CRITICAL BUT DIVERGENT ROLES FOR CD62L AND CD44 IN DIRECTING BLOOD MONOCYTE TRAFFICKING IN VIVO DURING INFLAMMATION.

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ABSTRACT

Using non-invasive in vivo imaging and experimental autoimmune uveoretinitis as a model we show for the first time, that the mechanisms controlling blood monocyte recirculation through peripheral and lymphoid tissues alter during inflammation. The recirculation of monocytes in mice with ocular inflammation, but not controls was found to be dependent upon CD62-L and CD44. Not only was rolling efficiency ablated or markedly reduced in antibody treated mice, the majority of labelled monocytes disappeared from the circulation within seconds, anti-CD44 treated monocytes homing to the lymph nodes and anti-CD62-L treated monocytes homing to the spleen. Our data indicate that while PSGL-1 has a partial role in the transmigration of monocytes into the inflamed retina, CD62-L has a key role in regulating recruitment of monocytes to lymphoid tissue from the blood during inflammation and that CD44 is required to maintain CD62-L**ve inflammatory monocytes within the circulation during inflammation. This effect was systemic, as sequestered monocytes accumulated in mesenteric as well as draining cervical lymph nodes, and inflammation dependent, as depletion of circulating blood monocytes was much reduced or absent in normal mice and accumulations of adoptively transferred monocytes in the lymphoid tissues did not occur.
INTRODUCTION

Non-differentiated monocytes are known to circulate in the blood and tissues for up to three days, and during that time they are recruited to the tissues where they differentiate into macrophages or myeloid dendritic cells\(^1-3\). Subsets of blood monocytes with differential migratory potential have been identified, and the capacity of monocytes to preferentially migrate to sites of inflammation has been linked to expression of the selectin CD62-ligand (CD62L), CC-chemokine receptor-2 (CCR-2) and CD14, whilst subsets that are CCR-2\(^{-}\) and CD16\(^{+}\) are recruited to tissues independently of inflammatory stimuli to become resident monocyte macrophages or myeloid DC \(^4\). More recently, we have shown that differentiation and recruitment of the inflammatory subset is independent of local inflammatory stimuli, requiring monocyte conditioning or differentiation over time within the circulation. Bone marrow derived monocytes (BM-Mo) transferred i.v. to mice with established ocular inflammation, required 24-48h \(\text{in vivo}\) conditioning before being able to roll on endothelium efficiently and migrate into the inflamed retina. This capacity to roll and migrate was largely lost after 72h in the circulation. In the retina, adoptively transferred, \(\text{in vivo}\) conditioned monocytes differentiated into CD11c\(^{+}\), B220\(^{+}\) DC, and F4/80\(^{+}\) macrophages, indicating that a permissive endothelium alone, is not sufficient for active recruitment of monocytes from the blood \(^3\).

Understanding the processes involved in mononuclear myeloid cell trafficking has considerable importance both for the targeting of antigen pulsed DC used as vaccines and for the control of inflammatory diseases \(^5,6\). Recruitment of mononuclear myeloid cells from the blood to the tissue and from the tissue to lymph nodes is controlled by adhesive interactions between the cell and the vascular or lymphatic endothelium. The mechanisms involved in recruitment of leukocyte subsets from the blood to the tissues and lymphoid organs have been extensively studied, primarily in \(\text{in vitro}\) model systems under defined molecular conditions. Multiple molecules, constitutively expressed or induced under inflammatory conditions, have been identified as playing a role in monocyte adhesion and diapedesis \(^7,8\). The multi-step paradigm that has emerged invokes weak interactions via selectins that allow leukocyte rolling on vascular endothelium that initiates an
adhesion cascade. This is triggered by chemokines and results in firm adhesion and spreading of the monocyte on the endothelium through regulation of avidity of $\beta_1$-integrins and $\beta_2$-integrins \cite{9,10}. Fast rolling is mediated by CD62-L \cite{11} and is regulated by vessel wall shear stress \cite{12}. Rolling through E- or P-selectin is slower, but is also shear stress dependent when rolling on P-selectin glycoprotein ligand-1 (PSGL-1) \cite{13}. This dependence on shear stress is believed to limit leukocyte interactions in the centre of vessels or in vessels with very high or very low wall shear stress, and the steady rolling generated enables the leukocyte to receive crucial activating stimuli from the endothelium. Some overlapping and redundancy appears to exist in the system \cite{14}, and mechanisms controlling monocyte trafficking to a Th1-type inflammatory lesion under physiological conditions of flow in vivo are not known.

