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Topical Glucocorticoid Therapy Directly Induces Up-Regulation of Functional CXCR4 on Primed T Lymphocytes in the Aqueous Humor of Patients with Uveitis

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Overexpression of the constitutive chemokine receptor CXCR4 has been shown to contribute to the accumulation of leukocytes at sites of chronic inflammation. Glucocorticoids are widely used to treat inflammatory disorders such as uveitis to considerable effect, yet paradoxically have been reported to increase CXCR4 expression in vitro. We show here that ocular lymphocytes isolated from patients with uveitis who had been treated with topical glucocorticoids expressed highly elevated levels of CXCR4. The up-regulation of CXCR4 could be reproduced in vitro by culture of CD4+ T cells with aqueous humor (AqH), indicating a role for the ocular microenvironment rather than preferential recruitment of CXCR4+ cells. Untreated uveitis and noninflammatory AqH up-regulated CXCR4 to a limited extent; this was dependent on TGF-β2. However, the highest levels of CXCR4 both in vivo and in vitro were found in the glucocorticoid-treated patients. Glucocorticoids appeared to be directly responsible for the induction of CXCR4 in treated patients, as the glucocorticoid receptor antagonist RU38486 inhibited the in vitro up-regulation by AqH from these patients. Dexamethasone selectively up-regulated CXCR4 in vitro, but not any of a wide range of other chemokine receptors. CXCL12, the ligand for CXCR4, was present in AqH under noninflammatory conditions, but the levels were low in untreated uveitis and undetectable in treated uveitis AqH. The importance of these results for the treatment of HIV patients with glucocorticoids is discussed as well as a role for glucocorticoid-induced CXCR4 up-regulation and CXCL12 down-regulation in controlling the migration of lymphocyte populations, resulting in resolution of inflammation.

Inflammatory disorders are characterized by the accumulation of leukocytes at the site of specific lesions. This requires the integration of several distinct processes including the recruitment, retention, and survival of cells (1,2). A key control for both recruitment and retention is the expression by inflammatory leukocytes of functional chemokine receptors that interact with locally expressed chemokines within the lesion (3,4). The dominant paradigm for chemokine/chemokine receptor function suggests a distinction between constitutive chemokine receptors that are involved in regulating physiological recirculation patterns for lymphocytes and other cells (5,6) and inflammatory chemokine receptors, which regulate the recruitment and retention of cells during immune/inflammatory responses (7).

Recent studies have questioned this clear functional dichotomy. CXCR4 is a constitutive chemokine receptor, which is widely expressed on leukocytes and is involved in the recirculation of naive lymphocytes through high endothelial venules into lymphoid tissue (8,9). It also plays a key role in the retention of stem cells, differentiating B cells, and neutrophils within bone marrow (10,11) and controls B cell positioning within lymph nodes, where its expression is regulated by IL-4 (12). CXCR4 is unusual in having a single monogamous ligand CXCL12 (stromal cell-derived factor 1 (SDF-1)4), which is distributed widely in tissues (13). Intriguingly, this chemokine receptor has recently been reported to enhance the active retention of highly differentiated primed T cells at sites of chronic inflammation (14,15). Naive T cells initially up-regulate expression of CXCR4 following stimulation, but primed cells lose this characteristic (14,15). However, even highly differentiated T cells show a potent increase in functional CXCR4 expression following exposure to any of the three isoforms of TGF-β (14).

These observations are particularly intriguing, because in vitro studies have indicated that expression of T cell lines to glucocorticoids may also induce potent up-regulation of CXCR4 expression (18,19), yet glucocorticoid therapy is routinely used in many inflammatory disorders to resolve the inflammation. This suggests a dichotomy, but currently there is a lack of in vivo evidence to

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*Abbreviations used in this paper: SDF-1, stem cell-derived factor 1; AqH, aqueous humor; z-VAD, benzoyloxycarbonyl-Val-Ala-Asp.
support the role of glucocorticoids in regulating CXCR4 expression. If the up-regulation of CXCR4 by glucocorticoids was confirmed in vivo, this would suggest a role for the molecule in regulating the migration of lymphocyte populations, resulting in resolution of inflammation. This question is also of particular significance because CXCR4 is a major coreceptor for T cell tropic strains of HIV-1, and high-level induction of CXCR4 by dexamethasone on CD4+ T cells in vitro has been shown to increase infection and replication of the virus (19).

