Tissue Distribution and Receptor-Mediated Clearance of anti-CD11a Antibody in Mice

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Non-Standard Abbreviations: Lymphocyte function associated antigen-1, LFA-1; Peripheral blood mononuclear cell, PBMC; Whole body autoradiography, WBA; Mean fluorescent intensity, MFI.
ABSTRACT

Efalizumab (Raptiva™) is a humanized monoclonal antibody specific for CD11a, the α-chain component of the lymphocyte function-associated antigen-1. In humans, the rate of efalizumab elimination from serum was related to the level of CD11a cell surface expression. These data suggested a role for the CD11a receptor itself in efalizumab clearance. Recently, we conducted a series of in vitro studies that suggested a role for CD11a-expressing T-cells in efalizumab clearance as mediated by cellular internalization and lysosome-mediated degradation (Coffey et al, 2004). To further study the mechanism of anti-CD11a clearance in vivo, we assessed the tissue distribution, cellular internalization, and subcellular localization of a rat anti-mouse CD11a monoclonal antibody in various tissues in mice. Anti-CD11a antibody primarily distributed to leukocytes and macrophages in the peripheral blood, spleen, and liver, with uptake in the lymph nodes and bone marrow following 72 h. At least a portion of the antibody was internalized and cleared by PBMC’s, lymphocytes, and splenocytes in a time-dependent manner in vivo. Internalized antibody co-stained with LysoTracker Red, suggesting that it was transported to lysosomes for degradation. Together, these data suggest that one clearance mechanism for anti-CD11a antibody in vivo is via receptor-mediated internalization and lysosomal degradation by CD11a expressing cells and tissues.
Psoriasis is an autoimmune disease characterized by the presentation of skin lesions resulting from epidermal thickening and hyperkeratosis (Linden and Weinstein, 1999). These lesions are highly vascularized and extensively infiltrated with inflammatory components (Greaves and Weinstein, 1995; Ortonne, 1996; Linden and Weinstein, 1999). Among these components are activated T-lymphocytes, which are found to play a significant role in the pathogenesis of psoriasis (Krueger et al., 1995; Gottlieb et al., 1995). T-lymphocyte infiltration to psoriatic lesions requires physical interaction with endothelial cells. This interaction is mediated in part through binding of the T-cell surface molecule LFA-1 with the endothelial cell surface protein ICAM-1 (Springer, 1990; Makagiansar et al., 2002). LFA-1 is a heterodimeric protein composed of CD11a and CD18. The anti-human CD11a-specific monoclonal antibody efalizumab (Raptiva™) is approved as a therapy for moderate to severe psoriasis on the basis of its ability to inhibit LFA1/ICAM1 interactions, and hence T-cell infiltration to psoriatic plaques (Werther et al., 1996; Bauer et al., 1999; Krueger et al., 2000; Gottlieb et al., 2000; Gottlieb et al., 2002; Lebwohl et al., 2003; Gordon et al., 2003). One mechanism behind the inhibition of LFA1/ICAM1 interaction appears to be the efalizumab-induced down modulation of circulating and psoriatic plaque lymphocyte cell surface CD11a (Bauer et al., 1999; Gottlieb et al., 2000).

In human psoriasis patients, the relationship between CD11a cell surface expression and serum levels of efalizumab was described by a CD11a-mediated clearance model for efalizumab (Bauer et al., 1999). In these studies, patients who received a dose of 0.3mg/kg efalizumab intravenously cleared the antibody from serum at an average rate of 322ml/day. This average rate of efalizumab clearance was reduced to 11ml/day in patients receiving 3mg/kg intravenously. Similar observations were made by Gottlieb et al (2000) who found that efalizumab saturated CD11a binding sites in human psoriasis patients when given intravenously.
at doses greater than 0.3mg/kg. Sub-saturating doses equal to or lower than 0.3mg/kg did not fully down modulate CD11a cell surface expression. These sub-saturating doses were also cleared at a faster rate than saturating doses (Bauer et al., 1999; Gottlieb et al., 2000). Further, when the efalizumab serum concentration following a saturating dose was reduced to 3 µg/ml or less, CD11a cell surface expression returned to normal and the clearance of efalizumab increased (Bauer et al., 1999; Gottlieb et al., 2000). These studies suggested a saturable, receptor-mediated clearance model for efalizumab.