Using experimental autoimmune uveoretinitis (EAU) as a model inflammation, we have been able to demonstrate mechanisms controlling specific T lymphocyte subset rolling, sticking and trans-endothelial cell migration within normal and diseased neurovascular post-capillary endothelial venules where shear stress levels are relatively high \cite{15-17}. A reduction in shear stress in retinal veins from about 30 dyn/cm$^2$ to 20 dyn/cm occurs up to 24h prior to leukocyte infiltration, and rolling and sticking efficiencies are negatively correlated with wall shear stress, providing a good model for studying leukocyte subset trafficking in vivo during inflammation. In this study we focus on the kinetics of monocyte trafficking in vivo, and in particular on the role of CD44 which is implicated in primary adhesive interactions between leukocytes and endothelium \cite{18} and has a major role in leukocyte homing in EAU \cite{19}, as well as CD-62L and PSGL-1 which have been shown to account for at least 90% of leukocyte rolling in vivo \cite{20}. In addition to mediating monocyte infiltration of the inflammatory site, we show for the first time, that CD62-L has a key role in regulating recruitment of monocytes to lymphoid tissue from the blood during inflammation and that CD44 is also required to maintain CD62-L$^+$ inflammatory monocytes within the circulation during inflammation. Unexpectedly, this effect was systemic as distant as well as draining lymph nodes...
were involved and inflammation specific, as no sequestration of the adoptively transferred, *in vivo* conditioned monocytes to lymphoid tissues was observed in normal mice.

**MATERIALS AND METHODS**

**Animals and retinal inflammation model.** Eight-12 week old wild type C57BL/6 mice and homozygous C57BL/6 mice expressing EGFP under the control of a chicken β-actin promoter and cytomegalovirus enhancer were maintained in the Medical Research Facility at Aberdeen University. Experimental autoimmune uveoretinitis (EAU) was induced in wild type C57BL/6 mice as described 21. Retinal inflammation occurred at day 16-18 pi and peaked at day 21-28 pi. All procedures were approved by Home Office Regulations for Animal Experimentation, UK.

**In vivo monocyte trafficking using scanning laser ophthalmoscopy (SLO).**

Lymphocyte depleted bone marrow monocyte (BM-Mo) were prepared from EGFP+ bone marrow cells as described 3. In vivo monocyte trafficking was studied using our SLO technique as described 22,23. This non-surgical technique minimises any leukocyte trafficking artifacts. The retinal vasculature is imaged through the intact cornea, and adoptively transferred leukocytes may be tracked as they enter via the retinal artery, traverse the capillary network and exit via the retinal vein. Mice are unharmed by the procedure, and can be re-scanned over several days if necessary. Briefly, mice were anaesthetized with an intramuscular injection of 0.4 ml/kg Hypnorm (Janssen-Cilag Ltd. Belgium) and 1 ml/kg Diazepam (Phoenix Pharmaceuticals Ltd. Gloucester, UK) intraperitoneally. Eight million (8 × 10⁶) EGFP+ BM Mo cells in 150 µl PBS were injected via the tail vein. After 48h to allow *in vivo* conditioning to an inflammatory phenotype, SLO images were recorded simultaneously on videotape (S-VHS) and digitally at 25 frames per second. For each eye, three regions of interest containing one to three veins/venules were recorded for at least 30 minutes. Video analysis was carried out off-line as described 17,24. This protocol excludes any neutrophils present in the EGFP+ BM-Mo transfers as neutrophils released from the bone marrow die rapidly.
and turn-over time within the circulation is normally no more than 24h. Rolling leukocytes and those not interacting with the endothelium were counted in each venule. Rolling cells were defined as those cells with a velocity below the critical velocity. The rolling efficiency was calculated as the percentage of rolling fluorescent cells among the total number of fluorescent cells entering a venule. The sticking efficiency was determined as the percentage of labelled monocytes that remained adherent for at least 20s. In antibody blocking experiments, baseline measurements were recorded for up to 15min before i.v injection of 30µg isotype control Ig (rat IgG) or 30µg anti-mouse mAbs CD44 (rat IgG2b IM7)\textsuperscript{25}, CD162 PSGL-1 (rat IgG 1 2PH1)\textsuperscript{26}, CD11a, LFA-1 (rat IgG2a M17/4)\textsuperscript{27}; CD62L, L-selectin (Rat IgG MEL-14)\textsuperscript{28} all from BD Biosciences.

