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The use of Flow Cytometry to assess a novel drug efficacy in Multiple Sclerosis

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Abstract

Applying different technologies to monitor disease activity and treatment efficacy are essential in a complex disease such as multiple sclerosis. Combining current assays with flow cytometry could create a powerful tool for such analyses. The cell surface expression level of CD74, the MHC class II invariant chain, is a potential disease biomarker that could be monitored by FACS analysis in order to assess disease progression and the clinical efficacy of partial MHC class II constructs in treating MS. These constructs, which can bind to and down-regulate CD74 cell-surface expression on monocytes and inhibit macrophage migration inhibitory factor (MIF) effects, can reverse clinical and histological signs of EAE. These properties of partial class II constructs are highly compatible with a flow cytometry approach for monitoring CD74 expression as a possible biomarker for disease activity/progression and as a treatment response marker.

Introduction

Multiple sclerosis (MS) is a complex demyelinating disease with an autoimmune origin (Sospedra and Martin 2005; Steinman 2001). Treating and managing such disease require informative data regarding the immunological processes that contribute to the pathogenesis of the disease and the clinical outcome of possible treatment reagents.

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Conflict of interest

Drs. Vandenberg, Benedek, Meza-Romero and OHSU have a significant financial interest in Artielle ImmunoTherapeutics, Inc., a company that may have a commercial interest in the results of this research and technology. This potential conflict of interest has been reviewed and managed by the OHSU and VAMC Conflict of Interest in Research Committees.

Biomarkers in MS could be used to predict, diagnose, follow disease activity and as treatment-response markers. There are only handful biomarkers that were proven to be clinically useful. Most of these biomarkers are protein-based, which measure humoral-immune responses and are evaluated by enzyme-linked immunosorbent assay (ELISA) (Freedman et al. 2005; Lennon et al. 2004; Polman et al. 2010).

One of the main challenges in this field of identifying new and reliable biomarkers is to correlate the changes in the biomarker levels to disease activity and to the treatment response. During the development of a new immunotherapeutic reagent flow cytometry could be a powerful technique that is used in the path from bench to bedside, especially if similar parameters that were used during the developing stage to assess drug efficacy in in-vitro and in animal models could be used as clinical biomarker.

We designed partial MHC class II $\beta 1\alpha 1$ constructs (termed Recombinant T-cell receptor ligands- RTL) with covalently tethered myelin peptides that could inhibit and treat experimental autoimmune encephalomyelitis (EAE) (Burrows et al. 1999; Sinha et al. 2010a; Vandenbark et al. 2003; Wang et al. 2006). The use of flow cytometry based assays enabled us to identify the predominant cell population that bind these constructs and to follow the changes in the partial MHC class II native cell surface receptor, the class II invariant chain (CD74), during the course of EAE and correlate the treatment response with this potential disease biomarker (Benedek et al. 2013). We further identified the DR $\alpha 1$ domain, but not the DR2 $\beta 1$ domain as the RTL component that binds and down-regulates CD74 (Meza-Romero et al. 2014; Vandenbark et al. 2013).

One such DR2 construct, RTL1000, containing the human MOG-35–55 peptide was recently tested successfully in a phase 1 clinical trial in MS subjects (Yadav et al. 2012). This construct is about to be tested in a phase 1/2 clinical trial in progressive MS subjects. Thus, measuring CD74 cell surface expression levels on CD11b+ monocytes by flow cytometry to collect interpretable, reproducible and reliable data in this study will further implicate CD74 as a possible biomarker for disease activity/progression and as a treatment response marker.

Partial MHC class II constructs

RTLs, MHC class II $\beta 1\alpha 1$ constructs of relevant class II molecules (HLA-DR*1501 for MS), were designed and developed with covalently tethered myelin peptides (Burrows et al. 1999). These constructs could interact directly with the cognate TCR and act as partial TCR agonists and trigger suboptimal downstream signaling, cytokine shift and loss of encephalitogenic response (Burrows et al. 2001; Sinha et al. 2009). RTL treatment of EAE was established previously in different models of the disease including DR*1501-Tg mice that develop EAE only after injection of mouse (m)MOG-35–55 peptide (Vandenbark et al. 2003), DR*1502-Tg mice that develop EAE after injection of human (h)MOG-35–55 peptide (Sinha et al. 2010b), MBP-TCR/DR2-Tg mice that develop EAE after injection of MBP-85–99 peptide (Vandenbark et al. 2013), C57BL/6 WT mice that develop EAE after injection of mouse (m)MOG-35–55 peptide and SJL/J mice that develop EAE after injection of PLP-139–151 peptide (Huan et al. 2004; Sinha et al. 2007). Recently, we developed

DR α 1 constructs that are covalently tethered to different myelin peptides. Because the DR α 1 domain is invariable and would not be recognized as an immunologically foreign antigen, treatment with DR α 1 constructs would not require HLA screening prior to injection. We demonstrated that these novel constructs could treat different central nervous system (CNS) diseases that involve migration of immune cells from the periphery to both the spinal cord (EAE) and brain (stroke) (Benedek et al. 2014; Meza-Romero et al. 2014).

