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Binding of recombinant T cell receptor ligands (RTL) to antigen presenting cells prevents upregulation of CD11b and inhibits T cell activation and transfer of experimental autoimmune encephalomyelitis

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Abstract

Recombinant T cell ligands (RTLs) ameliorate experimental autoimmune encephalomyelitis (EAE) in antigen specific manner. We evaluated effects of RTL401 (I-A^s α 1 β 1 + PLP-139-151) on splenocytes from mice with EAE to study RTL- T cell-tolerance-inducing mechanisms. RTLs bound to B, macrophages and DCs, through RTL-MHC- α 1 β 1 moiety. RTL binding reduced CD11b expression on splenic macrophages/DC, and RTL401-conditioned macrophages/DC, not B cells, inhibited T cell activation. Reduced ability of RTL- incubated splenocytes to transfer EAE was likely mediated through macrophages/DC, since B cells were unnecessary for RTL treatment of EAE. These results demonstrate novel pathway of T cell regulation by RTL bound APCs.

Keywords

EAE; RTLs; tolerance; APCs

1. Introduction

Recombinant T cell receptor (TCR) ligands (RTL) containing the membrane distal α 1+ β 1 domains of class II MHC molecules linked covalently to specific myelin peptides can induce

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T cell tolerance and reverse clinical and histological signs of experimental autoimmune encephalomyelitis (EAE) when administered i.v. or s.c. after onset of disease. RTLs containing the syngeneic MHC moiety in combination with the cognate encephalitogenic myelin peptide successfully treated monophasic EAE in Lewis rats (Burrows et al., 1998; Wang et al., 2003), chronic EAE in HLA-DR2 transgenic mice (Chou et al., 2004; Link et al., 2007; Vandenbark et al., 2003), relapsing EAE in SJL/J mice (Huan et al., 2004; Offner et al., 2005; Sinha et al., 2009b), and chronic EAE in C57BL/6 mice (Sinha et al., 2007). One such construct, RTL1000, containing the human MOG-35-55 peptide linked to the HLA-DR2 moiety, was recently tested successfully in a Phase 1 safety trial in Multiple Sclerosis (MS) subjects (Yadav et al., 2010). Understanding the mechanism of action of RTLs is necessary for exploiting their full therapeutic potential in MS.

RTL treatment is very effective at ameliorating clinical and histological signs of EAE when administered after disease onset, signifying that RTLs can reverse both inflammatory and neurodegenerative processes *in vivo*. The strong reduction of infiltrating immune cells and antigen-induced cytokine responses within the CNS of RTL-treated mice suggested that RTLs may interact directly with immune cells to modulate their function. RTLs are designed to interact with TCRs specific for the idiotope produced by the selected MHC/peptide combination, and we demonstrated that DR2-derived RTLs could induce changes in cytokine secretion patterns without proliferation in human T cell clones (Burrows et al., 2001). Moreover, RTL201 (comprised of the rat RT1B MHC moiety linked to Gp-MBP-72-89 peptide) could induce partial activation of the cognate A1 T cell hybridoma involving a CD3 ζ p23/p21 ratio shift, ZAP-70 phosphorylation, calcium mobilization, NFAT activation, and transient IL-2 production (Wang et al., 2003). TCR ligation with four-domain MHC class II/peptide idiotopes (and RTL ligation to a lesser degree) produces a transient and weak binding affinity that would not likely be detectable without additional cross-linking. Yet, our preliminary studies demonstrated RTL binding to splenic B cells through the MHC class II α 1 β 1 moiety in a manner independent of the bound antigenic peptide (Link, 2007). These findings raised the possibility that RTL modulation of T cell function could be mediated in part by an initial binding event to various antigen presenting cells (APCs) that might in turn present a tolerogenic idiotope to antigen-specific T cells. However, RTL binding to cellular receptors leading to T cell modulation in the context of ongoing inflammation remains largely uncharacterized.

In this study, we used fluorescent-labeled RTL401 (containing the I-A^s moiety linked to the PLP-139-151 peptide) to demonstrate that the RTL molecule binds strongly to APCs but not T cells in an antigenic peptide-independent manner. RTL conditioning of macrophages blocked upregulation of CD11b marker and inhibited proliferation and cytokine secretion induced in encephalitogenic T cells by free PLP-139-151 peptide. Moreover, using an adoptive transfer approach, we found that encephalitogenic T cells activated with RTL401-armed APC had reduced capacity to transfer EAE. Coupled with new data showing that B cells are not required for successful RTL therapy, our data demonstrate for the first time a novel cell-cell interaction by which RTL-armed myeloid APC deliver tolerogenic signals to cognate T cells to reverse the EAE disease process.

2. Material and methods

2.1. Animals

SJL/J female mice were obtained from Jackson Laboratories (Bar Harbor, ME) at 7–8 wk of age. PLP-139-151 TCR Tg 5B6 mice were a kind gift from Dr. Vijay Kuchroo, Harvard University and were bred in house. B cell deficient (μ MT knock-out, KO) mice on C57BL/6 background were obtained from Jackson Laboratories (Bar Harbor, ME) at 7–8 weeks of age. All the mice were housed in the Animal Resource Facility at the Portland Veterans

Affairs Medical Center (Portland, OR) in accordance with institutional guidelines. The study was conducted in accordance with National Institutes of Health guidelines for the use of experimental animals, and the protocols were approved by the Institutional Animal Care and Use Committee.