Given the role suggested for CXCR4 in promoting persistent inflammatory disease (14, 15), we wished to address whether it also plays a role in regulating a more self-limiting inflammatory response. Uveitis is an inflammatory condition characteristically affecting the anterior chamber of the eye (20). Many cases resolve rapidly, but a significant number of patients develop inflammatory damage to ocular structures, resulting in severe visual impairment. This condition is almost invariably treated (usually successfully) with topical glucocorticoids. The relationship between microenvironmental levels of TGF-β and induction of CXCR4 makes the eye a particularly intriguing target because of the high levels of TGF-β associated with the immune privilege characteristic of this organ (21, 22). As in many other inflammatory conditions, the leukocyte infiltrate in uveitis includes a predominance of CD45RO+, Fas, and HLA-DR expression (23, 27). Cells were maintained in CD4+ T lymphocytes in experimental models of uveitis (24, 25), but little is known of these factors in human disease. This study sought to determine the expression of functional CXCR4 by T lymphocytes infiltrating the anterior chamber of patients with uveitis, in untreated individuals, and those treated with topical steroids. Control samples were obtained from patients with no discernible inflammation undergoing surgery for age-related cata-
racts. The capacity of aqueous humor (AqH) to regulate CXCR4 expression was also examined to determine whether the inflammatory environment (pre- or posttherapy) was able to induce CXCR4 and whether this was a function of the disease or of the steroid therapy.

Materials and Methods

Patients, diagnosis, and AqH samples

AqH samples (~100 μl) were collected from patients with active uveitis using an insulin syringe. Samples were either taken before starting the standard treatment of a topical glucocorticoid preparation (untreated group) or within 1 wk of initiating this treatment (treated group). The untreated group (median age, 46.2 years; range, 21.6–71.4 years) consisted of 25 patients with acute anterior uveitis (24 idiopathic and 1 associated with ankylosing spondylitis) and 3 patients with panuveitis (2 idiopathic and 1 herpesvirus). The treated group (median age, 47.5 years; range, 31.8–66.7 years) comprised 14 patients with acute anterior uveitis (9 idiopathic, 4 herpesvirus, and 1 associated with ankylosing spondylitis) and 3 patients with idiopathic panuveitis. The number of AqH samples used for each of the experiments is shown in the figure legends. Sample collection followed the Helsinki Declaration of Helsinki and was approved by the West Birmingham Local Research Ethics Committee. Written informed consent was obtained from each patient. Noninflammatory control specimens were collected from individuals undergoing surgery for age-related cataracts (median age, 76.9 years; range, 57.4–85.3 years). Matched peripheral blood specimens were also taken from uveitis patients. AqH was centrifuged at 300 × g for 5 min, and the cell-free supernatant (subsequently referred to as AqH) was removed and frozen in aliquots at −70°C. Cell pellets were resuspended, counted, and analyzed by flow cytometry. Matched PBLS were isolated by density gradient centrifugation.

Maintenance of CD4+ T cell lines

CD4+ short-term T cell lines were derived from the peripheral blood of normal healthy volunteers as described previously (26). These cells share many features of the CD4+ lymphocytes observed in uveitis, including CD45RO, Fas, and HLA-DR expression (23, 27). Cells were maintained in RPMI 1640 (Sigma-Aldrich, Irvine, U.K.), streptomycin (100 μg/ml), pen-

icismillin (100 U/ml), glutamine (10 mM), and 10% heat-inactivated FCS, with two weekly stimulations with PHA-H15 (9 μg/ml; Murex Biotech, Dartford, U.K.) and gamma-irradiated (30 Gy) autologous EBV-transf-

formed B cells. IL-2 (Chiron, Harefield, U.K.) at 25 U/ml was added every 3–4 d. Cells were used for experiments 14 days after stimulation and viable cells were isolated by density gradient centrifugation.