Partial MHC class II constructs bind to cell surface CD74 on monocytes

A significant milestone in understanding the partial MHC class II construct mechanism of action was the identification by FACS of CD11b⁺ monocytes as the major cell population that bind the constructs after i.v. injection of naïve DR*1501-Tg mice with RTL342M (pDR2/mMOG-35–55)-FITC or RTL1000 (pDR2/hMOG-35–55)-FITC, with only modest binding by B-cells, T-cells and DC (Vandenbark et al. 2013). This cellular binding accounted for rapid partitioning of soluble RTL from the plasma to the blood cellular compartment after injection into mice with EAE and MS subjects and the identification of the binding components on the surface of these cells (Dahan et al. 2011).

Monocytes and macrophages play a pathogenic role in multiple sclerosis. EAE, a well-established model of MS, is characterized by extensive lymphocytic infiltration into the CNS (Ajami et al. 2011; Sospedra and Martin 2005). Several reports have demonstrated that monocytes are involved in the exacerbation of EAE, with monocyte depletion resulting in a marked suppression of clinical disease (Bauer et al. 1995; Brosnan et al. 1981; Huitinga et al. 1990).

The RTL binding proteins were identified by a pull-down assay using the RTL1000 construct. This analysis revealed at least four distinct bands in addition to RTL1000 that were either enriched (15kD and 18kD) or could not be detected without RTL1000 (31kD and 72kD). Elution of parallel bands from an unstained sample and analysis by LC-MS/MS identified the major RTL-binding proteins as H4 histone (14kD), H2A, H2B and H3 histones (18kD), CD74 (31kD) and MHC class II (72kD). The 72kD class II sequence was from the H-2E α 2 domain that was derived from the expressed DR2-transgene and not from the RTL1000 construct. To confirm involvement of CD74 as a major component of the RTL receptor, CD74 was immunoprecipitated from biotinylated DR*1501-Tg splenocyte membrane preparations. The moiety of RTL that binds CD74 was further determined to be the DR α 1 but not the DR2 β 1 domain (Vandenbark et al. 2013).

CD74 is a type II transmembrane glycoprotein whose structure contains a trimerization domain flanked by two highly unstructured regions (Cresswell 1996; Jasanoff et al. 1999). Earlier models suggested that the homotrimeric structure could bind up to three $\alpha\beta$ MHC class II heterodimers to form a nonameric complex, Ii₃($\alpha\beta$)₃ (Lamb and Cresswell 1992; Roche et al. 1991). More recent models propose a pentameric complex, with the Ii homotrimer chaperoning a single MHC class II heterodimer from the ER (Koch et al. 2011; Neumann and Koch 2005). While the structure of MHC class II bound to CD74 has not yet been solved, interactions between CD74 and the $\alpha\beta$ MHC class II heterodimer have been

mapped to at least three discrete extracellular locations and the transmembrane domains (Ashman and Miller 1999; Dixon et al. 2006; King and Dixon 2010).

The class II invariant chain not only chaperones peptide-loaded MHC class II molecules from intracellular compartments to the surface of antigen-presenting cells but when expressed as cell surface CD74 also functions as the receptor for macrophage migration inhibitory factor (MIF) (Leng et al. 2003). MIF engagement of CD74 leads to the recruitment and activation of CD44 and CXCR2/4 to initiate signaling pathways necessary for MAPK activation and cell motility (Bernhagen et al. 1993; Bernhagen et al. 2007; Calandra and Roger 2003; Fan et al. 2011; Liu et al. 2010; Schwartz et al. 2009; Shi et al. 2006).

CD74 expression is up-regulated in EAE and MS

We recently demonstrated enhanced CD74 cell surface expression on monocytes in mice with EAE and subjects with MS, which implicates its involvement in the disease course (Benedek et al. 2013)[10]. CD74 was shown to be up-regulated in peripheral blood monocytes by 3 hours after EAE induction, ~10 days before the appearance of definite clinical signs. In contrast, CD74 up-regulation on resting microglia and infiltrating monocytes and activated microglia in the CNS was correlated with the appearance of definite clinical signs of EAE. This up-regulation of CD74 expression in the CNS is associated directly with induction of inflammation by blood-borne monocytes. The attenuation in CD74 expression that was observed in the CNS could be associated with the attenuation in disease severity that occurs after the disease peak in the DR2-Tg mouse strain (Figure 1A and B).