This model of receptor-mediated clearance of efalizumab was tested in vitro using both efalizumab and human blood T-cells, as well as using the mouse surrogate antibody muM17 and T-cells purified from mouse lymph node (Coffey et al., 2004). Using each of these systems, we found that anti-CD11a antibody was internalized by T-cells, transported to lysosomes, and cleared from within the cells in a time-dependent manner. Total and intracellular clearance of efalizumab was inhibited with the inclusion of concanamycin A, an H+-ATPase inhibitor that attenuates lysosomal function, suggesting an important role for lysosomes in the intracellular clearance of anti-CD11a antibody. Results from these studies suggested that CD11a receptor-mediated internalization and lysosomal degradation of efalizumab may constitute one pathway for the in vivo clearance of this antibody.

The goal of the present study was to assess the in vivo distribution and receptor-mediated clearance of anti-CD11a antibody using M17, a rat anti-mouse CD11a monoclonal antibody, and muM17 (a murinized form of M17) in mice following intravenous administration. We report here the tissue distribution, cellular internalization, and subcellular localization of anti-CD11a antibody in mice. Further, we measured the relative capabilities of different PBMC and
splenocyte subtypes to bind and clear anti-CD11a antibody in vivo. Results from these studies suggest that this anti-CD11a antibody rapidly and specifically binds to leukocytes and macrophages in the peripheral blood, spleen, and liver in mice, with later uptake by the lymph nodes and bone marrow 72 h post injection. These data also suggest that anti-CD11a is cleared from within these tissues in a time-dependent manner, consistent with the reported pharmacokinetics of efalizumab.
METHODS

**Whole Body Autoradiography.** In all studies, the NIH principles for research involving animals was strictly upheld. Male CD-1 mice (28-32g) received a single IV bolus dose of the test article via the tail vein. Mice in Group 1 received 25 µCi of $^{125}$I-M17. Mice in Group 2 were dosed with 25 µCi of $^{125}$I-M17 mixed with 20 mg/kg M17. The specific activity for groups 1 and 2 were 25 µCi/µg and 0.05 µCi/µg, respectively. There were 4 mice per group; 2 sacrificed at 1 h and 2 sacrificed at 72 h post antibody injection for the analysis of antibody blood cell association and tissue distribution by whole body autoradiography (WBA) over time. The number of animals used in this study is justified given the high reproducibility of M17 distribution in mice. The selection of time points was based on an earlier pharmacokinetic study. The 1 h time point was within the distribution phase, the 72 h time point was within the terminal phase (data not shown). To block thyroid uptake of free iodine, mice from both groups were treated with sodium iodide by subcutaneous injection of 0.1 ml of a 5% (V/V) solution at 48 h, 24 h, and 0.5 h pre-dosing of $^{125}$I-M17 (Nakajo et al., 1983).

Blood from Group 1 and Group 2 animals was collected into heparinized plasma separator tubes. The radioactivity of the blood was measured in a gamma counter. The tubes were then centrifuged and the plasma was removed. Radioactivity in the cell pellets and plasma was measured, and percent cell-associated $^{125}$I-M17 was calculated from this data. For the analysis of tissue distribution by WBA, sacrificed animals were flash frozen in a dry ice/ethanol bath. Sagittal sections (20 µm) were cut at multiple levels using a cryostat microtome (2250 Cryomicrotome, LKB). Sections were exposed to Kodak X-Omat AR film (Eastman Kodak, Rochester, NY) for 12 days for autoradiography. Quantification of WBA was
made by phosphorimaging using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Each of these sections was exposed to phosphorimaging plates together with $^{125}$I-microscale standards (Amersham Pharmacia Biotech, Piscataway, NY). Using the blood pool in the heart, radioactivity was determined in order to derive the tissue to blood ratio of radioactivity. Images were prepared for presentation using Adobe Photoshop version 7.0.1. (San Jose, CA).

**Microautoradiography, Immunohistochemistry, and Transmission Electron Microscopy.**

Selected organs and blood were taken from animals either 1 h or 24 h after dosing with 240 µCi $^{125}$I-M17 as described above. Red blood cells were lysed with an ammonium chloride solution (168 mM ammonium chloride, 10 mM potassium bicarbonate, 0.1 mM EDTA) and washed with phosphate-buffered saline prior to preparation for microautoradiography or electron microscopy. For microautoradiography, tissues were fixed in 10% neutral-buffered formalin, paraffin embedded, and sectioned at 5 µm, according to standard laboratory procedures. Sections were dipped in Kodak NT3 photographic emulsion (Eastman Kodak, Rochester, NY), exposed for 24 weeks, developed, and then counterstained with hematoxylin and eosin.