**Ex-vivo tracking of monocytes in retina and lymphoid tissue.**

To test the effect of mAb treatment on monocyte infiltration of the EAU retina and other tissues, groups of 6 immunised mice (21-24 days pi) were injected i.v. with 8 x 10\textsuperscript{6} EGFP BM cells and then treated for 3 days with 30µg/mouse/day mAb or control rat anti-mouse IgG. These concentrations have been optimised in previous studies and had no effect on the recirculation of monocytes in control groups (data not shown)\textsuperscript{29}. To label retinal vessels, 50 µl of 2% Evans blue (Sigma) was injected via the tail vein and allowed to bind for 5-10 minutes before asphyxia with CO\textsubscript{2}. Tissues were then harvested and fixed in 2% (w/v) paraformaldehyde (Agar Scientific Ltd, Cambridge, UK). Retinal whole mounts were prepared as described elsewhere\textsuperscript{19}. Other tissues were cryo-embedded in OCT compound and frozen sections prepared. Both retinal whole mounts and tissue sections were observed using a confocal scanning laser microscope (LSM510 META, Carl Zeiss Ltd, Gottingen, Germany). For tissue sections both 488 nm and 543 nm wavelengths were used to distinguish between auto-fluorescence and EGFP\textsuperscript{+} cells. Three sections were obtained from each tissue and three images were taken randomly from each using ×20 objective lens. Images were analysed using Image Pro Plus system (Media Cybernetics, MD, USA), and data expressed as means and SEM of the number of EGFP\textsuperscript{+} cells per mm\textsuperscript{2}. Unfixed, frozen tissue sections were
labelled with Mel-14, IM7 or anti-eGFP antibody ab290 (rabbit polyclonal; AbCam, Cambridge, UK) using the APAAP technique as described.\textsuperscript{21}

**Flow cytometry.**

Single cell suspensions were blocked with 1% normal rat serum and immuno-stained with CD44 (IM7), CD162 PSGL-1 (2PH1); CD62L, L-selectin (MEL-14), CD11b (M1/70), CD11c (HL3), CD11a (M17/4), B220 (RA3-6B2), or isotype control IgG (BD Biosciences, Coley, Oxford, UK), or F4/80 (CI:A3-1, Serotec, Kidlington, Oxford, UK). Samples were analysed by LSR flow cytometry (BD Biosciences). Antibodies were conjugated to FITC, PE, APC, PerCP, PerCP-Cy5.5 or biotin as required. Biotin- labelled antibodies were detected by addition of SA-APC or SA-PE (1:400, BD Biosciences). Negative isotype controls, and single positive controls were performed to allow accurate breakthrough compensation. Gates and instrument settings were set according to forward and side scatter characteristics and populations gated to exclude dead or clumped cells. Data was collected from at least 3 individual animals in each group and expressed as means and SEM and compared using unpaired student $t$ test.

**RESULTS**

**In vivo kinetics of monocyte recirculation through the inflamed EAU retina and effect of blocking mAb to CD62-L, PSGL-1, CD44 and LFA-1.**

Figure 1 shows the kinetics of monocyte recirculation measured by SLO imaging of inflamed eyes. Data was recorded from mice with EAU 48h post monocyte transfer and the effects of mAb treatment on recirculation, rolling efficiency and sticking efficiency was measured. Analysis of SLO images showed that the normal rate of EGFP$^+$ monocyte re-circulation through inflamed vessels was 11.9 ± 5.6 cells per min. The rolling efficiency of these monocytes on the endothelium varied considerably, depending on the extent of inflammation (as assessed by vessel leakage of low concentration fluorescein dye co-injected with cells), but the average rolling efficiency (i.e. % of
transferred cells passing through vessel that rolled on endothelium) was 37.8 ± 15.7, similar to our previous observations in this model. The average sticking efficiency of monocytes adhering to endothelium was 8.7 ± 6.6. LFA-1 is essential for T cell trafficking but has been shown not to be involved in monocyte adhesion and diapedesis \textit{in vitro} we therefore included CD11α blocking mAb M17/4 as an additional irrelevant mAb control group for these studies.

After baseline measurements had been recorded, the role of specific adhesion molecules was tested by infusing blocking mAb i.v. and imaging continued for a further 15-20m. CD62-L and CD44, the hyaluronan (HA) receptor, are two major adhesion molecules on monocytes, and treatment with 30µg/mouse of anti-CD62-L mAb MEL-14 or anti-CD44 mAb IM7 had an immediate and dramatic effect \(p<0.01\) on the numbers of circulating monocytes passing through the retinal vessels. CD44 mAb removed virtually all cells from the circulation and rolling and sticking efficiencies were effectively reduced to nil. In some CD62-L mAb treated mice a few EGFP+ cells continued to circulate (5.8 ± 1.9 cells/min), but with significantly reduced rolling efficiency (5.1 ± 7.9\% \(p<0.01\)) and sticking efficiency \(p<0.01\). In contrast, rat IgG isotype control Ab or LFA-1 mAb had no significant effect on EGFP+ monocyte numbers in the circulation, or on their rolling and sticking efficiency within the inflamed vessels (Fig. 1).

CD62-P (P-selection) is upregulated on retinal venules, the principal sites of leukocyte adhesion and diapedesis in EAU, at the time of BRB breakdown and leukocyte infiltration of the retina. We therefore also examined the effect of blocking PSGL-1, a ligand for both P-and E-selectin, which has been shown to mediate monocyte/platelet aggregations, secondary tethering and integrin activation (Figure 1). Treatment with PSGL-1 mAb had no significant effect on circulating EGFP+ cell numbers, but rolling efficiency was significantly reduced \(p<0.05\). In this group PSGL-1 mAb also had an overall significant effect on sticking efficiency \(p<0.05\), but this was an “all or none” effect. This could reflect a threshold effect indicating that in some mice PSGL-1-independent molecular receptors were involved, possibly linked to haemodynamic
parameters (less severe disease and higher shear stress within the vessel) and/or tyrosine sulphation of the L-selectin ligand \(^{31}\).