Flow cytometric analysis of AqH cells and PBMC

AqH cells and matched PBMC were incubated with optimal concentrations of FITC-conjugated anti-CD8 (BD Biosciences, Oxford, U.K.), PE-conju-

gated anti-CXCR4 (R&D Systems, Abingdon, U.K.), ECD-conjugated anti-

CD4 (Beckman Coulter, High Wycombe, U.K.), and Tricolor-conjugated anti-CD45RO (Caligag Laboratories, Burlingame, CA). Cell labeling was performed in PBS (pH 7.4) containing 2% BSA for 20 min at 4°C. Cells were analyzed immediately, without prior fixation, by four-color flow cy-


tometry on a Coulter EPICS XL cytometer (Beckman Coulter).

Regulation of CXCR4 expression in vitro

CD4+ T cells were washed three times with RPMI 1640 to remove residual cytokines and serum factors. Cells were cultured for 18 h at 37°C in serum-

free medium containing RPMI 1640 with 1% low-endotoxin BSA (Sigma-

Aldrich) at 25,000 cells/well in Terasaki plates (Nunc, Roskilde, Denmark) with AqH added at 50% volume. Dexamethasone (Sigma-Aldrich) was added at the concentrations indicated. The glucocorticoid inhibitor RU38486 (mifepristone; Sigma-Aldrich) was added at 10−7 M. Thepan
caspase inhibitor benzylxoyacyrnon-Val-Ala-Asp (z-VAD, Calbiochem, San Diego, CA) was added at a final concentration of 100 μM from a stock solution of 50 mM in DMSO. Following culture, cells were sequentially incubated for 20 min at 4°C with optimal concentrations of anti-CXCR4 (12G5; R&D Systems) followed by FITC anti-mouse IgG (Southern Bio-
technology Associates, Birmingham, AL) or with PE-conjugated anti-

CXCR4 (see Fig. 7d). Cells were analyzed by flow cytometry for the ex-

pression of CXCR4, shown as a percentage of the median fluorescence intensity for cells cultured in medium alone.

Depletion of TGF-β and CXCL12 from AqH

All isoforms of TGF-β were depleted from AqH using multiple rounds of anti-TGF-β-coated magnetic beads. A mixture of rabbit anti-TGF-β (In-

sight Biotech, Wembley, U.K.)-coated M280 Dynabeads (Dyna, Oslo, Norway) and mouse anti-TGF-β (R&D Systems)-coated M450 Dynabeads (Dyna) were used to deplete TGF-β from AqH. As a control for the de-

pletion protocol, irrelevant Abs, rabbit IgG (DAKO Cytometry, Ely, U.K.), and mouse IgGl isotype control Ab (R&D Systems) were used.

Beads were coated with Ab overnight at 4°C, washed, and blocked with 1% casein/1% BSA (Sigma-Aldrich) in PBS. Three rounds (30 min each at 4°C) of depletion using 4 × 105 of each bead population were used. The concentration of TGF-β in the depleted samples was determined by ELISA (R&D Systems). All depletions showed a reduction in TGF-β of >90%. For CXCL12 depletion, anti-CXCL12 (K15C) (or irrelevant control) Ab was added to AqH at 30 μg/ml, incubated for 2 h at 4°C, and then depleted with three rounds of blocked M450 goat anti-mouse IgG Dynabeads (Dyn-

al). The depletion efficiency was >95% as measured by both immuno-

assay and the loss of CXCR4 down-regulation by exogenous CXCL12 on CD4+ T cells.