Macrophages were detected in tissue sections with a rat anti-mouse antibody (F480 Harlan/Serotec, Indianapolis, Indiana). Deparaffinized sections were digested with 0.4% pepsin at 37°C for 5 min, then blocked with 10% normal rabbit serum for 20 min. In addition, splenic marginal zone macrophages were stained with anti-mouse MOMA–1 (1:100) or rat IgG2a isotype control antibody. Sections were then incubated overnight at 4°C with either F480 (1:100) or rat IgG2b isotype control antibody (BD Biosciences Pharmingen, Franklin Lakes, NJ). Bound rat antibody was detected with biotinylated rabbit anti-rat antibody (Vector...
Laboratories, Burlingame, CA) using an ABC elite kit (Vector Laboratories, Burlingame, CA) and diaminobenzidine (Pierce, Rockford, IL) for substrate.

For electron microscopy, tissues were fixed using Carnovsky’s fixative (2.5% glutaraldehyde, 2% formaldehyde in cacodylate buffer) and processed for routine testing. Sections were mounted on coated grids and a monolayer of Ilford L4 photographic emulsion (Ilford Imaging USA Inc., Paramus, NJ) was applied to the grids and exposed. All images were prepared using Adobe Photoshop.

**Internalization and Intracellular Clearance of $^{125}$I-muM17.** Internalization and intracellular clearance of CD11a-specific antibody was assessed using muM17. muM17 is a chimeric antibody based on M17, and contains the M17 rat CD11a-specific variable region and mouse IgG constant regions to more closely resemble the fully humanized antibody efalizumab. Prior to experimental manipulation, twelve CD-1 mice (28-32g) received IP injections of 100 µl sodium iodide (200 mM) as described earlier. Mice were then divided into two groups. The first group of 9 mice was administered a single dose of 25 µCi $^{125}$I-muM17 (0.6 mg/kg) by tail-vein injection. Three mice from this group were sacrificed at each time point of 1, 6, and 24 h post antibody injection. The second group of 3 mice was similarly injected with 25 µCi $^{125}$I-muM17, but in the presence of twenty-fold excess non-radioactive muM17. One mouse from this group was sacrificed at each time point of 1, 6, and 24 h post antibody injection. At the conclusion of each time point, mice were delivered an IP injection of ketamine (75-80 mg/kg) and xylazine (7.5-15 mg/kg). Upon unresponsiveness, a cardiac stick was performed to remove an approximately 700 µl sample of blood. The blood was collected in a tube on ice containing heparin. Lymph nodes and spleen were also collected.
Single cell suspensions were generated from the lymph nodes and spleen using 0.44 µm cell-separation filters (Sigma: St. Louis, MO). Red blood cells were lysed in the blood and spleen cell suspensions using an ammonium chloride solution. Cells were divided into 2 aliquots, pelleted to remove supernatant, then re-suspended in either PBS, or a low pH acid wash (150 mM NaCl and HCl, pH=2.5 in nH2o) to remove external-bound muM17 before pelleting and re-suspending in 500 µl whole cell lyses buffer. Protein concentrations were determined using the Bradford method. muM17 internalization and intracellular clearance was assessed by SDS-PAGE (20 µg protein per condition) and phosphorimager analysis (Molecular Dynamics: Piscataway, NJ) of the radioactive protein. Images were prepared using Adobe Photoshop.

**Internalization and Subcellular Localization of muM17-488.** Seventeen CD-1 mice (28-32 g) were divided into 3 groups, the first group consisting of 2 mice that received no treatment. Whole blood collected from these mice was used to compensate the FACS Caliber. The second group consisted of 5 mice that received a tail vein injection of 3 mg/kg IgG-488 (1 mouse per time point). The third group consisted of 10 mice that received a tail vein injection of 3 mg/kg muM17-488 (2 mice per time point). At 1, 3, 6, 9, and 24 h post antibody injection, animals received an IP injection of ketamine (75-80 mg/kg) and xylazine (7.5-15 mg/kg). Upon unresponsiveness, approximately 700 µl whole blood was collected by cardiac stick, and the spleens were removed. A single cell suspension of the spleens was made as previously described. Animals were sacrificed by cervical dislocation while under anesthesia.