2.2. RTL401 construction

General methods for the design, cloning and expression of RTL (including RTL401 used in this study) have been described previously (Burrows et al., 1999; Huan et al., 2004; Offner et al., 2005). In brief, mRNA was isolated from the splenocytes of SJL mice using an OligotexDirect mRNA mini-kit (Qiagen, Valencia, CA). cDNA of the Ag binding/TCR recognition domain of murine I-A^s MHC class II β 1 and α 1 chains was derived from mRNA using two pairs of PCR primers. The two chains were sequentially linked by a 5-aa linker (GGQDD) in a two-step PCR with *Nco*I and *Xho*I restriction sites being added to the amino terminus of the β 1 chain and to the carboxyl terminus of the α 1 chain, respectively, to create RTL400. The PLP 139–151 peptide with a linker (GGGGS^{SLV}PRGSGGGG) was covalently linked to the 5' end of the β 1 domain of RTL400 to form RTL401. The murine I-A^s β 1 α 1 insert was then ligated into pET21d(+) vector and transformed into Nova blue *Escherichia coli* host (Novagen, Madison, WI) for positive colony selection and sequence verification. RTL400 and RTL401 plasmid constructs were then transformed into *E. coli* strain BL21(DE3) expression host (Novagen). The purification of proteins has been described previously (Burrows et al., 1999; Huan et al., 2004). The final yield of purified protein varied between 15 and 30 mg/L bacterial culture. The sequence of RTL400 and RTL401 and their MHC chain content is shown in Table 1A and B respectively.

2.3. EAE induction and RTL401 treatment

SJL/J and 5B6 mice were inoculated in the flanks with 0.2 ml emulsion containing 150 μ g of PLP-139-151 peptide and an equal volume of CFA containing 2 mg/ml of heat-killed *Mycobacterium tuberculosis*. B cell deficient (μ MT KO) mice were inoculated in the flanks with 0.2 ml of emulsion containing 200 μ g of MOG-35-55 peptide and an equal volume of CFA containing 4 mg/ml of heat-killed *Mycobacterium tuberculosis*. MOG peptide-immunized mice were also injected with 75 ng and 200 ng pertussis toxin (Ptx) intraperitoneally on days 0 and 2 relative to immunization. Mice were assessed for signs of EAE according to the following scale: 0, normal; 1, limp tail or mild hind limb weakness; 2, moderate hind limb weakness or mild ataxia; 3, moderately severe hind limb weakness; 4, severe hind limb weakness or mild forelimb weakness or moderate ataxia; 5, paraplegia with no more than moderate forelimb weakness; and 6, paraplegia with severe forelimb weakness or severe ataxia or moribund condition. At the onset of clinical signs of EAE (days 10–11), mice were divided into two groups and treated with vehicle (100 μ l 20 mM Tris-HCl in 5% dextrose) or with 100 μ l of 1 mg/ml RTL subcutaneously for indicated time points. Mice were monitored for changes in disease score until they were euthanized for *ex vivo* analyses.

2.4. Passive transfer of EAE

Female 5B6 mice were immunized with PLP-139-151/CFA as described in section 2.3 and spleens were harvested 7 days later. Transgenic 5B6 mice were chosen for this assay since majority of T cells in these mice expressed TCR specific for PLP-139-151. Splenocytes were cultured *in vitro* in the presence of 10 μ g/ml PLP-139-151 peptide, 50 μ g/ml RTL401 or 10 μ g/ml PLP-139-151 + 50 μ g/ml RTL401 (both RTL and peptide added simultaneously) in stimulation medium (RPMI containing 1% sodium pyruvate, 0.4% of 1:1000 β -mercaptoethanol, 1% L-glutamine, 2% FBS) for 72 h. This dose of RTL401 was chosen such that it contained at least half the molar concentration of PLP-139-151 as was added in cultures stimulated with free PLP-139-151 peptide, considering that on a molar basis, PLP-139-151 constituted ~10% of RTL401. Experiments were also carried out using 100 μ g/

ml RTL401 to provide an equimolar concentration of PLP-139-151 peptide. Neither dose of RTL401 was toxic to the cells. Cells were then washed extensively, and 10 million activated cells were injected i.p. into naive SJL/J recipient mice. The mice were assessed daily for signs of EAE according to the scale described above.

2.5. Fluorescent staining and antibodies

Four-color (fluoresceinisothiocyanate, phycoerythrin, propidium iodide, allophycocyanin) fluorescence flow cytometry analyses were performed to determine the phenotypes of cells following standard monoclonal antibody staining procedures. After staining, cells were washed with staining medium (1X PBS containing 0.5% bovine serum albumin, 0.02% sodium azide and 0.4% of 0.5M EDTA) and data were acquired immediately with a FACS Calibur (Becton-Dickinson, Mountain View, CA) and analyzed using FACS express software. Granulocytes specific antibody (Gr1, clone 1A8) was used to exclude them from all the analyses done on the FACS plots. All antibodies were purchased from BD PharMingen (San Diego, CA). Cells were stained with combinations of the following anti-mouse antibodies: CD3 PE (145-2C11), CD4 PE or APC (RM4-5), CD19 APC (1D3), CD11b FITC or APC (M1/70), CD11c PE or APC (HL3).