In order to ascertain the potential relevance of the above observations in MS, we determined whether subjects with MS had elevated levels of CD74. We evaluated stored PMBC samples from a cohort of eight HLA-DR2 positive MS subjects from our previous clinical study vs. eight HLA-DR2 positive, age and gender-matched healthy controls (HC) and found significantly higher expression levels of CD74 on CD11b⁺ monocytes in MS subjects vs. HC donors. These data correlate with the data obtained from the mouse MS model (Benedek et al. 2013) (Figure 1C).

We are currently continuing to assess the importance of the above observations in a larger cohort of MS subjects; we aim to determine by flow cytometry whether subjects with MS have elevated levels of CD74 on peripheral CD11b⁺ cells. This study includes evaluating the levels of CD74 in healthy control (HC) subjects, and subjects from each MS disease subgroup: clinical isolated syndrome (CIS), relapsing-remitting (RR-MS), secondary-progressive (SP-MS) and primary progressive (PP-MS), that are being treated with or without disease modifying treatments (DMT). In this regard, it would be interesting to evaluate CD74 expression levels on monocytes that are isolated from the cerebrospinal fluid (CSF) as a possible correlate of changes taking place within the CNS. Although, the frequency of CSF monocytes is reduced in MS subjects compared with HC, CD74 levels could be up-regulated on these cells (Cepok et al. 2001; Kowarik et al. 2014).

Partial MHC class II constructs down regulate CD74 expression on monocytes and inhibit MIF signaling effects

In-vitro treatment of CD11b⁺ cells with all the partial MHC class II constructs leads to down regulation of cell surface CD74, both on mouse cells and human cells. Using labeled constructs, we demonstrated an inverse correlation between CD74 expression and RTL binding to mouse monocytes which could be inhibited by an Fab construct that is specific for binding the DR α 1 domain (FabG4), (Figure 2A,B). These studies revealed a dose-dependent hierarchy of partial MHC class II constructs, the most active being those that are linked to the MOG-35–55 peptide (mouse or human). It is interesting to note that the DR2 β 1 domain, which does not bind CD74, did not down-regulate CD74 expression levels (Meza-Romero et al. 2014; Vandenbark et al. 2013). Furthermore, CD74 expression on human CD11b⁺ monocytes from HC and MS subjects was down-regulated after treatment with RTL1000 (Figure 2C). The binding of the labeled partial MHC class II constructs to GFP⁺ monocytes could also be visualized by fluorescent microscopy, as shown in Figure 2D.

In addition to down regulating CD74 expression levels, binding of the DR α 1 domain to CD74 inhibited MIF binding and signaling. Blocking of MIF binding, by partial MHC class II construct, either to immunoprecipitated mouse CD74 or to human PBMC was detected by western blot or by flow cytometry, respectively, using Alexa-488 labeled recombinant MIF protein. This inhibition of MIF binding resulted in profound downstream effects on EAE, including inhibition of cell migration to the CNS, secretion of pro-inflammatory cytokines and enhanced survival of activated monocytes (Benedek et al. 2013; Meza-Romero et al. 2014).

These effects were manifested after treating EAE mice with partial MHC class II constructs as a significantly increased number of infiltrating cells into the CNS, a reduced frequency of activated microglia and infiltrating monocytes (CD11b⁺CD45^{high}) and a less inflammatory milieu within the CNS.

Fab blockade of partial MHC class II down regulation of CD74 on monocytes neutralizes its treatment effects on EAE

To determine if down regulation of CD74 on monocytes was necessary for treatment of EAE, Fab1B11 selected for binding to 2-domain DR2 molecules and a control, FabD2, reactive to an irrelevant (2 domain DR4/GAD-555–567) construct were evaluated for their respective abilities to block RTL-induced down regulation of CD74 expression and thus to neutralize RTL treatment effects. Incubation of RTL342M (DR2/mMOG-35–55) with Fab1B11 but not FabD2 at a 1:1 or 1:2 molar ratio for 2h resulted in a significant (~60%) blockade of RTL342M-induced down regulation of CD74 expression on CD11b⁺ monocytes *in vitro* and a comparable (~60%) neutralization of the protective activity of RTL342M *in vivo* after 3 daily s.c. injections into mice with EAE. These combined selective effects of Fab1B11 vs. FabD2 demonstrate that RTL binding and down regulation of CD74 is necessary for its therapeutic activity on EAE (Vandenbark et al. 2013).