Measurement of CXCL12 and TGF-β2 in AqH

AqH samples (50–150 μl) were analyzed for the presence of CXCL12 (SDF-1) and active TGF-β2 using multiplex bead analysis (28). Monoclo-

nal capture Abs specific for CXCL12 and TGF-β2 (R&D Systems) were covalently coupled to separate fluorescent microspheres (Luminex, Austin, TX) ac-
cording to the manufacturer’s protocol. AqH was incubated with the mAb-

coupled capture beads for 2 h at 20°C. Washed beads were subsequently incubated with biotin-conjugated polyconal goat anti-human CXCL12 and TGF-β2 Abs (R&D Systems) for 2 h, followed by streptavidin-PE (Upstate Biotechnology, Milton Keyne, U.K.) at 40 pg/ml for 30 min. Samples were analyzed using a Luminex suspension platform. In some experiments, recombinant human CXCL12 and TGF-β2 (R&D Systems) were used to calibrate the assay. For each sample 100 beads were analyzed; the assay was sensitive to <10 pg/ml.

Transmigration assay

CD4+ T cells were washed three times with RPMI 1640 to remove cyto-
kines and serum factors. Cells were cultured for 20 h at 37°C in serum-free medium containing RPMI 1640 with 1% low-endotoxin BSA with the addition of 10−7 M dexamethasone as indicated. Viable cells were isolated by density gradient centrifugation.
by density gradient centrifugation and resuspended at 5 × 10^6 cells/ml in 0.5% fraction V BSA (Sigma-Aldrich) in RPMI 1640. Migration in response to chemokines was determined using 6.5-mm diameter, 5.0-μm pore size Transwell inserts (Corning Costar, Cambridge, MA) as described previously (29). A titration curve of the chemokine CXCL12 (R&D Systems) was prepared, equilibrated to 37°C, and 600 μl was added to the lower Transwell chamber. Control or dexamethasone-treated CD4^+ T cells (100 μl) were added to the upper Transwell chamber. Control wells containing no chemokine were included in each assay. After incubation for 4 h at 37°C, 5% CO_2, cells were carefully resuspended from the upper and lower chambers into a fixed volume of PBS containing 25 μg/ml propidium iodide (Sigma-Aldrich) and 25% FCS. Viable cell counts were performed using the fixed-volume counting facility of the Coulter EPICS XL cytometer. The number of viable cells present in each sample was determined using forward and 90° scatter characteristics along with exclusion of propidium iodide.

**Statistical analysis**

The Mann-Whitney U test was used to determine significant differences between nonpaired groups. Wilcoxon-matched pairs analysis was performed on paired samples. Correlations were calculated using Spearman’s correlation coefficient. The minimal level of confidence at which the results were judged significant was \( p < 0.05 \).

**Results**

**Expression of CXCR4 on intraocular lymphocytes in uveitis**

The chemokine receptor CXCR4 is typically expressed at high levels on naive CD45RA^+ T cells, but is down-regulated on primed T cells (CD45RO^-). In uveitis patients, AqH T cells were almost invariably CD45RO^- (Fig. 1a). Compared with matched CD45RO^- peripheral blood T cells, AqH T cells from these patients expressed significantly elevated levels of CXCR4 (Fig. 1a). The level of expression of CXCR4 on peripheral blood T cells was similar between different patients, but AqH T cells showed a wide range of expression. Intriguingly, only the glucocorticoid-treated patients showed significant increases in CXCR4 expression on AqH-derived T cells compared with peripheral blood cells from the same patients, both for CD4 and CD8 subsets (Fig. 1, b and c). The level of CXCR4 was significantly higher on T cells from the AqH of glucocorticoid-treated patients than those from the untreated group (CD4^+, \( p < 0.01 \); CD8^+, \( p < 0.05 \)). The expression of CXCR4 correlated closely between the CD4^- and CD8^- lymphocytes (\( r = 0.84, p < 0.01 \)). There was no significant difference in disease activity or severity between the treated and untreated patient groups (data not shown). Stratification by disease classification of uveitis subtype or disease severity indicated no significant association between either of these factors and CXCR4 expression (data not shown).