2.6. RTL binding assay

Splenocytes obtained from SJL mice were either incubated alone or with Alexa-488 labeled RTL401 for 1h at 37°C in 0.5ml of PBS+1% BSA. Cells were washed in PBS+1% BSA, and stained with fluorescent anti-CD3, -CD19, -CD11b or -CD11c antibodies. Data were acquired on a FACS Calibur and analyzed with FACS express (DeNovo Software, Los Angeles, CA). RTL binding to APCs was also confirmed with an adherence assay. Glass cover slips were coated with BSA or RTL401 for 1 hr at RT. RTL401-coated surfaces were blocked for 1 hr with BSA (5mg/ml) to prevent non-specific adhesion. CD19+ (B cells), CD11b+ (macrophages + dendritic cells, DC), CD11c+ (DC) or CD3+ (T cells), were sorted from SJL/J splenocytes and 3.5×10^5 cells/well were incubated with immobilized RTL or BSA for 30 min at 37°C.

2.7. T cell and APC co-culture

Spleens were collected from 5B6 mice 7 days after immunization (as described in section 2.3) with PLP-139-151. Cells were labeled with CD3, CD19 and CD11b MACS microbeads followed by sorting on an Automacs. Cell purity was >95%. Carboxy fluorescein succinimidyl ester (CFSE) labeled PLP-139-151-specific CD3+ T cells were co-cultured with CD3-depleted splenic APCs, or magnetic bead-sorted CD19+ or CD11b+ cells at a 1:2 ratio (for APCs) and a 1:1 ratio (for CD19+ and CD11b+ cells) in 96 well round-bottom plates for 5 days at 37°C in the presence of PLP-139-151, RTL401 or PLP-139-151+RTL401. PLP-139-151 and RTL401 concentrations used were as described earlier. More (sorted) APCs were added to the co-cultures as compared to CD19+ or CD11b+ co-cultures due to the presence of fewer CD11b+ cells (and more CD19+) in unfractionated APCs. Following incubation, cells were harvested, washed and stained with fluorescent-labeled anti-CD4 to study T cell proliferation concomitant with CFSE dilution. Culture supernatants were also collected for measuring secreted IL-17, TNF- α and IFN- γ cytokines using a Luminex assay.

2.8. In vitro splenocyte culture

Splenocytes were obtained from SJL/J mice 10 days post immunization. Immunizations were performed as mentioned in section 2.3. Cells were cultured either untreated or with previously mentioned doses of PLP-139-151, RTL401 or PLP-139-151+RTL401 at 37°C for

indicated time points. Cells were collected by scraping with cell scraper, washed extensively and subjected to CD11b fluorescent staining as described earlier.

2.9. CD11b expression on blood monocytes

SJL/J mice were immunized with PLP-139-151/CFA and scored as described in section 2.3. At disease onset mice were either treated daily with vehicle or 100µg of RTL401. Blood was collected at the indicated time points after treatment. Following RBC lysis, cells were stained with fluorescent-labeled anti-CD11b antibody. Data acquisition and analysis were carried out using a FACS Calibur and FACS Express, respectively. Monocytes were gated to study expression of CD11b+ cells.

2.10. Histopathology

Intact spinal cords were removed from mice that had received *in vitro* stimulated cells and the tissue was fixed in 10% Formalin. Spinal cords were dissected after fixation and embedded in paraffin before sectioning. The sections were stained with Luxol fast blue/periodic acid Schiff-hematoxylin to assess demyelination and inflammatory lesions, and analyzed by light microscopy.

2.11. Statistical analyses

Differences in percentages of cells expressing various cell surface markers, proliferation responses and cytokine responses were evaluated using Student's *t* test. Differences in mean, peak and cumulative EAE scores between RTL-treated and control groups were determined by the Mann-Whitney test. *p* values ≤0.05 were considered significant.

3. Results

RTL binds to APCs

Our preliminary studies demonstrated RTL binding to splenic B cells through the MHC class II $\alpha 1\beta 1$ moiety in a manner independent of the bound antigenic peptide. To further evaluate if other APCs bind RTL401, 10⁶ splenocytes were incubated with 10µg of Alexa488 labeled RTL401 in 0.5ml of PBS+1%BSA and washed in PBS+1%BSA. Binding of fluorochrome-labeled RTL to different cell types was evaluated by FACS Calibur. As depicted in Fig. 1A, RTL401 adhered to CD19+ B cells, CD11b+ macrophages/DC and CD11c+ DC, but did not bind to CD3+ T cells. Furthermore, the binding was concentration dependent but was independent of the presence of covalently bound peptide, since empty RTL400 also bound to APCs (data not shown). The binding of RTL to APCs was further illustrated microscopically. As is shown in Fig. 1B, immobilized RTL401 bound to B cells, macrophages and DC, but not T cells. Taken together with the FACS analysis, these data demonstrate selective RTL binding to APC in a peptide non-specific manner to as-yet uncharacterized receptors.