In groups where mAb treatment did not deplete circulating EGFP\(^+\) monocytes, rolling velocity was measured on inflamed endothelium (Fig. 2). The rolling velocity was recorded for 15 minutes before infusion of 30\(\mu\)g of PSGL-1 mAb or, LFA-1 mAb. Recording in the same vessels was continued for another 20 minutes. Average rolling velocity of EGFP\(^+\) monocytes in untreated or LFA-1 mAb treated inflamed vessels was 124.8 ± 16.3 \(\mu\)m/s, and this was significantly increased in PSGL-1 treated mice (231.4 ± 38.4 \(\mu\)m/s; \(p<0.01\)) (Fig 2A), consistent with dominant PSGL-1 dependent interaction between monocytes and endothelium with residual, possibly CD62L dependent, faster rolling interactions occurring when PSGL-1 N-terminus is blocked \(^{32,33}\). Our results are also consistent with previous \textit{in vitro} observations of significantly faster CD62-L dependent rolling of neutrophils when compared with P- or E-selectin dependent rolling \(^{11}\). These experiments were carried out under high shear stress (20-30 dyn/cm\(^2\)) equivalent to that found in EAU venules \(^{17}\), however, the velocity of PSGL-1 blocked monocyte rolling observed in the inflamed retinal venules here (231.4 ± 38.4 \(\mu\)m/sec) is approximately twice the velocity of CD62-L dependent neutrophil rolling observed \textit{in vitro} by Puri. \(^{11}\)

**Circulating monocyte depletion by CD62-L mAb MEL-14 and CD44 mAb IM7 is inflammation dependent**

Inflammatory processes impose activating phenotypes on both endothelium and leukocyte, so to identify antibody-dependent clearance mechanisms as a reason for the sudden loss of circulating EGFP\(^+\) monocytes after treatment with MEL-14 and IM7 mAbs, we examined the effect of these antibodies on the recirculation of monocytes through the retina in normal mice in comparison to EAU mice. Figure 3A&B shows that adoptively transferred EGFP monocytes re-circulated through the normal retina at approximately the same rate (25.7 ± 2.7 cells/min) as in inflamed EAU retinas (Fig.1). This value was not reduced after infusion of CD62-L mAb (24.8 ± 2.23 cells/min),
however, in CD44 mAb treated normal mice a significant reduction in re-circulating monocytes was observed 2-5 min after infusion (from 24.2 ± 2 to 16.4 ± 2.1 cells/min; \( p < 0.05 \)). Infusion of mAb can temporarily sequester target cells within tissues such as the lung, and when SLO measurements of re-circulating cells were recorded over time, a sudden dip in EGFP monocytes passing through the retina was seen in both controls and EAU mice 2m after mAb infusion (Fig. 3, C&D). This was a temporary effect in normal mice, as the rate of recirculating EGFP monocytes increased back to control levels after 12 minutes. In EAU mice the numbers of re-circulating monocytes in both CD62-L and CD44 mAb treated EAU mice continued to fall, and remained profoundly reduced compared to control (\( p < 0.01 \) in both groups), showing that the depletion of circulating monocytes observed in EAU mice was inflammation specific.

**Differential trafficking of monocytes to lymphoid tissues in EAU, but not control mice treated with CD62-L mAb MEL-14 or CD44 mAb IM7.**

To determine the fate of adoptively transferred monocytes, organs and tissues of groups of recipient EAU and normal control mice, were examined using flow cytometry and microscopy 30m and 24h after a single infusion of mAb, and at 72h after 3 infusions of mAb. Figure 4A-C shows that 30m post-infusion with MEL-14 anti-CD62L antibody, adoptively transferred monocytes were found dispersed throughout the venous sinuses of the spleen, but were restricted to the subcapsular sinuses of lymph nodes, whereas in IM7 anti-CD44 antibody treated mice, monocytes had entered the node and were present within the medullary sinus and in clusters around vessels. In the spleen, eGFP\(^{+}\) monocytes were also found throughout the venous sinuses and red pulp, and notably in large numbers clustered around and in the marginal zones of the lymphoid follicles (Fig 4C). This rapid accumulation of transferred monocytes within the lymphoid tissues, particularly in IM7 treated mice provides an explanation for the sudden loss of circulating fluorescent cells in the retina observed by SLO (Figs 1 and 3). After 24h these relatively large accumulations of eGFP expressing cells were no longer evident, only a few scattered cells being detectable using the APAAP.
technique. Using flow cytometry, we detected a drop from 88 ± 2.5% to 55 ± 18% in CD11b+ve monocytes in the blood of anti-CD44 mAb treated group at 30m post-infusion as predicted from SLO data (Figs 1 and 3), but this was not reflected in statistically significant increases or decreases of CD11b or eGFP+veCD11b monocytes in the spleen, (IgG control, 0.08± 0.04 %; CD62L, 0.07± 0.04%; CD44 0.04± 0.005%).

