TISSUE DISTRIBUTION AND RECEPTOR-MEDIATED CLEARANCE OF ANTI-CD11A ANTIBODY IN MICE

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ABSTRACT:
Efalizumab (Raptiva) is a humanized monoclonal antibody specific for CD11a, the ζ-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 after 72 h. At least a portion of the antibody was internalized and cleared by peripheral blood mononuclear cells, lymphocytes, and splenocytes in a time-dependent manner in vivo. Internalized antibody was costained 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 (Gottlieb et al., 1995; Krueger 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 LFA-1/ICAM-1 interactions and, hence, T cell infiltration to psoriatic plaques (Werther et al., 1996; Bauer et al., 1999; Gottlieb et al., 2000; Krueger et al., 2000; Gordon et al., 2003; Lebwohl et al., 2003). One mechanism behind the inhibition of LFA-1/ICAM-1 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 0.3 mg/kg efalizumab intravenously cleared the antibody from serum at an average rate of 322 ml/day. This average rate of efalizumab clearance was reduced to 11 ml/day in patients receiving 3 mg/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 0.3 mg/kg. Subsaturating doses equal to or lower than 0.3 mg/kg did not fully down-modulate CD11a cell surface expression. These subsaturating doses were also cleared at a faster rate than saturating doses (Bauer et al., 1999; Gottlieb et al., 2000). Furthermore, 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. Furthermore, we measured the relative capabilities of different PBMC and splenocyte subsets 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 postinjection. 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.

Materials and Methods

Whole Body Autoradiography. In all studies, the National Institutes of Health principles for research involving animals was strictly upheld. Male CD-1 mice (28–32 g) received a single i.v. bolus dose of the test article via the tail vein. Mice in group 1 received 25 μCi of 125І-M17. Mice in group 2 were dosed with 25 μCi of 125І-M17 mixed with 20 mg/kg M17. The specific activities for groups 1 and 2 were 25 μCi/μg and 0.05 μCi/μg, respectively. There were four mice per group; two were sacrificed at 1 h and two were sacrificed at 72 h after 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 earlier pharmacokinetic studies. 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 injected with 0.5 Ci of 125І-muM17 at ASPET Journals on April 12, 2017 dmd.aspetjournals.org Downloaded from


clearance of 125І-muM17 was assessed using WBA as described above. To block thyroid uptake of free iodine, mice from both groups were injected with 0.5 Ci of 125І-muM17. Mice in group 2 were similarly injected with 25 μCi of 125І-M17, but in the presence of 20-fold excess nonradioactive muM17. One mouse from this group was sacrificed at each time point of 1, 6, and 24 h post-antibody injection. The second group of three mice was similarly injected with 25 μCi of 125І-M17, but in the presence of 20-fold excess nonradioactive 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 i.p. 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-Aldrich, St. Louis, MO). Red blood cells were lysed in the blood and spleen cell suspensions using an ammonium chloride solution. Cells were divided into two aliquots, pelleted to remove supernatant, and then resuspended in either phosphate-buffered saline or low pH acid wash (150 mM NaCl and HCl, pH 2.5, in nH2O) to remove external-bound muM17 before pelleting and resuspending in 500 μl of whole cell lysis buffer. Protein concentrations were determined using the Bradford method (Bradford, 1976). muM17 internalization and intracellular clearance was assessed by SDS-polyacrylamide gel electrophoresis (20 μg of protein per condition) and PhosphorImager analysis (Amersham Biosciences Inc.) of the radioactive protein. Images were prepared using Adobe Photoshop.

Internalization and Subcellular Localization of 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 human antibody structure. Before experiment-
determination of internalization and subcellular localization. A single cell suspension was prepared from the spleens using a 0.44-μm cell separation filter (Sigma-Aldrich). Cells from the blood and spleen (approximately 10^6 cells) were treated with red blood cell lysis buffer, washed, and then incubated with 5 μM diethylaminoethylcarboxyamine 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(lysine)-coated plates and assessed for muM17-488 internalization and subcellular localization using wide field microscopy. Images were prepared using Adobe Photoshop.

PBMCs and splenocytes (0.5 × 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 of ammonium chloride solution for an additional 5 min at room temperature. The lysing reaction was terminated by washing twice with 2 ml of wash buffer (Hanks’ balanced salt solution with 0.1% sodium azide) and centrifugation to pellet the cells. Cells were resuspended in 500 μl of 2% formaldehyde in phosphate-buffered saline and stored at 4°C until all samples were collected for FACS analysis.

