AMD3465, a Novel CXCR4 Receptor Antagonist, Abrogates Schistosomal Antigen-Elicited (Type-2) Pulmonary Granuloma Formation

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CXCR4 is a major receptor for CXCL12 and is known to participate in multiple physiological systems. The present study tested a second generation CXCR4 antagonist, AMD3465, for effects on highly defined models of Th1- and Th2-cell-mediated hypersensitivity-type pulmonary granuloma formation. Type-1 and type-2 granulomas were induced, respectively, by intravenous challenge of sensitized CBA/J mice with *Mycobacteria bovis* purified protein derivative- or *Schistosoma mansoni* egg antigen-coated beads. Before challenge, mice were implanted with osmotic pumps releasing AMD3465 at 5 µg/hour (6 mg/kg/day). Compared to vehicle, AMD3465 had minimal effect on type-1 inflammation or cytokine responses in draining lymph nodes, but the type-2 inflammation was significantly abrogated with reductions in lesion size and eosinophil content as well as abrogated interleukin (IL)-5, IL-10, and IL-13 cytokine production in draining lymph nodes. The biased effect of AMD3465 correlated with greater CXCR4 ligand expression in the type-2 model. Treatment during a primary response impaired lymph node IL-2 production after both *Mycobacteria bovis* purified protein derivative and *Schistosoma mansoni* egg antigen challenge indicating an unbiased effect during immune induction. In summary, CXCR4 blockade inhibited eosinophil recruitment during type-2 granuloma formation and interfered with primary and secondary T-cell activation events in lymphoid tissue, suggesting potential therapeutic application for chronic hyper-

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Cysteine-x-cysteine chemokine receptor 4 (CXCR4), the receptor for CXCL12, previously known as stromal cell-derived factor 1 α (SDF-1 α), is reportedly expressed by epithelial cells,¹ naïve T lymphocytes,² and the Th2 subset of T-helper lymphocytes.³ The CXCR4 receptor is activated by the chemokine ligand CXCL12, which is constitutively expressed by a number of tissues, suggesting that CXCR4 and CXCL12 play a role in physiological homeostasis.^{4–6} It is known that CXCL12 is an important chemoattractant in T-lymphocyte circulation.⁴ In addition, CXCR4 strongly influences the migration and tissue target of leukocytes and plays an essential role in retention and homing of CD34⁺ stem cells in bone marrow.⁷ The importance of the receptor is revealed by the fact that mice with genetic deletion of the receptor or its ligand display impaired murine embryonic development of heart, brain, and large vessels.^{8–10} Studies to date suggest that the targeting of CXCR4 with specific chemical antagonists for therapeutic purposes would be promising. There is compelling evidence that disrupting CXCR4-CXCL12 interactions might be effective in diseases such as asthma,^{11,12} cancer,^{13,14} and arthritis.¹⁵ CXCR4 also acts as a co-receptor for HIV,¹⁶ thus making the receptor an attractive target for anti-HIV therapy.

The present study used a soluble CXCR4 receptor antagonist known as AMD3465. It is a related derivative of AMD3100, which has been shown to block HIV entry into cells,^{17,18} inhibit collagen type-1 model of arthritis in mice,¹⁹ and decrease CD4⁺ and CD8⁺ T-cell recruitment and airway hyperresponsiveness in cockroach antigen-

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induced model of asthma.¹² The clinical use of AMD3100 as a CXCR4 receptor antagonist in leukopenic cancer patients also showed benefit by enhancing leukocyte numbers in the blood. Specifically, combined with granulocyte colony-stimulating factor, AMD3100 can rapidly mobilize CD34⁺ hematopoietic progenitor cells and leukocytes in healthy patients and patients with multiple myeloma and non-Hodgkin's lymphoma.^{7,20} CXCR4 receptor antagonists were first identified in the mid-1980s and structurally were bicyclams,²¹ which consisted of two monocyclams (1,4,8,11-tetraazacyclotetradecane) connected by an aliphatic or aromatic linker. AMD3465, unlike the bicyclam AMD3100, had a monomacrocyclic *N*-pyridinylmethylene cyclam structure. This structure has decreased molecular charge and demonstrates a 10-fold superior antagonistic effect on CXCR4 and still maintained similar potency against X4 HIV strains when compared to the bicyclam prototype.²²