Using more sensitive detection technique of confocal microscopy to follow fate of eGFP+ve cells in the tissues we were able to show significant accumulations of adoptively transferred monocytes in lymphoid tissues of mice with EAU after 3 days treatment with CD62-L or CD44 mAb compared with control IgG, LFA-1 or PSGL-1 mAbs (Fig 4 D,E and Fig 5). Sectioning of lymph nodes, spleen, and other tissues revealed that in EAU mice treated with CD62-L mAb, EFGP+ cells had preferentially homed to the spleen (72.6 ± 13 cells/mm², p<0.01), with significantly reduced numbers being found in the cervical lymph nodes (c-LN: 2.8 ± 1.2 /mm², p<0.01) and mesenteric lymph nodes (m-LN: 5.6 ± 1.32, p<0.05) compared to IgG controls. Conversely, in mice treated with CD44 mAb IM7, EFGP+ cells preferentially homed to the lymph nodes (c-LN: 55.9 ± 17.3 cells/mm², p<0.05; m-LN 60.7 ± 16.2 cells/mm², p<0.01), with reduced numbers found in the spleen (17.5 ± 1.7 cells/mm², p<0.05) compared to IgG controls. In normal mice, (Fig. 5B) treatment with blocking mAb actually reduced numbers of monocytes in the lymphoid tissues. These observations are consistent with a role for CD62-L and CD44 in directing circulating monocytes to other non-lymphoid tissues such as bone marrow in the absence of inflammation 34. Very few cells were found in the lungs or livers in any of the groups indicating that cellular clearance via the reticulo-endothelial system was not a significant event with any of the mAbs tested.

**Effect of mAb treatment over 72h on monocyte number, phenotype and migration into inflamed retina.**
Monocytes as well as retinal antigen specific T cells are required for full expression of EAU, and mechanisms for monocyte migration into peripheral inflamed tissues rather than lymphoid tissues are likely to differ\textsuperscript{14}. We therefore examined the effect of blocking mAb on the migration of adoptively transferred monocytes into inflamed retina. Mice with EAU were treated with 30µg Ig/mouse/day for three days after infusion of donor EGFP\textsuperscript* monocytes. After 72h, blood samples were taken for FACS analysis, the mice killed and retinal whole mounts prepared for confocal microscopy. Figure 6A shows that 72h after injection, small numbers of transferred EGFP\textsuperscript* cells continued to circulate despite significant sequestration of cells in the lymphoid tissues of EAU mice treated with CD62-L or CD44 mAbs. EGFP\textsuperscript* monocyte numbers in the blood of CD62-L and CD44 mAb treated mice were depleted compared to control Ig treated mice, but this was significant only for the CD44 mAb treated group (p<0.01). Treatment with CD62-L, CD44 and PSGL-1 mAbs also significantly reduced the numbers of infiltrating EGFP\textsuperscript* monocytes in the retinas of EAU mice compared to isotype and LFA-1 mAb treated control groups (p<0.01), confirming that CD11a also has no role in inflammatory monocyte trafficking \textit{in vivo} (Fig. 6B).

Anti-CD44 treatment with mAb such as IM7 blocks HA binding function and has been shown to be effective in blocking T cell traffic and ameliorating inflammation in a number of animal models including EAU\textsuperscript{19}, however given the importance of soluble (shed) as well as membrane bound CD44 in regulating cell function, inhibition of leukocyte recirculation and infiltration of the inflammatory site may involve more than simple blocking of HA function. Other mechanisms proposed include regulation of function through activation and sulphation of the adhesion molecule or changes in cell surface expression through down regulation or shedding. Previous studies have indicated T cell trafficking is controlled by surface expression of CD62-L and CD44. High surface expression of CD62-L, characteristic of naïve T cells, is lost during inflammation as CD44 is upregulated. Equally, CD62-L is less important in inflammatory T cell recruitment than in lymph-node homing. We therefore analysed the expression of CD62-L, CD44, PSGL-1 and LFA-1 on circulating EGFP monocytes recovered from the circulation of EAU mice.
(Fig. 6C) and normal control mice (Fig. 6D) after 3 days mAb treatment. CD44 expression (Geo-MFI) was very much lower on monocytes from all groups of EAU mice compared with normal mouse groups suggesting that monocyte transmembrane CD44 was lost during inflammation. Treatment with the mAbs had relatively minor effects on surface expression of adhesion molecules examined. Statistical analysis showed some significant differences. Treatment with CD62-L mAb reduced CD44 expression and treatment with PSGL-1 mAb increased LFA-1 expression on monocytes from EAU but not control mice. However, whether these changes were sufficient to exert physiological effects is in doubt as Geo-MFI changes did not mirror in vivo functional changes observed in earlier experiments.