Whole blood and splentocytes from the muM17-488 treatment group collected at 3 h post-injection were reserved for wide field microscopy for determination of internalization and subcellular localization. A single cell suspension was prepared from the spleens using a 0.44 µm
Cells from the blood and spleen (approximately $10^6$ cells) were treated with red blood cell lyses buffer, washed, and then incubated with 5 µM DiI for 5 min at 37°C to label the cell membrane, or 50 nM LysoTracker Red (Molecular Probes; Eugene, OR) for 15 min at 37°C to label the lysosomes, and 100 nM Hoechst 33342 for 5 min at 37°C (Calbiochem; San Diego, CA) to label the nucleus. Labeled cells were applied to poly[L]lysine coated plates and assessed for muM17-488 internalization and subcellular localization using wide field microscopy. Images were prepared using Adobe Photoshop.

PBMC’s and splenocytes (0.5 x $10^6$ per condition) collected from each time point were incubated with antibody per the manufacturer’s recommendations. Briefly, cells were incubated with the antibodies for 30 min at room temperature, followed by the addition of 1 ml ammonium chloride solution for an additional 5 min at room temperature. The lysing reaction was terminated by washing twice with 2 ml wash buffer (HBSS with 0.1% sodium azide) and centrifugation to pellet the cells. Cells were re-suspended in 500 µl 2% formaldehyde in PBS and stored at 4°C until all samples were collected for FACS analysis.

The association of IgG-488 or muM17-488 with specific PBMC’s or splenocytes over time in vivo was made by labeling cells with fluorescent conjugated anti-CD3e, -CD4, and -CD8 to identify T-cell subsets; with anti-NK1.1 to identify natural killer cells; with anti-CD45R to identify B-cells; with anti-CD11b to identify monocytes; and with Ly-6G to identify neutrophils. The cell populations were separated using forward versus side scatter, and lymphocytes were identified and gated based on CD45 expression. Ten thousand events were acquired for each condition to determine the geometric mean fluorescent intensity (geo MFI). The FACS Caliber was compensated for a four-color analysis using the FITC, PE, PerCP-Cy5.5, and APC-
conjugated specific and non-specific IgG antibodies. The geo MFI data was imported to an Excel file for analysis.

muM17 and mouse IgG1 were conjugated to AlexaFluor-488 using the protocol supplied by the manufacturer (Molecular Probes: Eugene, OR). All other antibodies used for flow cytometric analysis were purchased from BD Biosciences Pharmingen (San Diego, CA). These included APC, PE, and FITC-conjugated IgG1κ (A19-3), PE-conjugated IgG2ακ (R35-95), PE-conjugated IgG2bκ (A95-1), PerCP Cy5.5-conjugated IgG2bκ (A95-1), APC-and PE-conjugated IgG1κ (145-2C11), PE-conjugated IgG2bκ (GK1.5), PE-conjugated anti-CD8α (53-6.7), PE-conjugated anti-CD11b (M1/70), PerCP Cy5.5-conjugated anti-CD45 (30-F11), PE-conjugated anti CD45R (RA3-6B2), PE-conjugated anti NK1.1 (PK136), and PE-conjugated anti-Ly-6G (RB6-8C5).
RESULTS

Blood Cell Association and Tissue Distribution of anti-CD11a Antibody. Following IV injection the majority of $^{125}$I-M17 in blood was associated with PBMC’s in mice from Group 1. At 1 h, 85% and 89% of the blood radioactivity was associated with PBMC’s. By 72 h, the percent of radioactivity associated with PBMC’s dropped to 64% and 68% in the $^{125}$I-M17 - treated mice. Mice in Group 2 that received $^{125}$I-M17 plus 20 mg/kg unlabeled M17 had substantially lower percentages of PBMC-associated radioactivity; 8% and 16% radioactivity associated with PBMC’s at 1 h, and 9% and 5% by 72 h. These data are consistent with the specific binding of $^{125}$I-M17, presumably to CD11a on blood leukocytes and platelets (Krensky et al., 1983; Nakajo et al., 1983; Strassmann et al., 1985), which was displaceable by the 20 mg/kg of unlabeled M17.