As part of a broader effort to identify critical chemokine receptors participating in hypersensitivity-type granuloma formation, we investigated the effect of AMD3465 in models of pulmonary mycobacterial (type-1) and schistosomal (type-2) antigen-elicited granulomatous inflammation and concurrent lymphoid tissue cytokine responses.^{23,24} These responses are representative of two major forms of T-cell-mediated granulomatous inflammation mediated by Th1 and Th2 helper cells. Our findings indicate the AMD3465 impaired type-2 to a greater extent than type-1 inflammation, respectively. The effect was associated with a significant reduction in local eosinophil recruitment and abrogated Th2-related cytokine production by *ex vivo* cultured draining lymph nodes. The CXCR4 antagonist profoundly reduced CXCR4 transcripts in lungs with type-2 lesions. The antagonist did not affect transcript levels of CXCL12 or unrelated chemokines and chemokine receptors. The observed biased effect was possibly related to the greater induction of ligand, CXCL12, in the lungs and lymph nodes during the type-2 response. Surprisingly, despite reducing local type-2 granulomatous inflammation, AMD3465 did not reduce local cytokine transcript levels, suggesting that local effector T-cell recruitment was not compromised. However, the effect on draining lymph nodes was profound, suggesting a regional effect on Th2 effector cell re-expansion, possibly by interrupting migratory events in lymph node microenvironments. These findings suggest that CXCR4 antagonism may prove highly effective in the treatment of established Th2 cell-mediated inflammatory conditions by abrogating both local inflammation and subsequent T-cell expansion.

Materials and Methods

Animals

Female CBA/J mice were obtained from Jackson Laboratories, Bar Harbor, ME. All mice were maintained under specific pathogen-free conditions and provided food and water *ad libitum*. For this study all mice were used at 6 to 8 weeks of age.

Elicitation of Primary and Secondary Type-1 or Type-2 Immune Responses

Secondary type-1 and type-2 lung antigen bead granulomas were generated as previously described.²³ Briefly, mice were sensitized with either a subcutaneous injection of 20 μ g of *M. bovis* purified protein derivative (PPD) (Department of Agriculture, Veterinary Division, Ames, IA) incorporated into 0.25 ml of CFA (Sigma-Aldrich, St. Louis, MO), or by an intraperitoneal injection of 3000 *S. mansoni* eggs in 0.5 ml of phosphate-buffered saline (PBS). After 15 days, mice were challenged by tail vein with 6000 Sepharose 4B beads covalently coupled to either PPD or soluble schistosome egg antigens (SEA). The SEA was prepared from isolated schistosome eggs as previously described.²⁵ Primary responses were elicited in naïve mice by intravenous injection of either 6000 PPD beads or 6000 SEA beads. Mice were then sacrificed 4 days after bead challenge.

Delivery of AMD3465

AMD3465 was dissolved in PBS and delivered using peritoneally implanted osmotic pumps as follows. Sensitized or naïve mice were subjected to isoflurane anesthesia, the abdomen was shaved and cleaned with 70% alcohol and providine. A 0.8-cm incision was made in the skin and peritoneum through which was implanted an osmotic pump (Alza Corp., Palo Alto, CA) containing AMD3465 to deliver at a constant rate. A dose response study included pump rates of 1 μ g/hour (1.2 mg/kg/day), 5 μ g/hour (6 mg/kg/day), and 25 μ g/hour (30 mg/kg/day) delivered throughout the 5-day study period. Control pumps contained PBS vehicle. Wounds were closed with two stainless steel surgical staples. After a 1-day rest, the mice were challenged intravenously with antigen-coated beads. Four days after challenge, lungs and lymph nodes were excised and cultures prepared as described below. In all experiments, portions of lungs were saved for transcript analysis, granuloma isolation, and fixed in 10% buffered formalin for morphometric analysis. Draining mediastinal lymph nodes were collected for culture.

