression was subsequently associated with recruitment of β-chemokine-producing pDCs (CCR6$^+$); and (iii) subsequent recruitment of CD4 T-cell targets to fuel the local expansion of infection in the cervical tissues. In this study, we systematically analyzed the locations, quantities, and kinetics of accumulation of different types of leukocytes in cervical tissues after vaginal inoculation.

Focusing on early mucosal events, we examined the spatial and temporal profiles of the expression of chemokines and chemokine receptors in the cervix. The two principal findings from this work are: (i) new evidence for the role of the cervical epithelium in initiating CD4 T-cell recruitment and (ii) a new amplification mechanism mediated by focally clustered macrophages and pDCs to sustain CD4 T-cell recruitment.

Here, we provide evidence for the following sequential events in CD4 T-cell recruitment (Figure 9): (i) cervical epithelium responds to inoculation by producing CCL3, CCL20, and CXCL8; (ii) the epithelial chemokines are spatiotemporally associated with and contribute to the recruitment of macrophages and pDCs, which co-cluster beneath the epithelium; (iii) the macrophages and pDCs themselves produce CCL3, CCL5, CXCL8, and CXCL10 to create a focal concentrated source of chemokines beneath the epithelium; (iv) this chemokine concentration gradient is spatiotemporally associated with the recruitment of β-chemokine-producing CD4 T cells along with more macrophages and pDCs to generate a positive feed-forward mechanism to sustain further increases in CD4 T-cell targets.

Bringing CD4 T cells, macrophages, and pDCs together in close proximity beneath cervical epithelium provides a mechanism to account for the association and likelihood of finding most of the vRNA$^+$ cells in these clusters. Note that because pDCs are the major producer of IFN-α in the cervical tissues as shown previously, the clusters may provide a site of high levels of interferon to select for transmitted founder viruses with relatively greater IFN-α resistance. Thus, clustering of recruited innate immune cells and CD4 T cells underneath the cervical epithelium represents a key early event to facilitate mucosal transmission.

Although this reconstruction of early mucosal events gives an explanation of the co-clustering of mainly vRNA$^+$ CD4 T cells that lack markers of activation, it raises the interesting question of why no more than about 5% of the vRNA$^+$ cells are macrophages even though they are CD4$^+$ CCR5$^+$. Perhaps macrophage-specific restriction factors, e.g., SAMHD1, the activation state of the macrophages,
and responsiveness to IFN-α produced by pDCs make the macrophages less permissive to productive infection.

This study further extends the concept of a spatiotemporal signaling axis from epithelium to innate immune cells, and then to CD4 T-cell targets to support local expansion of infection. The high dose challenge model used in order to increase the likelihood of sampling key events in FRT tissues most resembles a low level of CD4 T-cell recruiting chemokines in the cervicovaginal compartment associated with highly HIV-1-exposed seronegative individuals. In addition, a low level of CD4 T-cell recruiting chemokines in the cervicovaginal compartment is associated with highly HIV-1-exposed seronegative individuals.

Thus, interventions based on interrupting this signaling axis may guide potential strategies for preventing HIV transmission to women. We previously showed that one correlate of the maturation of protection by SIVmac239Anef vaccination is the formation of immune complexes that interact with the inhibitory receptor FcγR2b in the epithelium, which then generates factors that inhibit downstream events associated with a pro-inflammatory response and CD4 T-cell recruitment. Here, we show direct effects associated with SIVmac239Anef vaccination on inhibiting chemokine expression in the epithelium. This is consistent with the concept that the multiple overlapping and complementary pathways involved in the epithelial response to SIV can be targeted by prevention strategies such as vaccination and the microbicide, glycerol monolaurate, which is thought to protect by interfering with the epithelial outside-in signaling. Further exploration of approaches to disrupt these signaling pathways thus could provide additional novel strategies for prevention.

METHODS

Tissues from SIV-infected animals. New tissue sections were cut for the studies described here from archived genital tissues from previous studies of SIV high-dose vaginal infection and SIVmac239Anef vaccine. Briefly, in those studies, monkeys had been inoculated intravaginally with pathogenic SIVmac251 twice in a single day, with a 4-h interval between inoculations. Each inoculation contained 1 ml virus stock of 10⁶ TCID₅₀. Fresh tissues obtained at necropsy were fixed in 4% paraformaldehyde or SAFEFIX II (Fisher Scientific, Kalamazoo, MI), and embedded in paraffin as previously reported. In the cohort of SIV high-dose vaginal infection, necropsy was carried out at day 0, 1, 3, 4, 5, 6, 7, 14, and 28 post infection (d.p.i.). In the SIVΔnef vaccine study, necropsy was carried out at day 0, 4, 5, 7, 11, and 14 post challenge (d.p.c.) in vaccinated animals.

Immunohistochemistry. Single and double immunohistochemical staining, fluorescent immunohistochemical staining, and quantitative image analysis were performed as described elsewhere. The primary antibodies (Abs) used in this study are summarized in Table 2. In brief, tissue sections were deparaffinized in xylene and rehydrated in phosphate-buffered saline. After blocking in Background Sniper (Biocare Medical, Concord, CA), sections were incubated with primary Abs at 4°C overnight. Then, signals were amplified with either 2nd Ab-Biotin + ABC Kit (Vector Lab, Burlingame, CA) in immunohistochemistry or Alexa Fluor dyes (Invitrogen, Eugene, OR) in fluorescent immunohistochemical staining. Nuclei were counterstained with hematoxylin or TOTO-3 (Invitrogen) respectively. Images and montages were taken on Olympus BX60 and Olympus Fluoview FV1000 (Olympus, Center Valley, PA). To measure the kinetics of leukocyte accumulation, we manually counted cells in the entire cervical transition zones of all examined sections. The tissue areas and pixels/intensities were measured by the Aperio Scanscope System (Leica, Buffalo Grove, IL).