**Regulation of CXCR4 expression by the ocular microenvironment**

To determine whether the increased level of CXCR4 on AqH lymphocytes is likely to reflect up-regulation by factors present within the ocular microenvironment or alternatively preferential recruitment of CXCR4^hi^CD45RO^- T cells from peripheral blood, we cultured a short-term CD4^+ T cell line in vitro, with cell-free AqH isolated either from uveitis patients or noninflammatory control subjects undergoing cataract surgery. The expression of CXCR4 increased when the T cells were cultured with either noninflammatory control or uveitis AqH compared with medium alone (Fig. 2), suggesting that the ocular environment has an inherent ability to up-regulate CXCR4. All samples were able to up-regulate CXCR4 levels above those obtained when using medium alone (Fig. 2b), but AqH samples from the glucocorticoid-treated patients elevated CXCR4 to significantly higher levels than either control or untreated uveitis AqH (Fig. 2b). These results closely paralleled the in vivo expression of CXCR4 on ocular lymphocytes and suggest that the ocular microenvironment is responsible for the observed increase in CXCR4 expression. More significantly, the particularly high levels observed in glucocorticoid-treated patients appeared likely to be due to the steroid therapy itself rather than a disease-related process.

**Regulation of CXCR4 expression by glucocorticoids**

The results obtained indicated two possibly distinct phenomena: first, an inherent capacity of AqH to up-regulate CXCR4 expression on T cells and, second, a significantly higher level of induction by AqH from patients treated with topical glucocorticoid therapy. To assess the possible direct role of glucocorticoids in the up-regulation of CXCR4 in treated patients, we incubated the synthetic glucocorticoid dexamethasone with in vitro-cultured CD4^+ T cells across a wide range of concentrations (Fig. 3a), including those estimated to be present in AqH from glucocorticoid-treated patients (30). Dexamethasone showed potent induction of CXCR4 at pharmacologically relevant concentrations. Mature T cells are
largely resistant to the process of glucocorticoid-induced apoptosis, but at high concentrations dexamethasone can induce low levels of apoptosis in mature T cells (31). We therefore examined whether the up-regulation of CXCR4 by dexamethasone was in any way dependent on the induction of apoptosis in a proportion of T cells by treating cells with the pan caspase inhibitor z-VAD. This treatment completely abolished apoptosis as measured by caspase-3 activation (data not shown) but CXCR4 was still elevated by dexamethasone in the presence of z-VAD (Fig. 3a). Direct induction of apoptosis with an anti-Fas Ab failed to increase CXCR4 expression (data not shown). We also confirmed that all currently prescribed topical glucocorticoids (prednisolone acetate, prednisolone sodium phosphate, and betamethasone sodium phosphate) and also the endogenous glucocorticoid cortisol (but not cortisone) induced a similar up-regulation of CXCR4 (Fig. 3b).

The glucocorticoid-induced CXCR4 was functional, as shown by the increased migration of glucocorticoid-treated cells in response to the ligand CXCL12 over a range of concentrations (Fig. 3c). In addition to CXCR4, we analyzed the effect of dexamethasone on the expression of a wide range of chemokine receptors. Intriguingly, dexamethasone only up-regulated CXCR4 and had no effect on any of the other chemokine receptors studied (Fig. 4).

To address whether glucocorticoids in AqH from treated uveitis patients were directly responsible for increasing CXCR4 expression to high levels, we used the glucocorticoid receptor antagonist RU38486 (mifepristone). This antagonist effectively blocked up-regulation of CXCR4 by dexamethasone (Fig. 5a), except at the very highest dexamethasone concentration used. Crucially, addition of the antagonist significantly reduced the up-regulation of CXCR4 expression by AqH from glucocorticoid-treated uveitis patients, but had no significant effect on the lower level of CXCR4 up-regulation by noninflammatory control and untreated uveitis AqH (Fig. 5b). This result not only confirms that the enhanced CXCR4 up-regulation observed in glucocorticoid-treated patients is most likely to be a direct effect of the therapy, but also indicates that the inherent capacity to up-regulate CXCR4 observed for control AqH is unlikely to be mediated by endogenously produced cortisol.