Lymph Node Culture

After perfusion via the right heart ventricle with cold RPMI 1640, lungs excluding trachea and major bronchi and mediastinal lymph nodes were excised. Draining mediastinal lymph nodes were excised and teased into a single-cell suspension. The cells were washed by centrifugation and cultured in RPMI 1640 plus 10% fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin at 5×10^6 cells/ml in the presence or absence of 5.0 μ g/ml PPD or SEA. Cells were cultured in 35-mm dishes for 24 hours in a 37°C incubator with 5% CO₂ humidified atmosphere. Supernatants were collected by centrifugation and cytokine production measured by EIA.

Cytokine Measurement

Murine interleukin (IL)-2, -4, -5, -10, and -13, and interferon- γ were measured from cultured lymph node supernatants by EIA using commercially available reagents and standards (BD Pharmingen, San Diego, CA; R&D Systems, Minneapolis, MN; PeproTech, Rocky Hill, NJ). Sensitivities were between 15 to 50 pg/ml.

Morphometry

Individual excised lung lobes were inflated and fixed with 10% buffered formalin for morphometric analysis. Granuloma area was measured in a blinded manner in hematoxylin and eosin-stained sections of paraffin-embedded lungs using computer-assisted morphometry. A minimum of 20 lesions was measured from each lung. Only granulomas with full cross-sections of the bead nidus were measured.

Differential Analyses of Granuloma Cells

After perfusion via the right heart ventricle with cold RPMI 1640, lungs excluding trachea and major bronchi were excised. Perfused lung lobe samples were placed in 50 ml of RPMI 1640 and homogenized in a sterile Waring blender for 30 seconds. Intact granulomas were collected using a sterile number 100 stainless steel sieve and digested in RPMI-fetal bovine serum containing 1000 U/ml type IV collagenase for 25 minutes at 37°C. After washing three times, the dispersed granuloma cells were resuspended at 2.5×10^6 cells/ml in 1.0 ml of fetal bovine serum and then used for cytospin preparation (Thermo Electron Corp., Waltham, MA). A 200-cell differential analysis was performed on duplicate Wright-stained cytospin preparations.

Real-Time Reverse Transcriptase (RT)-Polymerase Chain Reaction (PCR) Analysis

Freshly excised lung tissue for RNA isolation was stored in RNA-Later (Ambion Inc., Austin, TX) at -20°C. Poly(A) pure mRNA was isolated from lung tissue using Poly(A) pure mRNA isolation kits (Ambion). DNA-free DNA removal kit (Ambion) was used to remove any contaminating genomic DNA. Each mRNA sample was reverse-transcribed in a 20- μ l reaction in a PCR tube using SuperScript II RNase H- reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA). Analysis of the transcripts was performed by real-time PCR using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). For this study, the comparative threshold cycle (CT) method recommended by the manufacturer was adopted. GAPDH gene expression was used as the endogenous reference. Primer-probe sets were purchased commercially (Applied Biosystems) with thermal cycling conditions programmed according to the manufacturer's instructions. Transcript levels were expressed as arbitrary units and calculated as previously described.²⁶ Briefly, arbitrary units were calculated from the fluorescent detection units. The original gene copy

number (Co) is related to fluorescence of the generated signal as follows: $Co = F \times E^{-1} \times I \times 2^{-n}$, where F is an arbitrary conversion constant, E^{-1} is amplification efficiency constant (~ 1 for manufacturer's real-time primers sets), I is the fluorescent intensity reading, and n is the amplification cycle number. Hence, the equation constitutes an arbitrary measure of original copy number that is directly related to the fluorescent product and inversely related to cycle number. Because E is approximately equivalent for the various primer sets, the expression levels among genes are comparable at orders of magnitude. For this study, we measured the mRNA expression levels of CXCR3, CXCR4, CCL2, CXCL12, IL-4, IL-5, and interferon- γ .

Statistics

The Student's t -test was used for comparison of AMD-treated and PBS control groups. Values of P greater than or equal to 0.05 were considered to indicate lack of statistical significance. Statistical analysis was performed for each chemokine and cytokine analysis. Statistically significant changes were generally observed when transcript levels were enhanced by twofold or more or decreased by more than 35%.