RTL401 prevented upregulation of CD11b integrin on myeloid cells

We studied expression of various activation markers, including CD11b integrin, on the macrophage subpopulation of splenocytes obtained from PLP-139-151 immunized mice by FACS staining. Total %CD11b expression on live gated monocytes (illustrated in Fig. 2A) did not change significantly among the groups after 24 and 48 h of incubation (Fig 2B). However, while PLP-139-151 stimulated cultures showed an increase in CD11b expressing cells over time (0.9%±0.7 at 24h vs. 1.27%±0.05 at 48h (*p*<0.01) vs. 2.7±0.2 at 72h (*p*<0.01)), the percentage of CD11b expressing cells in RTL401 or PLP-139-151+RTL401 stimulated cultures was significantly less after 72h of incubation (RTL401, 1.6±0.2; PLP-139-151+RTL401, 1.7±0.1, Fig 2B). Moreover, among CD11b-expressing cells within

PLP-139-151 stimulated cultures, there was a consistent cluster of CD11b^{high} cells that was significantly decreased in RTL401 and PLP-139-151+RTL401 treated groups at all time points studied (Fig. 2C). Cells were also mock cultured for this assay. Basal level of CD11b expression was observed in the un-stimulated cultures (Fig 2B) and there was no CD11b^{high} cells present in these cultures (Fig 2C). Granulocytes were excluded from all the analysis performed on FACS plots.

***In vivo* treatment with RTL401 decreased CD11b expression on blood monocytes**

Because of the ability of RTL401 to modulate CD11b integrin expression on macrophages *in vitro*, we further analyzed CD11b expression *ex vivo* on blood monocytes at different times after treatment with RTL401 or vehicle. As mentioned earlier, granulocytes were excluded from all the analysis. In vehicle-treated mice, CD11b⁺ live gated monocytes were 48%±6 one day after treatment, 38%±9 after 3 daily treatments and 41%±29 after 5 treatments (Fig. 2D). The corresponding percentages of CD11b⁺ cells were significantly lower at two earlier time points in RTL401 treated mice, with 24±12% positive cells after 1 treatment, 21%±6 positive cells after 3 treatments (Fig 2D).

RTL401 inhibits peptide-induced proliferation of activated T cells when co-cultured with primed splenic APCs and this is mediated by CD11b⁺ cells

To evaluate possible regulatory effects of RTL401-armed APC, T cell depleted splenocytes or purified APC subpopulations were co-incubated with carboxy fluorescein succinimidyl ester (CFSE) labeled T cells obtained from PLP-139-151 peptide-immunized mice. 5B6 TCR transgenic mice specific for PLP-139-151 peptide were used for these co-culture experiments to enrich for PLP-139-151 specific T cells. APCs and negatively selected CD3 positive (no touch) mouse T cells were sorted from spleens of immunized 5B6 mice using Automacs beads. Sorted APCs were co-cultured with CFSE labeled transgenic T cells in the presence of free PLP-139-151 peptide, RTL401 or PLP-139-151+RTL401 in 96 well round-bottom plates for 5 days. As expected, splenic APCs induced strong proliferation of CFSE labeled T cells (77% positive) in the presence of added PLP-139-151 peptide (Fig. 3A). Incubation of the APC + T cell mixture with RTL401 also induced a proliferation response that was significantly greater than medium alone (19% vs. 2%) but far less than peptide alone. Of critical importance, no additional proliferation of activated T cells occurred when free PLP-139-151 peptide was added to the RTL401-armed splenic APCs (19% positive, Fig. 3A). The ability of RTL401 to increase T cell proliferation responses and to inhibit proliferation induced by free PLP-139-151 peptide was dependent on the covalently tethered RTL-bound peptide, since incubation of the cell mixture with RTL400 (I-A^s α1β1 with no tethered peptide) had neither effect (Fig 3A, B and C). Unlike RTL401, RTL400 by itself was not able to induce T cell proliferation nor was the proliferation inhibited when PLP-139-151 was added along with RTL400 to the cultures. In accordance with this, we have shown previously that RTL400 has no clinical effect on PLP-139-151 induced EAE (Huan et al., 2004).