DISCUSSION

Differential trafficking is known to be required for localisation of effector leukocyte subsets, either to sites of inflammation or to lymphoid tissues, and tissue specific chemokine expression and receptor signalling has been identified as providing specificity to endothelial traffic signals, raising the concept of molecular codes specific for particular disease processes.\(^5\) Previously we have shown that a permissive endothelium alone is not sufficient to recruit monocytes from the circulation\(^3\), but data we present here supports the concept of differential monocyte trafficking in vivo in steady state and inflammation, with specific roles identified for CD62-L and CD44 in maintaining monocytes within the circulation during inflammation. Blockade of CD62-L caused retention of monocytes within the spleen whilst blockade of CD44 caused retention of monocytes within the lymph nodes. Surprisingly, this effect was inflammation specific as blockade of CD62-L and CD44 had no apparent effect on the ability of monocytes to recirculate in normal mice. These observations are also consistent with a role for CD62-L and CD44 in directing circulating monocytes to other non-lymphoid tissues such as bone marrow in the absence of inflammation\(^3^4\). Identifying signals controlling monocyte differentiation and function in inflammation versus steady
state will be important for identifying disease associated therapeutic targets that do not affect normal protective immunity.

For monocytes, two major subsets have been identified with differing chemokine and adhesion molecule expression that govern trafficking potential to inflammatory sites. Recently we showed that acquisition of the inflammatory monocyte phenotype is a time limited property of monocytes independent of external inflammatory signals. Bone marrow derived monocytes adoptively transferred into normal mice re-circulated freely for at least 72h and viable monocytes could be found in both lymph nodes and spleen throughout the sampling period. When injected into mice with EAU, adoptively transferred monocytes did not migrate into the inflamed retinal tissue until 24-48h after transfer, despite repeated trafficking through inflamed retinal vessels. This time frame coincided with acquisition of maximum rolling efficiency and maximal expression of CCR2 and LFA-1 by monocytes. These data indicate that an inflamed endothelium and associated chemokine and cytokine microenvironment is not sufficient to recruit circulating monocytes, and new monocytes leaving the bone marrow require in vivo conditioning over a defined time period before switching to a phenotype that enables homing to inflammatory sites. In the experiments described here we have included a 48h in vivo conditioning period to allow inflammatory monocyte maturation/differentiation before taking SLO measurements or administering mAb treatments to examine mechanisms controlling monocyte recirculation and homing to an inflammatory site in vivo.

The main physiological function of selectins is to allow high affinity rolling interactions between leukocytes and the endothelium under flow, mediating signal transduction and firm adhesion. For these experiments we examined the role of CD62-L as it is expressed by most monocytes and has been implicated in mediating inflammatory monocyte migration. PSGL-1 was also chosen as the interaction between P/E selectin and PSGL-1 induces changes in integrin function and increased adhesion of monocytes to endothelium in vitro, but to date there is no record of its role in mediating monocyte function in DTH inflammation in vivo. CD44 was also
included in this study as both CD44 and its ligand hyaluronan are upregulated in the EAU eye\textsuperscript{16}, and although implicated in lymphocyte function in both in the uveitic eye and other DTH models, no specific role for CD44 in monocyte trafficking has been identified. Antibodies to CD11a were included to confirm that LFA-1 has no role in monocyte adhesion and diapedesis \textit{in vivo} in our model, and provided a useful additional negative control for our assays. VLA-4 has been implicated in monocyte trafficking in other models, but inclusion of this integrin was considered beyond the scope of this study as VLA-4 has been shown not to be involved in monocyte attachment under flow\textsuperscript{36}, and is not specifically up-regulated in retinal venules in EAU\textsuperscript{16}.

Our experiments show that while PSGL-1 has a partial role in regulating monocyte migration into the inflamed retina, CD62-L and CD44 were absolutely required for effective monocyte trafficking to the retina in EAU. The accumulation of monocytes in the spleens of CD62-L mAb treated EAU but not control mice is unexplained. Immuno-staining of lymph nodes and spleens from normal and EAU mice showed no obvious differences in expression of CD44 or CD62L (data not shown). This is perhaps not surprising as in this organ specific inflammatory model, the focus of inflammation is limited to the retina of the eye. The restriction of eGFP$^+$ve cells to the subcapsular region of the lymph nodes in MEL-14 treated mice is consistent with a defect in the ability of these cells to traverse HEV, particularly during inflammation. In contrast, the spleen has no HEV so in MEL-14 treated animals monocytes were able traffic freely through this tissue.