**TGF-β-dependent control of CXCR4 expression**

The moderate induction of CXCR4 by control AqH and the lower level observed for untreated uveitis AqH were not associated with ligation of the glucocorticoid receptor, as they were unaffected by the antagonist RU38486. Several cytokines have been reported to up-regulate CXCR4, including IL-4, IL-7, and all isoforms of TGF-β. Preliminary experiments showed no detectable IL-4 or IL-7 in either control or uveitis AqH, but TGF-β2 was detected in...
11 of 12 control samples taken at cataract surgery and in 8 of 23 uveitis patients. AqH from untreated uveitis patients, but not those treated with topical glucocorticoids, showed a significant association between TGF-β2 levels and the capacity to induce CXCR4 expression on T cells (r = 0.54, p < 0.05; Fig. 6a). No TGF-β1 was detected in any of the samples tested (data not shown). We assessed a possible role for TGF-β in the up-regulation of CXCR4 in AqH by depleting one-half of each of a number of AqH samples with anti-TGF-β Abs coupled to magnetic beads; this resulted in the depletion of >90% of TGF-β as measured by ELISA in all samples shown. The remaining sample was mock-depleted using irrelevant Ab to provide the control. Depletion of TGF-β significantly reduced the ability of eight of nine noninflammatory and all untreated uveitis AqH to up-regulate CXCR4 expression (Fig. 6b). AqH from glucocorticoid-treated patients showed some evidence of TGF-β-dependent up-regulation of CXCR4, but also significant TGF-β-independent up-regulation, consistent with a direct action of glucocorticoids as shown above.

**Regulation of CXCR4 up-regulation by CXCL12**

The sole ligand for CXCR4, CXCL12, was found at levels up to 1 ng/ml in noninflammatory AqH, but was greatly reduced in uveitis AqH and was effectively absent from the glucocorticoid-treated group (Fig. 7a). Addition of CXCL12 was able to prevent the up-regulation of CXCR4 by TGF-β2 (Fig. 7b), although the concentration required was 100 times greater than that detected in AqH. Addition of CXCL12 (100 ng/ml) was able to reduce the ability of AqH to up-regulate CXCR4 (Fig. 7c). However, depletion of CXCL12 from AqH of either untreated or glucocorticoid-treated uveitis patients had no effect on CXCR4 levels (Fig. 7d). These results collectively indicate that the bioactive levels of CXCL12 in AqH are below those able to significantly modulate the expression of CXCR4 on CD4+ T cells. Addition of TGF-β2 to AqH was able, in some cases, to further increase the up-regulation of CXCR4 (Fig. 7d). For glucocorticoid-treated uveitis this could be explained by an additive effect of TGF-β2 and dexamethasone on CXCR4 expression (Fig. 7e).

**Discussion**

The chemokine receptor CXCR4 has been classified as a member of the constitutively expressed group of receptors (4–6), because it plays a key role in the retention of precursor cells in bone marrow (10, 11), the positioning of B lineage cells in germinal centers (12), and also in the traffic of naive T cells through high endothelial venules into secondary lymphoid tissue (8, 9). However, recent studies have suggested that CXCR4 also plays an important role in chronic inflammation at sites such as the rheumatoid synovium (14, 16) and the inflamed airway (15); a CXCR4 antagonist (AM3100) ameliorated inflammation in experimental models of both conditions (32, 33). Naive T cells express high levels of CXCR4, which are up-regulated further during the first few cycles of activation of such cells (14). In contrast, primed T cells express very low levels of this receptor both at rest and following activation (14, 17). At sites of inflammation however, the expression of CXCR4 can be induced on even highly differentiated primed T cells by a number of cytokines including IL-4, IL-7, and TGF-β (14, 19). The receptor under such circumstances is functional and can mediate adhesion to matrix and active migration (14, 19). The high levels of TGF-β known to be expressed in the eye as part of the mechanism of immune privilege (21, 22) suggested that induction of CXCR4 may play a significant role in the retention of T cells in the eye when privilege is breached during the inflammatory condition uveitis. Our initial observations confirmed that both CD4 and CD8 T cells in the AqH of uveitis patients expressed very high...
levels of CXCR4 and that AqH was itself capable of inducing CXCR4 expression on cells from a primed T cell line. However as expected, control samples of AqH obtained from patients undergoing cataract surgery were also effective at mediating CXCR4 up-regulation. Indeed, the control samples were quite consistent in the level of CXCR4 induced, whereas uveitis patients varied considerably from significantly higher than controls to very little induction at all. The observation that TGF-β depletion significantly diminished the CXCR4 induction by AqH from uninflamed control samples and also untreated uveitis patients was not surprising; TGF-β2 is present at high levels in normal AqH (21, 22), but falls during inflammation (34). For some AqH, addition of TGF-β2 resulted in an increase in CXCR4 up-regulation (Fig. 7d), again suggesting suboptimal levels of TGF-β2 in some patients with uveitis. Analysis of AqH lymphocytes, from uveitis patients who had yet to be given glucocorticoid therapy in the present study, showed no significant increase in CXCR4 expression above the level found on peripheral blood cells. We were only able to analyze CXCR4 on patients with high levels of inflammation (and therefore low levels of TGF-β2) due to the very low cell numbers in AqH from less inflamed cases.