We further studied which APC population (CD19⁺ B cells vs. CD11b⁺ macrophages and DC) might be responsible for inhibiting RTL401-mediated T cell proliferation. CD11b⁺, CD19⁺ and CD3⁺ mouse T cells were sorted from spleens of immunized 5B6 TCR Tg mice using Automacs beads. Sorted splenic APCs were co-cultured with CFSE-labeled transgenic T cells in the presence of PLP-139-151 peptide, RTL401 or PLP-139-151+RTL401 in 96 well round-bottom plates for 5 days. CD11b⁺ macrophages/DC and CD19⁺ B cells both induced strong proliferation of CFSE-labeled T cells in the presence of added peptide as expected (92–93%, Figs. 3B&C). However, only the CD11b⁺ cells (Fig. 3B), but not the CD19⁺ cells (Fig. 3C) mirrored the T cell activation pattern observed using mixed splenic APC, i.e. modest induction of proliferation with RTL401 (19% positive) and inhibition of

additional proliferation induced by free PLP-139-151 peptide (16% positive) (Fig 3B). This activation pattern was dependent on using CD11b⁺ cells from PLP-139-151 primed mice, since naïve CD11b⁺ cells obtained from unimmunized mice induced a similar level of proliferation of Tg 5B6 T cells in the presence of added PLP-139-151 peptide (59%), RTL401 (58%) or PLP-139-151+RTL401 (43%, data not shown). Of interest, addition of RTL401 to primed CD19⁺ B cells provided sufficient PLP-139-151 peptide to induce a full T cell proliferation response (92%, Fig. 3C), suggesting distinct RTL-dependent pathways in B cells vs. myeloid cells. These key results demonstrate that between the two APC subpopulations tested, CD11b⁺ cells fully accounted for the inhibitory activity of RTL401 on PLP-139-151-induced T cell activation observed in the mixed splenic APC population. In addition to inhibition of T cell proliferation, RTL401-armed macrophages/DC also significantly inhibited (>50%) secretion of pro-inflammatory cytokines, including IFN- γ , TNF- α and IL-17 (Fig. 4).

RTL-armed splenocytes suppress transfer of EAE by primed T cells

To study whether RTL exposure *in vitro* could alter the ability of T cells to transfer EAE, splenocytes from immunized TCR transgenic 5B6 mice were cultured with PLP-139-151 peptide, RTL401 or both agents for 72hrs and the cells transferred into WT SJL/J recipients. As expected, cells cultured in the presence of PLP-139-151 peptide induced EAE in recipient mice, with peak disease of 3.2 ± 1.8 units and cumulative disease index (CDI) of 30.4 ± 20 (Fig. 5A). In contrast, cells cultured in the presence of RTL401 alone transferred minimal EAE, with peak disease of 0.9 ± 1.2 and CDI of 6.5 ± 11.7 . Of paramount importance, cells activated with PLP-139-151 peptide in the presence of RTL401 had a significantly reduced capacity to transfer EAE compared to cells stimulated with PLP-139-151 peptide alone, with peak disease of 1.8 ± 1.8 and CDI of 17.6 ± 16.9 (Fig. 5A). To rule out the possibility that a lower molar concentration of RTL-derived PLP-139-151 peptide present in the 50 μ g/ml dose of RTL401 might lead to suboptimal activation of T cells in cultures stimulated with RTL401 alone, we also cultured cells in the presence of 100 μ g/ml of RTL401 that contained an equimolar concentration of PLP-139-151 compared to 10 μ g/ml free peptide. Cells obtained from this culture did not transfer EAE at all (data not shown), suggesting an RTL401 dose-dependent effect. Spinal cords of mice receiving cells stimulated with RTL401 or PLP-139-151+RTL401 showed fewer inflammatory lesions and loss of myelin compared to mice receiving PLP-139-151 stimulated cells (white arrows in spinal cords of mice receiving PLP-139-151 stimulated cells, Fig 5B). These unique results demonstrate that RTL-arming of splenic APC *in vitro* down-modulates the disease-causing potential of encephalitogenic PLP-139-151-primed T cells.

CD19⁺ B cells are not required for RTL treatment of EAE

Although RTL401 clearly binds to both CD11b/c⁺ macrophages/DC and CD19⁺ B cells (Fig. 1), the RTL401-armed B cells directly induced strong proliferation and failed to inhibit 5B6 T cell activation induced by the PLP-139-151 peptide *in vitro* (Fig. 3), suggesting lack of a tolerogenic function. To further evaluate the role of B cells in the RTL therapeutic mechanism, RTL551 (designed to modulate MOG-35-55 specific T cells in C57BL/6 mice) was employed for treatment of B cell deficient μ MT knockout mice with EAE. As shown in Fig. 6, RTL551 was highly effective for treating EAE induced in B cell deficient mice, reducing the peak disease score from 4.5 ± 0.6 in vehicle-treated mice to 1.6 ± 0.4 in RTL401-treated mice and the cumulative disease index (CDI) from 34.5 ± 6.5 in vehicle-treated mice to 10.2 ± 2.0 in RTL-treated mice ($p < 0.01$). These data suggest that B cells are not essential for successful RTL therapy of EAE.