Partial MHC class II constructs can inhibit EAE in an antigenic peptide-independent manner

The ability of various partial MHC class II constructs to modulate cell surface expression of CD74 is reflected in their clinical efficacy in treating EAE. The RTL342M (DR2/mMOG-35–55) and RTL340 (DR2/MBP-85–99) constructs have comparable therapeutic activity when treating EAE induced with their cognate encephalitogenic peptides. However, RTL constructs containing MOG-35–55 peptides differ from RTL340 containing MBP-85–99 in their remarkable ability to treat EAE induced by non-cognate encephalitogenic peptides. That is, whereas RTL340 containing MBP-85–99 peptide could not treat mice with MOG peptide-induced EAE, both RTL342M and RTL1000 constructs containing MOG-35–55 peptide could fully treat mice with MBP peptide-induced disease. These treatment effects on EAE of different RTL constructs were directly related to their ability to down-regulate CD74. Indeed, 10-fold higher doses of the “empty” DR2 construct, RTL302-5D, that possessed a weaker ability to down-regulate CD74, produced a significant peptide-independent treatment effect on mMOG-35–55 induced EAE (Vandenbark et al. 2013).

A similar hierarchy of efficacy was observed with the DR α 1 and the DR α 1–MOG constructs, in which the latter had a significantly more potent effect in down regulating CD74 on human CD11b⁺ monocytes and was 50 $\times$ more potent in treating EAE (Meza-Romero et al. 2014). Subsequent comparisons established a significant structure-activity relationship (SAR) between CD74 levels on CNS-derived CD11b⁺ cells and EAE severity measured by the cumulative disease index (CDI), which clearly established RTL342M as the most effective construct and predicted dose-dependent therapeutic activity of other constructs commensurate with their ability to modulate CD74 levels (Figure 3). The SAR is further supported by the lack of treatment effect of the DR2 β 1 construct, which does not bind to CD74 nor down regulate its expression levels. We could further demonstrate that there was a significant correlation between the disease severity (CDI) and levels of demyelination in spinal cord (manuscript in preparation). Hence, FACS assessment of CD74 cell surface down regulation by the different partial MHC class II construct could predict their clinical efficacy and could be correlated to demyelination levels in EAE. Although this sort of SAR could not be confirmed using CNS monocytes from MS patients, it is conceivable that CD74 expression levels on CSF cells might correlate with MS disease severity.

Challenges in application of CD74 as a biomarker in the upcoming RTL1000 Phase 2 clinical trial

Most patients with MS initially have a relapsing remitting course during which focal inflammatory lesions composed of T-cells and macrophages episodically form within the CNS, resulting in focal demyelination and axonal injury. These inflammatory lesions cause clinical relapses when they develop in functionally critical CNS pathways. Most patients (85%) start with a relapsing remitting course (RRMS), although 15% have primary progressive MS (PPMS) in which there is gradual progression of impairment and disability from the onset without an initial relapsing remitting phase. Ten to 15 years after the onset of

their MS, over 50% of patients with relapsing remitting MS (RRMS) develop secondary progressive MS (SPMS). The steady clinical worsening in secondary progressive MS appears to be the result of progressive degeneration of axons and neurons, possibly secondary to the effects of soluble mediators of inflammation released by activated microglia within the CNS (Sospedra and Martin 2005; Steinman 2001). While there are several FDA approved therapies approved for the treatment of RRMS, the only approved drug for SPMS is a chemotherapy drug, mitoxantrone, which has limited benefit and is highly toxic (Gonsette 2003).

The upcoming clinical trial will investigate the use of a RTL1000 as a unique approach to treating SPMS. In this Phase 2 study, RTL1000 at 20 mg or 60 mg per dose is to be administered every 4 weeks over 96 weeks in order to assess the safety and efficacy in participants with secondary progressive MS. Change in whole brain volume measured by MRI, which has been shown to be a key metric of disease progression in MS, will serve as the primary outcome in this study. In addition, one of the secondary objectives of this study will be determined if RTL1000 efficacy could be correlated to the expression of CD74 on monocytes in human subjects that were treated with RTL1000.