Results

AMD3465 Abrogates Type-2 Granuloma Formation and Eosinophil Mobilization

In a previous report, intraperitoneal dose regimens in the range of 1 to 10 mg/kg/day of AMD3100 caused significant effects in a murine asthma model.¹² Based on these precedents and using osmotic pumps so as to achieve steady-state release, we performed a dose response study using 1.2 mg/kg (1 μ g/hour), 6 mg/kg (5 μ g/hour), and 30 mg/kg (25 μ g/hour) of the second generation antagonist AMD3465. Treated animals showed no signs of toxicity. Figure 1 shows the effect of treatment on mycobacterial (type-1) and schistosomal (type-2) antigen-elicited granuloma formation. Although AMD3465 treatment did not significantly affect type-1 lesion size, type-2 lesion areas were reduced by 34% and 46% at the 6 and 30 mg/kg doses. In addition, the numbers of eosinophils in type-2 lesions appeared reduced.

Table 1 shows the differential cellular analysis of dispersed granulomas in the control and treated mice. Type-1 lesion cell populations showed no change at the lowest dose, but the neutrophil proportion was enhanced at the higher doses and at the 30 mg/kg dose there was a decrease in large mononuclear cells. In type-2 lesions, treated mice displayed a nearly 40% reduction in relative eosinophil content at the 6 and 30 mg/kg doses confirming the histological impression. In addition, large mononuclear cells were reduced at the highest dose similar to that observed with type-1 lesions, suggesting that at very high doses AMD3465 impaired monocyte as well as eosinophil recruitment. Treatment did not appear to reduce relative lymphocyte content.

Figure 1. Effect of AMD3465 on type-1 (PPD) and type-2 (SEA) antigen bead-elicited, anamnestic lung granuloma formation. Groups of PPD- and SEA-sensitized mice were implanted with osmotic pumps delivering vehicle or AMD3465 at 1 (1.2 mg/kg), 5 (6 mg/kg), or 25 (30 mg/kg) $\mu\text{g}/\text{hour}$. A day later, mice were challenged with respective antigen beads. Four days after challenge, lungs were harvested for histological evaluation. **A:** Day 4 granuloma cross-sectional area; Bars are means $\pm$ SEM. **Dashed line** indicates area occupied by the bead. Five to six mice per group. **Asterisks** indicate significant differences compared to vehicle control. **B:** Histological appearance of type-2 bead granulomas in control and treated mice shows reduction in eosinophils. H&E stain. Original magnifications, $\times 400$; $\times 1000$ (insets).

Effects of AMD3465 treatment on peripheral blood leukocyte populations were also assessed. In both models, treatment significantly increased circulating lymphocyte numbers at the highest (30 mg/kg) dose by nearly twofold (type-1, vehicle = $0.90 \pm 0.03 \times 10^3/\mu\text{L}$, AMD3465 = $1.65 \pm 0.21 \times 10^3/\mu\text{L}$; type 2, vehicle = $0.88 \pm 0.03 \times 10^3/\mu\text{L}$, AMD3465 = $1.42 \pm 0.02 \times 10^3/\mu\text{L}$). This effect may have been related to interference with lymphocyte recirculation. Significant changes in circulating monocytes and eosinophils could not be established. However, in the type-1 but not the type-2 model, we observed twofold and fourfold increases in circulating neutrophils at the 6 and 30 mg/kg doses, respectively (vehicle = $0.38 \pm 0.03 \times 10^3/\mu\text{L}$, AMD3465 (6 mg/kg) = $0.79 \pm 0.04 \times 10^3/\mu\text{L}$, AMD3465 (30 mg/kg) = $1.80 \pm 0.14 \times 10^3/\mu\text{L}$). This observation could explain the higher proportion of neutrophils recruited to PPD lesions at these doses. The finding would be consistent with the capacity of CXCR4 antagonists to promote mobilization of bone marrow-derived leukocytes especially when given in concert with GM-CSF. The greater effect in the type-1 model may be related to higher levels of colony-stimulating factors that we have observed during PPD bead challenge. In view of the notable distortion in leukocyte populations and potential nonspecific effects at the highest dose, subsequent studies were performed at the 6 mg/kg dose unless otherwise indicated.