4. Discussion

Previously we have shown that RTL treatment reduces encephalitogenicity of T cells *in vivo*, leading to remission from EAE both in chronic (C57BL/6 and DR2 mice) and relapsing remitting (SJL/J mice) models of EAE (Offner et al., 2008). This study, for the first time, demonstrates RTL-mediated functional effects on pathogenic T cells after binding to APCs, including CD19⁺ B cells, CD11b⁺ and CD11c⁺ macrophages and dendritic cells, but not CD3⁺ T cells. RTL binding to APC was not dependent on covalently tethered peptide, as empty RTL400 also bound to these cells with equal intensity. Thus, the robust cell binding event described in the current study involves a putative RTL receptor that differs from direct ligation of the T cell receptor by soluble cognate RTLs reported in previous studies using human T cell clones and T cell hybridomas. Our inability to detect direct RTL binding to CD3⁺ cells in this study may indicate the absence of the putative RTL receptor on T cells, a low frequency of peptide-specific T cells (typically <1/1000 splenocytes in an unimmunized mouse) and a low binding avidity of TCR for the RTL idiotope not likely to be detectable using FACS or immobilization on glass (Davis et al., 1998). Indeed, peptide-specific binding of RTL1000 (containing human DR2 covalently bound to MOG-35-55 peptide) to membrane preparations containing cognate TCR (from our H2-1 hybridoma) are transient and of low avidity when assessed by Biacore measurements (AAV manuscript in preparation).

To evaluate the phenotypic effects of RTL401 on CD11b⁺ cells, we cultured splenocytes obtained from immunized SJL/J mice in the presence of free PLP-139-151 peptide, RTL401 or both reagents. Initially, we focused on expression of the CD11b (Mac-1, integrin) molecule itself, known to be both a complement receptor (CR3) and a cell adhesion molecule present on the surface of macrophages that undergoes upregulation from preformed cytoplasmic stores upon activation (Nicholson et al., 2007). CD11b integrin was found to increase progressively over 72 h in PLP-139-151 stimulated cell cultures (0.9%–2.5%), whereas no increase occurred in RTL-treated cultures at the 72h time point (Fig. 2B). Moreover, there were significantly lower percentages of the CD11b^{high} subset in RTL401-treated cultures at all time points (Fig. 2C). Finally, RTL401 treatment of EAE *in vivo* resulted in a profound significant reduction of CD11b upregulation on blood monocytes during the course of EAE (Fig. 2D).

CD11b integrin was found to be critically important for EAE induction (Bullard et al., 2005), and therefore, downregulation of CD11b on monocytes by RTL401 could have a direct consequence on EAE progression. Gordon et al (Gordon et al., 1995) found that anti-CD11b administration delayed the onset and reduced the severity of EAE, and it is well established that although T cells initiate EAE, the effector mechanisms provided by infiltrating CD11b⁺ macrophages and endogenous microglia can lead to inflammation and demyelination in the CNS (Benveniste, 1997). Mature macrophages and dendritic cells are prominent infiltrates in multiple sclerosis and EAE. CD11b⁺ macrophages are even more significant for causing damage in EAE as microglia are not appropriately activated in the absence of blood borne macrophages (Bauer et al., 1995). This also suggests that possible interaction between infiltrating macrophages and T cells might be responsible for the activation of microglia in the CNS. Inhibition of CD11b upregulation on monocytes can block their antigen presentation, migration and adhesion to vascular endothelium leading to reduced accumulation in the CNS. Indeed, we demonstrated in previous studies that RTL treatment of EAE resulted in significant reduction of inflammatory infiltrates in the spinal cords (Huan et al., 2004; Sinha et al., 2009a; Sinha et al., 2009b; Sinha et al., 2007; Wang et al., 2006). Taken together, we propose a model where RTL401 prevents upregulation of CD11b integrin on myeloid cells in the blood early in the disease and blocks any subsequent entry of CD11b⁺ cells into the CNS. This could in turn result in suboptimal activation of

already infiltrated cells and abrogation of the inflammatory cascade in the CNS, eventually leading to attenuation from EAE. It should be noted that strong binding of RTL was also observed with CD11c⁺ dendritic cells and it is conceivable that like CD11b, CD11c integrin might also be down-regulated by RTL401, an issue that will be addressed in future studies.

The *in vitro* experimental paradigm used to assess RTL effects on encephalitogenic T cells was designed to mimic *in vivo* RTL treatment of established EAE. Thus, TCR Tg T cells from PLP-139-151 immunized 5B6 mice were co-cultured with primed splenic APC from the same mice in the presence of free PLP-139-151 peptide, RTL401 or free peptide + RTL401 and evaluated for proliferation and cytokine release, and ability to transfer EAE to naïve recipient SJL/J mice. PLP-139-151 was included with RTL401 to see whether the presence of free peptide might influence and/or reverse RTL effects on T cells. The major findings from these studies included: 1) Both myeloid and B cell APCs could induce strong T cell proliferation responses when pulsed with free PLP-139-151 peptide as expected; 2) B cells could induce comparably strong T cell proliferation responses when pulsed with RTL401 (but not RTL400 lacking the PLP-139-151 peptide), demonstrating an ability to process and present the covalently tethered PLP peptide derived from RTL401. In contrast, CD11b⁺ myeloid cells had a greatly reduced capacity (by ~80%) to induce T cell proliferation responses with RTL-derived PLP-139-151 peptide; 3) T cell proliferation and cytokine responses to free PLP-139-151 peptide were strongly inhibited (50–80%) by RTL401-conditioned, T cell depleted splenocytes and purified myeloid cells but not purified B cells from PLP-139-151 peptide-primed mice (no effect by myeloid cells from naïve mice); 4) The regulatory effects of RTL401-conditioned myeloid cells fully reflected the strong inhibition observed using unfractionated splenic APC (that included B cells), indicating a dominant effect of RTL-conditioned myeloid cells; and 5) The inhibitory effects of RTL401 on T cell responses to PLP-139-151 peptide *in vitro* were reflected by a significant reduction in severity of passively transferred EAE and reduced inflammatory lesions in spinal cord tissue.