Representative autoradiograms of cryosectioned CD-1 mice are shown in Figs. 1 and 2, depicting sagittal sections from Group 1 mice at 1 h and 72 h, respectively. Quantification of radioactivity in the sagittal sections showed tissue to blood ratios of greater than unity in spleen (8.7 and 6.4) and liver (5.9 and 2.8) at both 1 h and 72 h, respectively, with reduction of radioactivity from both organs observed at 72 h (Fig. 3). Specific binding of $^{125}$I-M17 was also found in the bone marrow and lymph node at 72 h with tissue to blood ratios of 1.6 and 1.3, respectively (Fig. 3). The co-administration of 20 mg/kg of unlabeled M17 with $^{125}$I-M17 in Group 2 lowered the tissue to blood ratio of radioactivity in the spleen, liver, bone marrow, and lymph node to below 1 at both time points (Fig. 3). There was no evidence of specific uptake of radioactivity by any other organ using whole body autoradiography.
Tissue Distribution and Cellular Localization. Microautoradiography, immunohistochemistry, and electron microscopy of animals dosed with $^{125}$I-M17 demonstrated the association of $^{125}$I-M17 with cells known to express CD11a in the liver, spleen, and blood (Figs. 4-6, respectively) (Krensky et al., 1983; McCaffrey and Berridge, 1986; Avraham et al., 1993; Philippeaux et al., 1996; Jacobs et al., 1997). Since the identities of cells in light-level microautoradiography were obscured by the intensity and large number of silver grains, immunohistochemistry was performed with the anti-macrophage markers, F480 and MOMA-1 (specific for splenic marginal zone macrophages), on adjacent sections of tissue and the patterns were compared. $^{125}$I-M17 was associated with Kupffer cells in the liver (Fig. 4), as well as interstitial marginal zone and red pulp macrophages in the spleen (Fig. 5). CD11a has been previously identified on Kupffer cells (Jacobs et al., 1997) and on tissue macrophages (Kuypers and Roos, 1989).

Electron microscopy of blood taken at the 24 h time point showed $^{125}$I-M17 associated with leukocytes and platelets as anticipated (Fig. 6A), based on the known cellular distribution of the antigen (Krensky et al., 1983; Nakajo et al., 1983). $^{125}$I-M17 labeling of lymphocytes was also observed in the spleen (Fig. 6B), and liver (Fig. 6C).

Internalization and Intracellular Clearance of muM17 in vivo. Internalization and intracellular clearance of $^{125}$I-muM17 by PBMC’s, lymph nodes, and spleen was also assessed (Fig. 7). The left, center, and right panels represent PBMC, lymph nodes, and spleen, respectively, harvested at 1 h (lanes a and b), 6 h (lanes c and d), and 24 h (lanes e and f) post antibody injection. The inclusion of 10-fold excess non-radioactive muM17 in the intravenous injection abolished detectable binding to the cells (data not shown). The top row represents total...
\^{125}\text{-muM17} binding to cells and the bottom row represents internalized \^{125}\text{-muM17}. In both the PBMC and lymph nodes there appeared to be greater accumulation of intracellular \^{125}\text{-muM17} by 6 h relative to 1 h; in contrast to the spleen where we observed a marked reduction in the level of intracellular \^{125}\text{-muM17} by 6 h. Roughly 70\% of the antibody internalized by PBMC’s and splenocytes at 1 h was cleared from the cells by 24 h. These data suggest that muM17 undergoes an \textit{in vivo} cell-mediated internalization and intracellular clearance.

\textbf{Internalization and Subcellular Localization of muM17 \textit{in vivo}.} Wide field fluorescent microscopy was used to assess the \textit{in vivo} internalization and subcellular localization of muM17-488 by mouse PBMC’s and splenocytes (Fig. 8). The two panels in the left column of Fig. 8 represent internalization of muM17-488 by PBMC’s (top panel) and splenocytes (bottom panel). The 2 rows with panels denoted as a, b, and c represent co-staining of muM17-488 with the lysosome marker, LysoTracker Red, within PBMC’s (top series of panels) and splenocytes (bottom series of panels). muM17-488 internalization is represented in panels a. Staining with LysoTracker Red is identified within these cells in panels b. Panels c represent a merged image of panels a and b. These images suggest that muM17-488 is internalized within PBMC’s and splenocytes \textit{in vivo}, and that at least a portion of the internalized antibody is found within lysosomes.