AMD3465 Selectively Reduces CXCR4 Transcript Expression in Lungs with Secondary Type-2 Granuloma Formation

Using quantitative real-time RT-PCR transcript analysis, we measured levels of transcripts for CXCR4, its ligand CXCL12, as well as for unrelated receptors and chemokines. This analysis was performed on lungs from treated and untreated mice with secondary type-1 or type-2 granuloma formation. As shown in Figure 2, AMD3465 treatment caused an $\sim 90\%$ reduction in CXCR4 transcript levels in lungs with type-2 lesions. There was a trend to reduced transcripts in type-1 lesions, but this did not achieve statistical significance. Transcripts for the CXCR4 ligand CXCL12 were likewise not significantly affected; however, it was noted that levels were nearly twofold higher in lungs with type-2 compared to type-1 lesions. The latter showed no induction of transcripts over constitutive levels in unchallenged lungs (Figure 2, dashed line). Transcripts for the CXCR3 chemokine receptor and the CC chemokine CCL2 were unaffected by the treatment, indicating a specific effect of AMD3465 on CXCR4. This effect would be consistent with impaired recruitment of CXCR4⁺ cells such as eosinophils, which are known to express and up-regulate CXCR4 during inflammation.^{27,28}

Because the effect of AMD3465 might have been attributable to impaired Th2 effector cell recruitment,³ we

Table 1. Effect of AMD3465 Treatment on the Cellular Composition of Secondary Type-1 (PPD) and Type-2 (SEA) Pulmonary Antigen-Bead Granulomas

Group	AMD3465 dose (mg/kg)	Lymphocytes (%)	Large mononuclears (%)	Eosinophils (%)	Neutrophils (%)
Type-1 (PPD)	0 mg/kg	35.1 $\pm$ 8.8	48.1 $\pm$ 6.4	1.2 $\pm$ 1.0	15.6 $\pm$ 4.4
Type-1 (PPD)	1.2 mg/kg	42.3 $\pm$ 7.0	41.7 $\pm$ 3.1	1.8 $\pm$ 0.5	14.0 $\pm$ 4.0
Type-1 (PPD)	6.0 mg/kg	31.1 $\pm$ 11.2	40.8 $\pm$ 13.1	1.0 $\pm$ 1.4	26.6 $\pm$ 2.2*
Type-1 (PPD)	30.0 mg/kg	37.9 $\pm$ 3.8	31.9 $\pm$ 2.2*	1.1 $\pm$ 1.3	29.0 $\pm$ 6.0*
Type-2 (SEA)	0 mg/kg	36.3 $\pm$ 5.4	31.2 $\pm$ 6.0	21.2 $\pm$ 2.4	11.2 $\pm$ 6.6
Type-2 (SEA)	1.2 mg/kg	39.7 $\pm$ 7.5	33.7 $\pm$ 2.5	18.7 $\pm$ 9.0	8.7 $\pm$ 3.8
Type-2 (SEA)	6.0 mg/kg	41.8 $\pm$ 4.6	36.1 $\pm$ 7.1	13.0 $\pm$ 4.5*	8.8 $\pm$ 3.9
Type-2 (SEA)	30.0 mg/kg	52.3 $\pm$ 9.9	19.7 $\pm$ 1.5*	13.0 $\pm$ 9.0*	15.3 $\pm$ 13.7

* $P < 0.05$, values are means $\pm$ SD.

Figure 2. Effect of AMD3465 on chemokine and chemokine receptor transcript expression in lungs with type-1 (PPD) and type-2 (SEA) antigen bead-elicited, anamnestic granuloma formation. Groups of PPD- and SEA-sensitized mice were implanted with osmotic pumps delivering vehicle or AMD3465 at 5 μ g/hour (6 mg/kg). A day later, mice were challenged with respective antigen beads. Four days after challenge, lungs were harvested for transcript analysis. Relative transcript levels are expressed as arbitrary units (AU) as described in Materials and Methods. Bars are means $\pm$ SD. Five to eight mice per group. Dashed lines indicate chemokine transcript levels in unchallenged lungs.

likewise measured transcripts for the Th2-related cytokines IL-4 and IL-5. As expected, these cytokines showed biased expression in the type-2 response but were unaffected by the CXCR4 antagonist treatment (Figure 3). This finding suggested that local recruitment of effector cytokine-producing cells was not significantly impaired and would be consistent with our finding of unimpaired lymphocyte mobilization to granulomatous lungs.