The *in vitro* T cell activation studies discussed above suggested important functional differences between B cells and myeloid cells, despite comparable binding of RTL by both APC lineages. Unlike myeloid cells, B cells could induce maximal T cell proliferation with RTL401-derived PLP-139-151 peptide that was comparable to free peptide alone. Moreover, RTL-armed B cells failed to inhibit T cell proliferation responses. These differences may well be linked to the ability of the RTL401 to prevent upregulation of the CD11b integrin present on myeloid cells but not B cells. Unlike PLP-139-151 stimulated cultures, failure to detect an increase in CD11b in RTL401 stimulated cultures suggests suboptimal activation and reduced effector functions of the macrophages. Indeed anti CD11b antibody has been shown to inhibit phagocytosis by neutrophils (Rubel et al., 1999) indicating its role in antigen uptake and therefore subsequent presentation of peptides, although evidence of a similar role of CD11b on macrophages is currently lacking. Failure of T cells to respond to PLP-139-151 with coordinate down-regulation of CD11b supports reduced APC function in the presence of RTL. Considering the antigen specificity of RTL mediated treatment of EAE, we believe that only cognate RTL is able to prevent upregulation of CD11b integrin on primed cells accompanied with inhibition of T cell proliferation. Nonetheless, this will be tested in future studies by using RTL with different target specificity than the immunizing peptide.

To further evaluate the role of CD19⁺ B cells in RTL therapy, we induced EAE in B deficient μ MT KO mice on the C57BL/6 background and treated the mice at disease onset with RTL551. The data demonstrated unequivocally that RTL551 was highly effective in reversing clinical signs of EAE, indicating that B cells are not necessary for RTL treatment effects on EAE. Given the ability of RTL-armed CD11b⁺ myeloid APC to inhibit T cell

activation and transfer of EAE, it is likely that the RTL551 treatment of μ MT KO mice was mediated through residual macrophages and/or dendritic cells. These results do not eliminate possible regulatory effects of RTL-armed B cells, however, since it is conceivable that B cells and myeloid APC represent distinct but redundant RTL-mediated inhibitory pathways.

In conclusion, our results demonstrate for the first time a novel pathway of T cell regulation that involves binding of RTLs to the surface of myeloid APC and down-regulation of the CD11b macrophage marker that may contribute to the reduced ability of the macrophages to induce T cell activation and transfer of EAE. In contrast, B cells could not inhibit T cell activation after RTL-binding and were found to be unnecessary for successful RTL therapy of EAE.

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Figure 1. RTL401 binds CD19⁺ B, CD11b⁺ macrophages & CD11c⁺ dendritic cells but not CD3⁺ T cells

A. Ten μg of Alexa488-labeled RTL401 was incubated with naïve SJL/J mouse splenocytes (black line) at 37°C. Cells were washed and data were collected on the FACS Calibur gated to detect CD19⁺CD3⁻CD11b⁻ B cells, CD3⁺CD19⁻CD11b⁻ T cells, CD11b⁺CD3⁻CD19⁻ macrophages and CD11c⁺CD3⁻CD19⁻ dendritic cells (DCs). Three experiments were performed with similar results. Dashed line represents SJL/J splenocytes with no added RTL. **B.** Naïve SJL/J splenocytes were sorted by Automacs for CD19⁺ B cells, CD11b⁺ macrophages, CD11c⁺ dendritic cells and CD3⁺ T cells; 3.5×10^5 cells/well were incubated for 30 min at 37°C over surfaces that had been coated with either BSA or RTL401 (100 $\mu\text{g}/\text{ml}$). After washing to remove non-adherent cells, bound lymphocytes were imaged on a Zeiss Axiovert 200M microscope.

Figure 2. RTL401 prevents upregulation of CD11b integrin

A. FACS plot showing CD11b and CD11b^{high} subpopulations. Percentages shown are CD11b^{high}/CD11b⁺ cells. **B.** CD11b positive cells in splenocytes obtained from immunized SJL/J mice were mock cultured or incubated with PLP-139-151, RTL401 or PLP-139-151+RTL401. At the indicated time points, cells were washed and stained with fluorescent anti-CD11b antibody. Data presented are the mean of results from three individual mice. Significant differences ($p \leq 0.05$) between PLP-139-151 peptide stimulated cells vs. RTL401 and RTL401+peptide-treated groups were determined using the Student's *t* test. Experiment was done twice with similar results. **C.** Among CD11b⁺ cells, PLP139-151 stimulated cultures had a greater percentage of CD11b^{high} expressing cells at all the time points studied as compared to other groups. **D.** CD11b upregulation on blood monocytes is prevented by RTL401 treatment. SJL/J mice were immunized with PLP-139-151/CFA and treated daily with vehicle or 100 μ g RTL401 at disease onset for 1, 2 or 5 days. Blood was collected at the indicated time points, RBC were lysed and white blood cells were subjected to fluorescent staining. CD11b expression was evaluated on live gated monocytes. Data

presented are from cells pooled from three mice. Repeat experiment was performed with similar results.