\textbf{Flow Cytometric Evaluation of muM17 Cellular Clearance \textit{in vivo}.} The relative contribution of different PBMC and splenocyte cell populations to muM17-488 binding and cellular clearance over time was explored by FACS analysis. The control antibody IgG-488 did not appreciably bind to any cell populations over 24 h (data not shown). In the blood, between 40 and 50\% of muM17-488 bound to mouse CD3, CD4, and CD8 positive T-cell subsets at 1 h
was absent by 6 h. This was increased to an approximate 70% reduction of muM17-488 by T-cell subsets by 24 h (Figs. 9A and 9B). Similarly, approximately 60% of the total antibody bound to mouse blood B-cells at 1 h was absent from the cells following 24 h in vivo (Figs. 9A and 9B). These results are consistent with the rate of in vivo intracellular clearance of $^{125}\text{I}$-muM17 observed from mouse PBMC’s (Fig. 7), and suggest that at least a portion of the bound antibody observed by FACS is cleared via internalization and intracellular degradation over time.

Blood NK cells, monocytes, and neutrophils, on the other hand, demonstrated binding to muM17-488, but exhibited no clear evidence of clearance of the antibody over 24 h (Figs. 9A and 9B). At 1 h post antibody injection, the muM17-488 MFI associated with NK, monocyte, and granulocyte cell populations was greater than that of T-lymphocytes, which was greater than that of B-lymphocytes (Fig. 9A). Assuming only limited clearance of muM17-488 at this time point, these data suggest that the MFI of muM17-488 associated with these mouse blood cell types is a reflection of the level of cell surface CD11a expression, consistent with observations made in humans and pigs (Desroches et al., 1990; Alvarez et al., 2000).

muM17-488 binding and clearance from splenocyte CD3+, CD4+, and CD8+ T-cells, B-cells, monocytes, and neutrophils was also assessed (data not shown). The binding of muM17-488 to mouse splenocytes was dramatically reduced relative to that observed in the blood cell populations, presumably a consequence of the specific sequestration of muM17-488 by PBMC’s at this dose. Nevertheless, the specific binding of muM17-488 to splenocytes relative to IgG-488 was observed, most noticeably to spleen monocytes and neutrophils. The relative binding of muM17-488 to spleen cell subsets was similar to that observed in the blood. Further, as was observed in the blood, muM17-488 was not noticeably cleared by spleen monocytes or
neutrophils, but rather more so by the T- and B-cell subsets. Similar to observations made in the blood, roughly 50% of the antibody bound at 1 h was absent from splenocyte B- and T-lymphocytes over 24 h (data not shown).
DISCUSSION

The species specificity of efalizumab for human and chimpanzee CD11a limited an understanding of the distribution and clearance mechanisms for this antibody. To better understand the distribution and mechanism of clearance of an anti-CD11a antibody, we used M17, a rat anti-mouse CD11a MAb, as a surrogate and determined the in vivo tissue distribution of the antibody over 72 h. M17 bound specifically to PBMC’s, liver, and spleen within the first hour following intravenous injection. By 72 h, specific binding to lymph node and bone marrow cells was observed, as well as the disappearance of radioactive material from the blood, liver, and spleen. The specific displacement of $^{125}$I-M17 by unlabeled M17 antibody indicated that binding to cells in these compartments was specific and saturable. Only limited distribution of antibody to the kidney was observed, suggesting a hepatic more so than renal contribution to the clearance of this antibody, consistent with known routes of monoclonal IgG elimination (Fukumoto and Brandon, 1982; Henderson et al., 1982). Consistently, hepatic clearance of efalizumab appears to be greater than that observed with other monoclonal antibodies, perhaps due to the presence of CD11a on Kupffer cells.

Anti-CD11a antibody M17 was associated with Kupffer cells in the liver, as well as interstitial marginal zone macrophages and leukocytes in the liver and spleen. The antibody was also associated with all PBMC’s analyzed in the blood. Kupffer cells and tissue macrophages are known to express LFA-1 (Jacobs et al., 1997; Kuypers and Roos, 1989). The tissue and cellular distribution of anti-CD11a antibody in mice reported here is consistent with the known distribution of LFA-1 expression (Krensky et al., 1983; McCaffrey and Berridge, 1986; Avraham et al., 1993; Jacobs et al., 1997). Given the relative size of the liver and spleen it appears that
tissue-associated lymphocytes may support the clearance of anti-CD11a antibodies such as M17, muM17, and efalizumab.