Toluidine staining of mast cells. Tissue sections were deparaffinized in xylene, rehydrated in water, and stained in toluidine blue solution for 2–3 min. Then, the slides were thoroughly washed in distilled water, quickly dehydrated in 100% ethanol, and cleared in xylene. The toluidine blue solution contains freshly mixed 1% toluidine blue O/70% ethanol and 1% NaCl/H₂O pH2.3 (1:9 v:v) (Sigma-Aldrich, St Louis, MO). In situ hybridization. SIV RNA was detected in paraffin-embedded tissues by in situ hybridization as previously described. Briefly, sections were deparaffinized in xylene, rehydrated in phosphate-buffered saline, and permeabilized sequentially in HCl, digitonin, and protease K. The sections were then acetylated and hybridized to 35S-labeled SIV-specific riboprobes. After
washing and digestion with ribonucleases, the sections were coated
with nuclear-track emulsion before exposure and development. For
fluorescent in situ hybridization, digoxigenin-labeled SIV-specific
riboprobes were used, and followed by sequential staining with Goat
anti-digoxigenin Abs (Roche, Indianapolis, IN) and Donkey anti-Goat
Abs conjugated with Alexa Fluor 555 (Invitrogen).34,35

Statistical tests. The Wilcoxon rank-sum test was used to measure the
variations in leukocytes over the course of infection. Statistical analyses
were carried out using Prism 4 software.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper
at http://www.nature.com/mi

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DISCLOSURE
The authors declared no conflict of interest.

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TABLE 2 Primary Abs used in this studya

| Antigens | Clones and producers | Fixation | Antigen retrieval conditions |
|----------|----------------------|----------|-----------------------------|
| CD4b     | 1F6 Vector (VP-C318) | PFA      | 1 m M EDTA buffer pH 8 98 °C 20 min |
| CD8a     | 1A5 Vector (VP-C325) | PFA      | 10 m M citrate buffer pH 6 98 °C 20 min |
| CD68     | KP1 DAKO M0814       | PFA      | 10 m M citrate buffer pH 6 98 °C 20 min |
| CD163    | Vector VP-C374       | PFA      | 10 m M citrate buffer pH 6 98 °C 20 min |
| BDCA-2   | 104C12.08 Dendritics DDX0041 | SAFEFIX II | 1 m M EDTA buffer pH 8 98 °C 20 min |
| CD123    | V-18 Santa Cruz sc-681 | PFA | 10 m M citrate buffer pH 6 98 °C 20 min |
| c-Kit    | Novus Biologicals NBP1-85593 | SAFEFIX II | 10 m M citrate buffer pH 6 98 °C 20 min |
| CCL3     | Neomarkers RB-10489-P | PFA      | 10 m M citrate buffer pH 6 98 °C 20 min |
| CCL4     | R&D AF-271-NA        | PFA      | 10 m M citrate buffer pH 6 98 °C 20 min |
| CCL5     | R&D AF-278-NA        | PFA      | 10 m M citrate buffer pH 6 98 °C 20 min |
| CCL20    | 2069D.05/Dendritics & # DDX0420 | PFA | 1 m M EDTA buffer pH 8 98 °C 20 min |
| IL-8     | Abcam ab7747-500     | PFA      | 10 m M citrate buffer pH 6 98 °C 20 min |
| IP10     | R&D AF-266-NA        | PFA      | 10 m M citrate buffer pH 6 98 °C 20 min |
| CCR5     | AIDS Reagents 4914    | PFA      | 1 m M EDTA buffer pH 8 98 °C 20 min |
| CCR6     | R&D MAB195           | PFA      | 10 m M citrate buffer pH 6 98 °C 20 min |
| CXCR1    | Novus Biologicals NBP1-88143 | SAFEFIX II | 1 m M EDTA buffer pH 8 98 °C 20 min |
| CXCR2    | Novus Biologicals NBP1-02412 | PFA | 10 m M citrate buffer pH 6 98 °C 20 min |
| CXCR3    | R&D 49801 MAB160     | PFA      | 10 m M citrate buffer pH 6 98 °C 20 min |
| BMK13    | Abcam 77842          | PFA      | 10 m M citrate buffer pH 6 98 °C 20 min |
| CD138    | AnaSpec 53317        | PFA      | 10 m M citrate buffer pH 6 98 °C 20 min |
| DC-SIGN  | R&D MAB161           | PFA      | 10 m M citrate buffer pH 6 98 °C 20 min |
| S100B    | Thermo Scientific MA5-15359 | PFA | 10 m M citrate buffer pH 6 98 °C 20 min |
| Fascin   | Vector Lab VP-F703   | PFA      | 10 m M citrate buffer pH 6 98 °C 20 min |
| CD83     | Vector Lab VP-C368   | PFA      | 10 m M citrate buffer pH 6 98 °C 20 min |
| CD1a     | DAKO 3571            | PFA      | 10 m M citrate buffer pH 6 98 °C 20 min |
| Langerin | Vector Lab VP-L552   | PFA      | 10 m M citrate buffer pH 6 98 °C 20 min |
| Elastase | DAKO 0752            | PFA      | 10 m M citrate buffer pH 6 98 °C 20 min |
| NKG2A    | Epitomics T3308      | PFA      | 10 m M citrate buffer pH 6 98 °C 20 min |
| Tryptase | DAKO M7052           | PFA      | 10 m M citrate buffer pH 6 98 °C 20 min |

aIsotype controls were performed on all examined animals (See Supplementary Figure S1 online).
bCD4 Ab concetration was optimized to only stain CD4+ T cells that have higher CD4 expression than macrophages36 (See Supplementary Figure S2).

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