MEL-14 mAb at high concentrations (100\textmu g per mouse) has been reported to affect cell trafficking, particularly of lymphocytes\textsuperscript{29} but a dose of 30\textmu g per mouse per day appeared to have no significant effect on monocyte trafficking in our control mice. Sialomucin (CD43) as well as CD62-L is involved in monocyte migration into lymph nodes from the blood via high endothelial venules (HEV)\textsuperscript{14} and our data would indicate that CD62-L is not necessary, or largely redundant in monocyte trafficking in the normal mouse, consistent with data from L-selectin-deficient mice\textsuperscript{37}. The inflammation specific nature of sequestering would imply that monocyte expressed CD62-L or its endothelial cell expressed ligands have undergone modification in response to the EAU
inflammatory response in the host allowing the adhesive interactions necessary for monocyte adhesion and migration\textsuperscript{35}. Our previous observation that rolling is an acquired characteristic of adoptively transferred CD62-L\textsuperscript{+} monocytes in mice with EAU, and that inflamed endothelium alone does not permit monocyte rolling\textsuperscript{3} would suggest that it is monocyte-expressed ligand changes as well as endothelial-cell expressed ligand changes that are critical for rolling. Flow cytometric analysis of CD62-L expression on monocytes retrieved from the circulation of normal and untreated EAU mice showed no significant differences in CD62-L expression (percentage and geometric MFI; data not shown), indicating that factors other than receptor density may determine L-selectin dependent monocyte rolling.

CD44 deficient mice also show no obvious immunological defects until challenged with infection or inflammation\textsuperscript{38}, and posttranslational modification of CD44 can alter function at different stages of disease\textsuperscript{18}. Our data is therefore consistent with previous studies that have suggested that CD44 is not important for normal T cell trafficking to lymph nodes, and that physiological changes associated with inflammation alter hierarchy of adhesion molecule function to allow selective recruitment of T cells to lymphoid or inflammatory sites, and is the first indication that CD44 also has a specific role in monocyte trafficking during inflammation. While distribution of monocytes in the MEL-14 treated mice can be rationalised as an effect on MEL-14 dependent trafficking at the HEV. The reduced numbers of eGFP\textsuperscript{++} cells in the spleens is more difficult to understand. CD44 clearly has a role in trafficking, particularly during inflammation, and monocytes were able to enter the spleen rapidly after adoptive transfer in IM7 treated mice. The localisation of large numbers of these cells both with the red pup and around the marginal zone at 30 m post transfer and later loss in inflammation could have two explanations: i) the cells were targeted for clearance; ii) the cells became sequestered in another compartment (such as the bone marrow). We believe the second explanation is more likely as IM7 is widely used as a blocking antibody in functional studies, cells were not found in the lungs or liver in any number and the simple clearance of antibody opsonised cells would be independent of inflammation.
In conclusion, we demonstrate for the first time that the mechanisms controlling monocyte recirculation through peripheral and lymphoid tissues via the blood alters during inflammation, and we also show that the effect is systemic. Differential trafficking is known to be required for the selective recruitment of naïve or effector T cells either to sites of inflammation or to lymphoid tissues. Our data suggests that differential trafficking of monocytes also occurs during inflammation, and that regulation of ligand density or post-translational modification of CD44 and CD62-L may be involved. Thus post-translational modification of CD44 is required to maintain monocytes within the circulation during inflammation and mediate homing to the inflammatory site, and CD62-L may be less important during inflammation but necessary for recruitment to the lymph node. PSGL-1 was also shown to be involved in monocyte rolling and recruitment to an inflammatory site. The SLO data also supports our earlier hypothesis that monocytes must undergo maturation and differentiation in the circulation before they acquire the ability to roll on inflamed endothelium and that changes in monocyte receptor function are crucial events enabling inflammatory monocytes to traffic to the site of inflammation. To date there is little information on monocyte trafficking in vivo during inflammation. This study highlights the dynamic relationship between CD44 and CD62-L in controlling monocyte trafficking in vivo. Understanding the mechanisms controlling differential trafficking of immature and inflammatory monocytes as well as naïve and effector T cells will be required for effective control chronic inflammation in the future.

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JL directed the research and wrote the paper. AM operated the SLO and HX carried out the SLO analysis, prepared the figures and contributed to the manuscript. IC assisted with experimental design and contributed to the manuscript. RD prepared and scored the tissue sections and provided technical support.

The authors have no conflicting financial interests.
FIGURE LEGENDS

Figure 1. Effect of monoclonal antibodies on monocyte trafficking in retinal vessels in EAU.
Blocking antibodies to CD62L, CD44 and PSGL-1 significantly reduced rolling and sticking of monocytes in inflamed vessels. Freshly isolated eGFP bone marrow derived monocytes (8x10^6) were injected i.v. into mice immunised 21-24 days previously with peptide to induce EAU. After 48h, cell trafficking in the retinal vasculature was analysed by SLO. Retinal images were recorded for 15m and then mice were injected i.v. with 30µg/mouse of rat anti-mouse antibody and recording continued for a further 20m. Data was then compared before and after antibody treatment. (A) Recirculation of adoptively transferred cells was expressed as the number of transferred eGFP monocytes detected in the same section retinal vessel before and after antibody infusion. (B) Rolling efficiency, expressed as the percentage of rolling fluorescent cells among the total number of fluorescent cells entering a venule before and after antibody infusion. (C) Sticking efficiency, expressed as the percentage of fluorescent monocytes within the same venule that remained adherent for at least 20s. *, p<0.05; **, p<0.01; Student’s paired t test; n>16 vessels from 3 mice.