The association of IgG-488 or muM17-488 with specific PBMCs or splenocytes over time in vivo was made by labeling cells with fluorescent conjugated anti-CD3ε, -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 fluorescence intensity (MFI). The FACScalibur was compensated for a four-color analysis using the fluorescein isothiocyanate-, PE-, PerCP-Cy5.5-, and APC-conjugated specific and nonspecific IgG antibodies. The geometric MFI data were 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). All other antibodies used for flow cytometric analysis were purchased from BD Biosciences PharMingen. These included APC-, PE-, and fluorescein isothiocyanate-conjugated IgG1κ (A19-3), PE-conjugated IgG2α (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 i.v. injection, the majority of ^125^I-M17 in blood was associated with PBMCs in mice from group 1. At 1 h, 85% and 89% of the blood radioactivity was associated with PBMCs. By 72 h, the percentage of radioactivity associated with PBMCs 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% 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 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 coadministration of 20 mg/kg 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 macro-
FIG. 3. Tissue to blood ratio of radioactivity in mice at 1 and 72 h after i.v. administration of $^{125}$I-M17 (group 1) or $^{125}$I-M17 plus 20 mg/kg M17 (group 2). The dashed 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; Ln, lymph node.

FIG. 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 i.v. 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.

FIG. 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 i.v. 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.
phages 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 $^{125}\text{I}-\text{M17}$ 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). $^{125}\text{I}-\text{muM17}$ 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 $^{125}\text{I}-\text{muM17}$ by PBMCs, lymph nodes, and spleen were also assessed (Fig. 7). The left, center, and right panels of Fig. 7 represent PBMCs, 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 nonradioactive muM17 in the intravenous injection abolished detectable binding to the cells (data not shown). The top row represents total $^{125}\text{I}-\text{muM17}$ binding to cells and the bottom row represents internalized $^{125}\text{I}-\text{muM17}$. In both the PBMCs and lymph nodes there appeared to be greater accumulation of intracellular $^{125}\text{I}-\text{muM17}$ by 6 h relative to 1 h, in contrast to the spleen, where we observed a marked reduction in the level of intracellular $^{125}\text{I}-\text{muM17}$ by 6 h. Roughly 70% of the antibody internalized by PBMCs and splenocytes at 1 h was cleared from the cells by 24 h. These data suggest that muM17 undergoes an in vivo cell-mediated internalization and intracellular clearance.

Internalization and Subcellular Localization of muM17 in Vivo. Wide field fluorescent microscopy was used to assess the in vivo internalization and subcellular localization of muM17-488 by mouse PBMCs and splenocytes (Fig. 8). The two panels in the left column of Fig. 8 represent internalization of muM17–488 by PBMCs (top panel) and splenocytes (bottom panel). The two rows with panels denoted as a, b, and c represent costaining of muM17-488 with the lysosome marker, LysoTracker Red, within PBMCs (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 PBMCs and splenocytes in vivo, and that at least a portion of the internalized antibody is found within lysosomes.

Flow Cytometric Evaluation of muM17 Cellular Clearance in Vivo. The relative contribution of different PBMC and splenocyte cell populations to muM17–488 binding and cellular clearance over time...
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 PBMCs, liver, and spleen within the first hour after 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 PBMCs analyzed in the blood. Kupffer cells and tissue macrophages are known to express LFA-1 (Kuppers and Roos, 1989; Jacobs et al., 1997). 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. Internalized muM17 was internalized and cleared from within PBMCs 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 PMBCs 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 pa-

Fig. 9. Flow cytometric analysis of muM17-488 clearance by mouse PBMCs in vivo over time. Data represent muM17-488 binding to CD3+ T cells (filled circles), CD4+ T cells (gray squares), CD8+ T cells (open triangles), CD45R+ B cells (X’s), NK1.1+ NK cells (filled diamonds), CD11b+ monocytes (open circles), and Ly-6G+ neutrophils (filled triangles). A, data are plotted as the average MFI ± standard deviation (StDev) on the x-axis over time. B, data are plotted as the average percentage 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 were performed post-muM17-488 injection (n = 2).
RECEPTOR-MEDIATED CLEARANCE OF ANTI-CD11a ANTIBODY


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 PBMCs express cell surface CD11a, with NK cells, monocytes, granulocytes, and a subset of CD8<sup>+</sup> T cells expressing the highest levels, B cells expressing the lowest levels, and CD4<sup>+</sup> and a different subset of CD8<sup>+</sup> 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, whereas B cells possessed the least capacity for binding muM17. Binding of muM17 to CD4<sup>+</sup>- and CD8<sup>+</sup>-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.

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, iotinated 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 lymphoas seen by colocalization 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 eflizumab clearance that involves receptor-mediated internalization and degradation.

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