Segregation of samples from uveitis patients into those treated with topical glucocorticoids and untreated patients revealed that the highest levels of CXCR4 on AqH-derived T cells were invariably seen in treated patients and that the highest levels of induction in vitro were also mediated by AqH from treated patients. There was no difference in disease severity or activity between the two groups of patients; indeed all patients were subsequently treated with topical glucocorticoids. The clinical imperative for AqH aspiration is the identification of viruses that may mediate ocular inflammation before specific antiviral therapy (35). However, all patients additionally receive topical glucocorticoids regardless of etiology. The issue of whether patients in this study had been treated with glucocorticoids when the AqH sample was obtained was determined by whether aspiration could be performed at the initial presentation or was deferred until after the initiation of glucocorticoid therapy.

The capacity for glucocorticoid-treated AqH to mediate CXCR4 up-regulation was markedly diminished by the specific glucocorticoid-receptor antagonist RU38486; along with the close association between treatment and CXCR4 level, this indicates that the high levels of expression in treated patients were mediated directly by the action of glucocorticoids. The ideal experiment would clearly have been to perform all analyses on each patient pre- and posttherapy; however, repeat aspiration is not clinically (or ethically) justified. As well as TGF-β2 and glucocorticoids, there may be other molecules present in AqH that affect the observed up-regulation of CXCR4. CXCL12, the ligand for CXCR4, was able to abrogate the up-regulation induced by TGF-β2, dexamethasone,
or AqH. However, the levels required to achieve this were significantly greater than those found in AqH. Indeed, depletion of CXCL12 from AqH did not significantly alter the resulting CXCR4 induction, suggesting that CXCL12 does not play a significant role in the regulation of CXCR4 by AqH. Other cytokines that are known to affect CXCR4 expression include IL-2, IL-7, IL-15, and IFN-γ. We could not detect IL-2, -7, or -15 in any of the AqH used in these studies (data not shown). Although IFN-γ was found in uveitis AqH, levels did not correlate with CXCR4 expression, and addition of IFN-γ to in vitro cultures did not influence the up-regulation of CXCR4 by either TGF-β2 or dexamethasone (data not shown). Clearly, we cannot rule out a role for other, as yet unknown, molecules in the regulation of CXCR4 expression. Our experiments have addressed the role of CXCR4/CXCL12 in ocular inflammation; however, it is clear that there are many inflammatory chemokines that will be controlling the recruitment and retention of leukocytes in the eye.

In vitro experiments have previously indicated that T cell expression of CXCR4 can be up-regulated by the synthetic glucocorticoid dexamethasone (19). As far as we are aware, the present report is the first evidence that glucocorticoid therapy leads directly to CXCR4 up-regulation in vivo. We confirmed that dexamethasone and all other currently prescribed topical glucocorticoids up-regulate CXCR4 on T cells in vitro and indeed the endogenous glucocorticoid cortisol (but not the inactive precursor molecule cortisone) was also able to mediate this up-regulation.