Figure 2. Monocyte rolling in inflamed retinal vessels and is increased by blocking antibody to PSGL-1, but not LFA-1.
Freshly isolated eGFP bone marrow derived monocytes (8x10^6) (A&B) were injected i.v. into mice immunised 21-24 days previously with peptide to induce EAU. After 48h, cell trafficking in the retinal vasculature was analysed by SLO. Retinal images were recorded for 15m and then mice were injected i.v. with 30µg/mouse of (A) rat anti-mouse antibody to PSGL-1, or (B) LFA-1 and recording continued for a further 20m. Rolling velocity of transferred eGFP expressing monocytes or T cells expressed as µm/sec was calculated as described in materials and methods and for monocytes, data compared before and after antibody treatment. *, p<0.05; **, p<0.01; Student’s paired t test; n>36 randomly chosen rolling cells in venules of 3 mice.
Figure 3. Depletion of circulating EGFP monocytes in the retinal vasculature by blocking mAb CD62L and CD44 is inflammation dependent.

Freshly isolated eGFP bone marrow derived monocytes (8x10^6) were injected i.v. into control mice or mice immunised 21-24 days previously with peptide to induce EAU. After 48h, cell trafficking in the retinal vasculature was analysed by SLO. Retinal images were recorded for 10m. Mice were then injected i.v. with 30µg/mouse of rat anti-mouse antibody and recording continued for a further 20m. Cells per minutes passing through the same section of retinal vessel were compared before and after antibody treatment in control mice (A&B). C & D Re-circulation of adoptively transferred cells was analysed at 2 minute intervals (expressed as the number of transferred eGFP monocytes detected in the same section retinal vessel) of control and EAU mice before and after antibody infusion. *, p<0.05; **, p<0.01; Student’s paired t test; n>16 vessels from 3 mice in each group.

Figure 4. Localisation of adoptively transferred monocytes in lymph node and spleen differs with antibody treatment.

Freshly isolated eGFP bone marrow derived monocytes (8x10^6) were injected i.v. into control mice or mice immunised 21-24 days previously with peptide to induce EAU., Mice were then treated with mAb for up to 3 days. Groups of mice were sacrificed at 30min and 24h after a single treatment or after 3 treatments. Tissue samples were then snap frozen, cryosectioned and immunostained using the APPAP technique (A-D) for the presence of eGFP+ cells using a specific antibody, or examined by confocal microscopy (E & F). LN, lymph node; Sp, spleen). Original magnification x400 (A-D).
Figure 5. Adoptively transferred monocytes in anti-CD62L treated mice home to the spleen, and in anti-CD44 treated mice monocytes home to the lymph nodes in EAU, but not control mice.

Effect of monoclonal antibody treatment on trafficking of adoptively transferred monocytes into secondary lymphoid and other tissues in control and EAU mice by confocal microscopy. Freshly isolated eGFP bone marrow derived monocytes (8x10^6) were injected i.v. into mice immunised 21-24 days previously with peptide to induce EAU (A), or control mice (B). Mice were then injected i.v. with 30µg of control IgG or blocking antibody per mouse per day for 3 days. Tissue samples were then snap frozen, cryosectioned and numbers of eGFP+ cells present in the tissues enumerated (A) and (B). c-Node, cervical LN; m-Node, mesenteric LN. *, p<0.05; **, p<0.01; Student’s paired t test; n>12 randomly chosen x 20 fields of view in tissue sections from 3 mice per group.

Figure 6. Blocking antibodies to CD62L, CD44 and PSGL-1 but not LFA-1 significantly reduce numbers of adoptively transferred monocytes entering inflamed retina.

Freshly isolated eGFP bone marrow derived monocytes (8x10^6) were injected i.v. into mice immunised 21-24 days previously with peptide to induce EAU. Mice were injected i.v. with 30µg of control IgG or blocking antibody per mouse for 3 days. Numbers of circulating cells remaining in the blood were analysed by flow cytometry (A), Transferred cells that had infiltrated the retinas were then counted by confocal microscopy of retinal wholemounts (B), **, p<0.01 compared with IgG control; student’s t test; n=12. Density of expression of adhesion molecules analysed in both EAU (C) and control mice (D) after mAb treatment expressed as Geometric mean fluorescent index.(Geo-MFI). *, p<0.05; **, p<0.01 compared with IgG control; student’s t test; n=3.
References


(31) Kanamori A, Kojima N, Uchimura K et al. Distinct sulfation requirements of selectins disclosed using cells that support rolling mediated by all three selectins under shear flow. L-


Critical but divergent roles for CD62L and CD44 in directing blood monocyte trafficking in vivo during inflammation

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