AMD3465 Abrogates Th2 Cytokine Production by ex Vivo Cultured Draining Lymph Nodes of Mice with Secondary Type-2 Granuloma Formation

We next examined the effect of AMD3465 treatment on events in draining lymph nodes. Antigen bead challenge of sensitized mice normally initiates an anamnestic memory T-cell activation and expansion in draining lymph nodes. Four days after bead challenge, draining mediastinal lymph nodes of treated and control mice were excised, dispersed, and cultured in the presence of specific antigen. Because *ex vivo* cultures were free of antagonist, any changes would reflect effects that had occurred during *in vivo* treatment. Figure 4 shows the levels of cytokines in 24-hour cultures. Control, PBS-treated, type-1 and type-2 cultures, respectively, displayed Th1- and Th2-dominant cytokine profiles, indicat-

ing that pump implantation did not disrupt the expected response. In the type-1 model, AMD3465-treated mice displayed no statistically significant effect on cytokine levels. In contrast, treatment impaired Th2-associated cytokines in the type-2 model, reducing IL-5, IL-10, and IL-13, by 60 to 70%. However, IL-2 and IL-4 were not significantly affected, suggesting that these cytokines were more resistant to treatment and possibly derived from different cells. To assess dose-dependent effects, we similarly measured key cytokines at other treatment doses. As shown in Figure 5, even at 30 mg/kg, interferon- γ levels in type-1 cultures were unaffected while comparable reductions of IL-5 and IL-13 occurred at the 6 and 30 mg/kg doses in the type-2 model. In addition, significant impairment of IL-4 occurred at the highest dose. These results indicated that even throughout a broad dose range the type-1 response was primarily resistant to AMD3465 treatment and correlated well with our histological observations.

One possibility for the greater effect of AMD3465 on the type-2 lymph node response was differential induction of the ligand, CXCL12, as we observed in the lungs with type-2 (SEA) granuloma formation. To test this possibility, we sequentially harvested draining lymph nodes from mice with type-1 and type-2 secondary granuloma formation throughout an 8-day period and directly measured CXCL12 transcript levels using quantitative RT-PCR analysis. As shown in Figure 6, CXCL12 transcript induction was notably greater during the SEA response,

Figure 3. Effect of AMD3465 on Th2-associated cytokine transcript expression in lungs with type-1 (PPD) and type-2 (SEA) antigen bead-elicited, anamnestic granuloma formation. Groups of PPD- and SEA-sensitized mice were implanted with osmotic pumps delivering vehicle or AMD3465 at 5 μ g/hour (6 mg/kg). A day later, mice were challenged with respective antigen beads. Four days after challenge, lungs were harvested for transcript analysis. Relative transcript levels are expressed as arbitrary units (AU) as described in Materials and Methods. Bars are means $\pm$ SD. Five to eight mice per group.

reaching sixfold greater than baseline constitutive levels of naïve, unchallenged mice by day 4. This finding suggested that CXCL12-CXCR4 interactions might participate to a greater degree during an established Th2-dominant secondary lymph node response.

AMD3465 Abrogates IL-2 Production by ex Vivo Cultured Draining Lymph Nodes during Primary Type-1 and Type-2 Antigen Bead Challenge but Fails to Affect Innate Granuloma Formation

The above studies indicated that AMD3465 could temper Th2 granuloma formation in the lung as well as influence anamnestic activation events in draining lymph nodes. Inasmuch as CXCR4 is expressed by mature dendritic cells^{29,30} and CXCL12 has been implicated in T-cell costimulation,³¹ we hypothesized that CXCR4 antagonism might interfere with antigen presentation events in lymphoid tissues. To explore this possibility, we tested the effect of AMD3465 treatment on the primary response to PPD and SEA bead challenge. Naïve mice were implanted with osmotic pumps then challenged with antigen beads 1 day later. As shown in Figure 7, beads elicited local innate granuloma formation that was unaffected by antagonist treatment. In contrast, when drain-