Upon binding of anti-CD11a antibody to various CD11a-expressing cells, a portion of the antibody was internalized and cleared from within the cells in a time-dependent manner. Iodinated muM17 was internalized and cleared from within PBMC’s and splenocytes over a period of 24 h. Similarly, by wide field fluorescent microscopy we detected the internalization of CD11a-specific antibody across the plasma membrane of PMBC’s and splenocytes, confirming that the antibody was internalized by cells in vivo. By 3 h post antibody injection, the internalized antibody was localized to subcellular organelles that stained positive with LysoTracker red. When considered with the observation that a portion of anti-CD11a antibody is internalized and targeted to lysosomes in vitro and cleared over time by purified human and mouse T-cells in a lysosome-dependent manner (Coffey et al., 2004), these data suggest that a portion of the antibody is targeted for degradation by lysosomes in vivo as well. Hence, the saturable receptor-mediated clearance of efalizumab in psoriasis patients reported upon by Bauer et al (1999) and Gottlieb et al (2000) likely involves CD11a receptor-mediated cellular internalization and lysosomal degradation.

The mechanism of anti-CD11a antibody internalization is presumed to be mediated via binding to the CD11a receptor itself, followed by internalization of both the antibody and receptor through a process similar to receptor-mediated endocytosis (Wileman et al., 1985). Hence, all cell types that express the cell surface CD11a receptor may participate in binding and clearing of anti-CD11a antibody from the serum. In humans and porcine, all PBMC’s express cell surface CD11a, with NK cells, monocytes, granulocytes, and a subset of CD8+ T-cells.
expressing the highest levels, B-cells expressing the lowest levels, and CD4+ and a different subset of CD8+ T-cells expressing intermediate levels (Desroches et al., 1990; Alvarez et al., 2000). The relative contribution of these different blood cell types in mice to muM17-488 binding and cellular clearance was explored by FACS analysis.

By FACS analysis, monocyte, NK, and neutrophil subsets appeared to exhibit greater binding to the anti-CD11a antibody, while B-cells possessed the least capacity for binding muM17. Binding of muM17 to CD4, and CD8 positive T-cells was intermediate relative to the other cell types. Although all cell types assessed did bind muM17, only the B- and T-lymphocytes appeared to clear the antibody over the course of 24 h, suggesting a specific clearance mechanism. The differences in the ability of these cell types to clear anti-CD11a antibody may be related to cellular differences in CD11a expression and turnover rates, internalization of antibody-CD11a complexes, or the capacity to degrade these complexes. Hence, it appears that the cell type expressing the target antigen may ultimately determine whether the antibody is taken up and targeted for a degradation pathway or not. The exact mechanism and relevance for this difference in binding and clearance of anti-CD11a antibody by these cell subsets is presently unclear and warrants future investigation.

Although the FACS data alone (Fig 9) do not directly define the exact mechanism of clearance of anti-CD11a antibody observed by T- and B-lymphocytes, when taken into context with the studies presented here, as well as previous studies conducted in vitro (Coffey et al, 2004), the data do suggest that cellular internalization and lysosomal degradation play a role in the total clearance of anti-CD11a antibody. First, in vitro studies previously defined the specific uptake and lysosome-dependent intracellular clearance of anti-CD11a antibody by purified T-
cells (Coffey et al., 2004). Second, iodinated anti-CD11a antibody was internalized in vivo and cleared from within the cells in a time dependent manner (Fig 7). Third, by injecting fluorescent conjugated anti-CD11a antibody in mice, we observed the specific internalization of antibody by CD11a-expressing tissues followed by trafficking to the lysozome as evidenced by co-localization with LysoTracker Red (Fig 8). Hence, it appears that both in vitro and in vivo, a portion of anti-CD11a antibody is internalized and targeted to lysosomes for intracellular clearance by CD11a expressing tissues and cells. Although the contribution of this pathway to total anti-CD11a clearance remains undefined, our results provide evidence for an in vivo model of efalizumab clearance that involves receptor-mediated internalization and degradation.
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REFERENCES


FOOTNOTES

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LEGENDS FOR FIGURES

**Figure 1:** Sagittal Section from a CD-1 Mouse 1 Hour after IV Administration of $^{125}$I-M17.
Photograph (A) and Autoradiogram (B) depicting the tissue distribution of $^{125}$I-M17 1 h after i.v. injection by WBA. The lines identify the heart, liver, spleen, and kidney.