Figure 3. Primed splenic APCs and CD11b+ cells incubated with RTL401 inhibit proliferation of PLP-139-151 primed TCR transgenic T cells

A. T cell depleted APCs were isolated from the spleens of PLP-139-151/CFA-immunized 5B6 mice by labeling with specific microbeads and sorting on Automacs. Sorted APCs were cultured with CFSE-labeled primed T cells from the same mice at a 1:2 ratio (T:APC) in the presence of free PLP-139-151 peptide, RTL400, RTL401, PLP-139-151+RTL400 or PLP-139-151+RTL401. After five days of culture, cells were washed and evaluated by FACS Calibur for CD4 expression and CFSE dilution. **B&C.** Similar experiments from PLP-139-151/CFA-immunized mice were done with 5B6 T cells cultured with sorted CD11b+ (**B**) and CD19+ (**C**) cells plus antigens, except that the T:CD11b and T:CD19 ratio was 1:1. Data presented are representative of three separate experiments.

Figure 4. RTL401 inhibits inflammatory cytokines induced by PLP-139-151 peptide
 Supernatants from 1:1 (T:CD11b) mixed cell cultures stimulated with free PLP-139-151 peptide, RTL401 or PLP-139-151+RTL401 for 48hr were evaluated for IL-17, TNF- α & IFN- γ . Note decreased levels of cytokines induced by RTL401-armed macrophages. Data presented are representative of two separate experiments.

Figure 5. Transfer of EAE to WT recipients is reduced when antigen primed cells are cultured in the presence of RTL401

A. TCR Tg 5B6 mice reactive to PLP-139-151 were immunized with PLP-139-151/CFA. Splenocytes were obtained 7 days post immunization and were cultured *in vitro* for 3 days in the presence of PLP-139-151, RTL401 or PLP-139-151+RTL401. Cells were washed extensively and 10 million blasting cells were transferred into WT SJL/J mice. Mice were then observed for induction of EAE. Data presented are the mean of four experiments for each group, with 5–6 mice per group. Significant differences between control and treated groups were determined using the Mann-Whitney test (* $\#$, $p \leq 0.05$). **B.** Fixed, paraffin-embedded spinal cord sections from SJL/J mice 21 days after cell transfer. Spinal cords from mice that received PLP-139-151 stimulated cells showed dense mononuclear infiltration (arrow) and loss of myelin (loss of blue staining) in the regions adjacent to the mononuclear infiltrate. Spinal cords from mice that were given cells activated by RTL401 or PLP-139-151+RTL401 showed reduced inflammatory cells with no apparent loss of myelin. Magnification: $\times 4$.

Groups	Incidence	Onset	Peak	CDI
Vehicle	5/5	12.0 ± 0.7	4.5 ± 0.6	34.5 ± 6.5
RTL551	5/5	12.4 ± 0.7	1.6 ± 0.4*	10.2 ± 2.0*

*vehicle vs. RTL551; $p < 0.01$

Figure 6. RTL551 can treat EAE in the absence of B cells

B cell deficient (μ MT) mice were immunized with MOG-35-55 peptide in CFA plus Ptx and were treated with 5 daily doses of 100 μ g RTL551 or vehicle at onset of clinical signs of EAE. Note significant reduction ($p \leq 0.05$) in Peak EAE and CDI scores in RTL-treated mice (evaluated by the Mann-Whitney test). The experiment was repeated twice with similar results.

Table 1**Table 1A. Sequence of RTL molecules used in this study**

RTL	Sequence
400	MGGDS (Leader) ERHFVFQFKGECYFTNGTQIRISVDRYIYNREEYLRFDSDVGEYRAVTELGRP DAEYYNKQYLEQTRAELDTVCRHNYEGVETHSLRRLGGQDDIEADHVGYY GTTVYQSPGDIGQYTHEFDGDEWFYVDLDKKETIWMLPEFGQLTSFDPQGGL QNIATGKYTLGILTKRSNSTPATN
401	MGGDS (Leader) HSLGKWLGHDPKF (PLP-139-151) GGGGS�VPRGSGGGG (linker) ERHFVFQFKGECYFTNGTQIRISVDRYIYNREEYLRFDSDVGEYRAVTELGRP DAEYYNKQYLEQTRAELDTVCRHNYEGVETHSLRRLGGQDDIEADHVGYY GTTVYQSPGDIGQYTHEFDGDEWFYVDLDKKETIWMLPEFGQLTSFDPQGGL QNIATGKYTLGILTKRSNSTPATN

B. Secondary structure content of RTL molecules used in this study

RTLs	Alpha helix	Antiparallel beta sheet	Parallel beta sheet	Turn	Others	Total	RMSE
RTL400	0.13	0.27	0.01	0.22	0.37	1.00	0.198
RTL401	0.14	0.27	0.03	0.21	0.35	1.00	0.206