Figure 4. Effect of AMD3465 on cytokine production by cultured draining lymph nodes from mice with type-1 (PPD) and type-2 (SEA) antigen bead-elicited, anamnestic granuloma formation. Groups of PPD- and SEA-sensitized mice were implanted with osmotic pumps delivering vehicle or AMD3465 at 5 μ g/hour (6 mg/kg). A day later, mice were challenged with respective antigen beads. Four days after challenge draining lymph nodes were harvested, dispersed, and cultured in the presence of respective PPD and SEA antigens. Cytokine levels were measured by standard EIA. Bars are means $\pm$ SD. Five to eight mice per group. Asterisks indicate significant differences compared to vehicle control.

ing lymph nodes were assessed for cytokine production, the treatment reduced IL-2 levels by 70 to 80% with either PPD or SEA bead challenge (Figure 8). Other cytokines measured were not affected or at levels too low to detect differences. The specific effect on IL-2 production might indicate interference with antigen presentation events by dendritic cell subpopulations, but this phenomenon will require further investigation. In any case, the findings indicate that CXCR4 antagonism disrupts both PPD and SEA antigen-elicited T-cell activation events during a primary challenge

Discussion

This study compared the effect of a second generation, highly specific CXCR4 antagonist, AMD3465, on well-defined models of Th1 (type-1) and Th2 (type-2) cell-mediated granuloma formation.^{23,24,32} Our findings indicate that the type-2 schistosomal, but not the type-1 mycobacterial, antigen-elicited secondary response, was abrogated by systemic treatment suggesting that chronic allergic-type inflammation might be more sensitive to CXCR4 blockade than Th1 cell-mediated responses. This effect correlated with greater overall expression of the CXCR4 ligand, CXCL12, in challenged lungs and lymph nodes during the type-2 response. Our findings are in good agreement with the study of Lukacs and col-

Figure 5. Dose response effect of AMD3465 on Th1- and Th2-associated cytokine production by cultured draining lymph nodes from mice with type-1 (PPD) and type-2 (SEA) antigen bead-elicited, anamnestic granuloma formation. Groups of PPD- and SEA-sensitized mice were implanted with osmotic pumps delivering vehicle or AMD3465 at 1, 5, or 25 μ g/hour. A day later mice were challenged with respective antigen beads. Four days after challenge draining lymph nodes were harvested, dispersed, and cultured in the presence of respective PPD and SEA antigens. Cytokine levels were measured by standard EIA. Bars are means $\pm$ SD. Three to six mice per group. Asterisks indicate significant differences compared to vehicle control.

leagues,¹² demonstrating amelioration of cockroach antigen-elicited allergic airway responses in mice treated with AMD3100. Like that study, we observed abrogated eosinophil recruitment; however, unlike that study we were unable to demonstrate a clear deficit in Th2 cytokine production in the lungs. This difference might be related to different effects of mucosal challenge with soluble

antigen versus an interstitial challenge with immobilized antigen. Interstitial challenge elicits a more intense innate response with a greater variety of chemokines that could potentially ligate additional T-cell chemokine receptors. Although a local reduction in Th2 cytokine production was not shown, we did demonstrate a profound reduction of Th2-related cytokines in the draining lymph node cul-

Figure 6. Relative and temporal induction of CXCL12 transcripts in draining lymph nodes of mice with type-1 (PPD) and type-2 (SEA) anamnestic lung granuloma formation. Before (day 0) and after challenge, draining lymph nodes were sequentially harvested and analyzed for CXCL12 transcript levels. Relative transcript levels are expressed as arbitrary units (AU) as described in Materials and Methods. Bars are means $\pm$ SD derived from five individual mice. Dashed line indicates levels in the nodes of naïve unchallenged mice.