Although our investigations have focused on the up-regulation of CXCR4, glucocorticoids are known to regulate many aspects of inflammation. Despite this, the precise mechanisms by which they mediate their anti-inflammatory effects are poorly defined (31, 36–38). The glucocorticoid receptor is a cytoplasmic molecule, which dimerizes when it is ligated by glucocorticoid. The receptor–ligand complex has two principle modes of action: first, dimerized receptor can migrate to the nucleus, where it binds to the glucocorticoid response element motif in the promotor region of a number of genes, to induce or repress transactivation (39, 40). Second, the complex can bind to and modulate the activity of transcription factors such as AP-1 and NF-κB (41–44), independently of receptor dimerization. The induction of thymocyte apoptosis appears to be dependent on direct DNA binding of dimerized receptor, because thymocytes from mice that are homozygous for a dimerization-defective glucocorticoid receptor (GRdim/dim) are resistant to glucocorticoid-induced apoptosis (45). In contrast, the anti-inflammatory effects of glucocorticoids appear to be largely independent of binding to the glucocorticoid response element motif, as GRdim/dim mice maintain a potent anti-inflammatory response to glucocorticoids (46). The up-regulation of CXCR4 expression induced by IL-4 in T cells appears to involve activation of the cAMP pathway (47, 48). cAMP leads to the activation of protein kinase A, which in turn phosphorylates the transcription factor CREB (49, 50). Mutation of a proposed cAMP response element identified in the CXCR4 promotor diminished production of the chemokine in response to dibutylryl cAMP treatment (51). In separate studies, dexamethasone has been reported to induce cAMP generation in T cell lines (52), suggesting that this pathway is also likely to be the mechanism by which glucocorticoids up-regulate CXCR4.

The observation that glucocorticoid therapy, which is the most potent anti-inflammatory treatment available for most conditions, strongly up-regulates expression of CXCR4 indicates that the proposed role of this receptor in the pathology of chronic inflammation is likely to be complex (14–16). The strongest evidence in favor of a direct pathogenic role for CXCR4 in inflammation was provided by studies of CXCR4 antagonists (32, 33). The apparent dichotomy presented by glucocorticoid-mediated CXCR4 up-regulation may be resolved by the parallel reduction that we observed in the availability of the sole ligand CXCL12 (SDF-1α). The absence of significant CXCL12 in uveitis contrasts sharply with the presence of CXCL12 in persistently inflamed sites such as the rheumatoid synovium (53–55), but is consistent with the ability of proinflammatory cytokines such as TNF-α or IL-1 to down-regulate the production of CXCL12 by stromal cells (56). In the absence of ligand, the CXCR4-mediated retention in inflamed tissue may be lost despite high levels of receptor. Inflammatory cells may subsequently sequester in the draining lymphoid tissue, where CXCL12 levels are high (8, 9, 12). Indeed, a long-standing clinical observation concerning high-dose systemic glucocorticoid therapy may well reflect the physiological consequences of CXCR4 up-regulation: patients treated with bolus doses of glucocorticoid systemically invariably show a marked reduction in circulating T cells within hours (57, 58). These cells are known to be transiently sequestered within bone marrow and lymphoid tissues, both of which express extremely high levels of CXCL12 (10, 12). An alternative explanation for the retention of cells mediated by moderate levels of CXCR4 in inflammation but loss of cells from inflammatory lesions associated with the high levels of CXCR4 induced by glucocorticoid therapy may reflect the threshold levels of receptor engagement required to differentially mediate adhesion and migration (14).

The observation that CXCR4 levels of CD4+ T cells in AqH are superinduced by topical glucocorticoids is of considerable clinical significance. In vitro studies have shown that CD4+ T cells that have been induced to express high levels of CXCR4 by glucocorticoids are highly sensitive to infection with HIV-1 virus and facilitate viral replication (19). Indeed, a strong inverse relationship between endogenous cortisol levels in patients with AIDS and the number of circulating CD4+ T cells has been reported previously (59); it is likely that this association reflects CXCR4 induction. The current study suggests considerable caution should be used before the use of any form of glucocorticoid therapy in HIV-infected patients.

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References