**Figure 2:** Sagittal Section from a CD-1 Mouse 72 Hours after IV Administration of $^{125}$I-M17.
Photograph (A) and Autoradiogram (B) depicting the tissue distribution of $^{125}$I-M17 72 h after i.v. administration of $^{125}$I-M17 by WBA. Arrows identify the heart, liver, spleen, and kidney.

**Figure 3:** Tissue to Blood Ratio of Radioactivity in Mice at 1 and 72 Hours post IV Administration of $^{125}$I-M17 (Group 1) or $^{125}$I-M17 plus 20 mg/kg M17 (Group 2). The dotted line represents a tissue to blood ratio of 1. Two animals per group were included in this assessment. Mean values on the y-axis are represented as tissue to blood ratio +/- standard deviation. Li = Liver, S = Spleen, B = Bone marrow, and Ln = Lymph node.

**Figure 4:** Anti-CD11a Binding to Kupffer Cells in Mouse Liver. Microautoradiography and immunohistochemistry with an anti-macrophage marker, F480, of a liver section from a CD-1 mouse 24 h after IV administration of $^{125}$I-M17. A: Prominent uptake of silver grains by Kupffer cells, with some non-cell associated signal in the blood vessel lumens. B: Kupffer cells identified by immunohistochemical staining. Black arrows identify positively stained cells.

**Figure 5:** Anti-CD11a Binding to Marginal Zone and Red Pulp Macrophages in Mouse Spleen. Microautoradiography and immunohistochemistry with an anti-macrophage marker MOMA-1 of
a spleen section from a CD-1 mouse 24 h after IV administration of $^{125}$I-M17. A: Predominate labeling in the marginal zone and red pulp, as confirmed in B, which shows macrophage staining with MOMA-1, an antibody specific for marginal zone macrophages. Black arrows identify positively stained cells.

**Figure 6:** Cellular Distribution of Anti-CD11a in Mouse Blood, Liver, and Spleen. Electron microscopic autoradiography of blood (A), liver (B), and spleen (C) from CD-1 mice 24 h after IV administration of $^{125}$I-M17. A: Labeling of granulocytes and platelets. B: Labeling of a resident lymphocyte in the liver. C: Labeling of a resident lymphocyte in the spleen. Black arrows identify positively stained cells.

**Figure 7:** muM17 is Internalized and Cleared by Mouse Cells *in vivo*. The left, center, and right panels represent internalization of muM17 by PBMC’s, lymph nodes (Ln), and spleen, respectively, at 1 h (lanes a and b), 6 h (lanes c and d), and 24 h (lanes e and f) following intravenous administration of the antibody. The top series of panels represent total cellular binding of $^{125}$I-muM17. The bottom series of panels represent internalized $^{125}$I-muM17. 2 animals were assessed per time point.

**Figure 8:** Internalization and Lysosomal Transport of muM17 *in vivo*. Wide field fluorescent microscopy of muM17-488 internalization and subcellular localization in blood (top row) and splenocytes (bottom row) *in vivo*. The first column on the left side shows internalization of muM17-488 *in vivo* by blood and splenocytes. The second series of 3 columns with panels a, b, and c represent co-staining of muM17 with lysosomes *in vivo*. a = internalized muM17-488
(green fluorescence), b = lysosomes stained red with LysoTracker red, c = merged images of panels a and b.

**Figure 9:** Flow cytometric analysis of muM17-488 clearance by mouse PBMC’s *in vivo* over time. Data represent muM17-488 binding to CD3+ T-cells (filled circles) CD4+ T-cells (gray squares), CD8+ T-cells (empty triangles), CD45R+ B-cells (X’s), NK1.1+ NK cells (filled diamonds), CD11b+ monocytes (empty circles), and Ly-6G+ neutrophils (filled triangles). A: Data are plotted as the average MFI +/- standard deviation (StDev) on the y-axis over time. B: Data are plotted as the average percent change in MFI +/- StDev over time. The x-axis represents the time points 1, 3, 6, 9, and 24 h when whole blood was collected and analyses performed post muM17-488 injection (N=2).
Figure 3

[Bar chart showing tissue:blood ratios for different groups at 1 hour and 72 hours.]
Figure 9

A

B