Figure 7. Effect of AMD3465 on primary type-1 (PPD) and type-2 (SEA) antigen bead-elicited, lung granuloma formation. Naïve mice were implanted with osmotic pumps delivering vehicle or AMD3465 at 5 μ g/hour (6 mg/kg). A day later mice were challenged with PPD or SEA antigen beads. Four days after challenge lungs were harvested for histological analysis. Bars are day 4 granuloma cross-sectional area; means $\pm$ SEM. The primary response is less than the secondary (Figure 1) and eosinophils are not a significant component of primary type-2 lesions. Dashed line indicates area occupied by the bead. Five to six mice per group.

Figure 8. Effect of AMD3465 on cytokine production by cultured draining lymph nodes from mice with type-1 (PPD) and type-2 (SEA) antigen bead-elicited primary (innate) granuloma formation. Naive mice were implanted with osmotic pumps delivering vehicle or AMD3465 at 5 μ g/hour (6 mg/kg). A day later mice were challenged with PPD or SEA antigen beads. Four days after challenge draining lymph nodes were harvested, dispersed, and cultured in the presence of respective PPD and SEA antigens. Cytokine levels were measured by standard EIA. Bars are means $\pm$ SD. Five to six mice per group. Asterisks indicate significant differences compared to vehicle control.

tures indicating that AMD3465 impaired anamnestic T-cell activation in draining lymph nodes. This is an important observation because it suggests that CXCR4 might play a role in expansion of Th2 memory and that long-term treatment with AMD3465 could lead to eventual erosion of Th2 cell-mediated hypersensitivity.

In the lung, the abrogating effect of AMD3465 on Th2 cell-mediated inflammation manifested mainly as decreased eosinophil mobilization in SEA antigen bead granulomas and is consistent with the report of Nagase and colleagues²⁷ who showed down-regulation of CCR3 and enhancement of CXCR4 expression by eosinophils at sites of inflammation. Our observation of reduced CXCR4 transcript expression in granulomatous lungs after AMD3465 treatment would be consistent with impaired mobilization of CXCR4⁺ eosinophils. We previously demonstrated a role for the CCR3 ligand, CCL7, in eosinophil recruitment.³³ In the present study, we demonstrate a role for the CXCR4 ligand, CXCL12. Our findings would be also consistent with the two-stage model of eosinophil recruitment proposed by Nagase and colleagues²⁷ in which CCR3 agonists first localize eosinophils and then CXCR4 agonists mediate arrest in the tissue interstitium.

Our observation that AMD3465 impaired lymph node cytokine responses supports a role for CXCR4 in lymphoid tissue function. A number of studies have provided evidence for CXCR4 participation in T-cell, B-cell, and dendritic cell migration as well as T-cell co-stimulation and B-cell follicle organization in lymphoid tis-

sue.^{29,31,34–38} Thus, the impaired IL-2 production after primary challenge with PPD or SEA is likely the result of interference with naïve T-cell and/or dendritic cell mobilization. Unlike the primary response, the secondary memory response displayed a biased inhibitory effect of CXCR4 antagonism on the Th2-dominant lymphoid response, which correlated with biased expression of CXCL12 in the latter. This observation is intriguing because it implies that Th1 and Th2 memory responses in lymphoid tissue are subject to very different demands. In view of the greater role of Th2 cells in B-cell help and follicle formation, it is tempting to speculate that secondary antigen presentation might be linked to activation events in or near germinal center dark zones where CXCL12 (SDF-1) is reportedly at high concentration.³⁴ In contrast, Th1 effector-memory responses might depend more on deep paracortical lymph node or peripheral organ antigen presentation. There is some circumstantial evidence supporting this hypothesis. For example, in an autoimmune gastritis model Katakai and colleagues³⁹ noted that Th1 cells were preferentially located to the affected organ whereas Th2 were more dominant in draining lymph nodes. Clearly, more detailed Th1 and Th2 localization studies will be required to explore this notion.

In summary, we have demonstrated that administration of the reversible monocyclam CXCR4 antagonist AMD3465 causes biased abrogation of eosinophil-rich, Th2-mediated hypersensitivity-type inflammation. Moreover, the agent appeared to block anamnestic Th2 activation in draining lymph nodes suggesting that chronic treatment could ultimately degrade the Th2-cell-mediated hypersensitivity response even in face of chronic antigen challenge.

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