This objective is in line with the increasing interest in identifying biomarkers in MS that could predict therapeutic response. This research avenue is very complex due to the understanding that not all patients are likely to have the same response to a certain treatment. Although this field is rapidly evolving there are number of challenges that investigators and clinicians are still facing: 1) In order to validate the biomarker the clinical study should have enough statistical power and be reproduced and replicated. 2) It is advantageous that the biomarker would be analyzed with a minimal invasive procedure and also would require minimal and quick processing. However, there are critical steps while processing the samples for flow cytometry analysis that could affect viability, yield and immunological functions of the cells which include different shipping conditions of blood samples (temperature, time of blood processing, and freezing and thawing procedures) if the analysis is not performed in the same facility. In addition, the detected signal could vary between processed (PBMC) and unprocessed (whole blood cells) samples: CD74 cell surface expression levels on CD11b⁺ monocytes differ between whole blood cells vs. Ficoll isolated PBMC from the same subject (Figure 4A). Furthermore, the processing time of the sample could affect the biomarker levels: CD74 expression levels on CD11b⁺ monocytes decrease if the sample is kept at room temperature and processed and analyzed 24 hr vs. 48 hr later (Figure 4B). Hence, although it may sound trivial, no matter what the biological source of the samples being analyzed is, the most important step is following the same protocol in each of the different centers that are participating in the clinical trial. 3) Most of the MS subjects that are enrolled in such clinical trials are already being treated with disease modifying therapies (DMTs). Thus, it could be difficult to distinguish between the DMT effect and the drug effect on the biomarker. In order, to gain insight on DMT's effect on CD74 expression levels in MS subjects, we are currently conducting a clinical study to assess CD74 expression on CD11b⁺ monocytes in different MS subtypes that are either treated or not treated with DMTs.

Conclusions

Multiple sclerosis is a complex autoimmune disease with only a handful of clinically available therapies that are mostly effective for RR-MS subjects and not for progressive patients. There are even fewer clinically available biomarkers that could be used to monitor disease activity and treatment response. We have implicated the binding, down regulation of CD74 and blocking of MIF binding on CD11b⁺ monocytes as the crucial early event in partial MHC class II construct treatment of EAE as demonstrated in Figure 5. We further suggest assessment of CD74 levels as an ex-vivo biomarker for disease activity as well as efficacy of these constructs. Flow cytometry is a powerful and versatile technique for analysis of different parameters of individual cells. We envision that modulation of CD74 will provide significant therapeutic effects in MS and monitoring CD74 expression levels by flow cytometry could be used to guide optimal dose selection and to determine treatment efficacy in different patients.

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References

- Ajami B, Bennett JL, Krieger C, McNagny KM, Rossi FM. Infiltrating monocytes trigger EAE progression, but do not contribute to the resident microglia pool. *Nat Neurosci.* 2011; 14:1142–1149. [PubMed: 21804537]
- Ashman JB, Miller J. A role for the transmembrane domain in the trimerization of the MHC class II-associated invariant chain. *J Immunol.* 1999; 163:2704–2712. [PubMed: 10453012]
- Bauer J, Huitinga I, Zhao W, Lassmann H, Hickey WF, Dijkstra CD. The role of macrophages, perivascular cells, and microglial cells in the pathogenesis of experimental autoimmune encephalomyelitis. *Glia.* 1995; 15:437–446. [PubMed: 8926037]
- Benedek G, et al. Partial MHC class II constructs inhibit MIF/CD74 binding and downstream effects. *Eur J Immunol.* 2013; 43:1309–1321. [PubMed: 23576302]
- Benedek G, et al. A novel HLA-DRalpha1-MOG-35–55 construct treats experimental stroke. *Metabolic brain disease.* 2014; 29:37–45. [PubMed: 24122483]
- Bernhagen J, et al. MIF is a pituitary-derived cytokine that potentiates lethal endotoxaemia. *Nature.* 1993; 365:756–759. [PubMed: 8413654]
- Bernhagen J, et al. MIF is a noncognate ligand of CXC chemokine receptors in inflammatory and atherogenic cell recruitment. *Nat Med.* 2007; 13:587–596. [PubMed: 17435771]
- Brosnan CF, Bornstein MB, Bloom BR. The effects of macrophage depletion on the clinical and pathologic expression of experimental allergic encephalomyelitis. *J Immunol.* 1981; 126:614–620. [PubMed: 6256443]
- Burrows GG, Chang JW, Bachinger HP, Bourdette DN, Offner H, Vandenbark AA. Design, engineering and production of functional single-chain T cell receptor ligands. *Protein engineering.* 1999; 12:771–778. [PubMed: 10506287]
- Burrows GG, et al. Rudimentary TCR signaling triggers default IL-10 secretion by human Th1 cells. *J Immunol.* 2001; 167:4386–4395. [PubMed: 11591763]
- Calandra T, Roger T. Macrophage migration inhibitory factor: a regulator of innate immunity. *Nat Rev Immunol.* 2003; 3:791–800. [PubMed: 14502271]

- Cepok S, et al. Patterns of cerebrospinal fluid pathology correlate with disease progression in multiple sclerosis. *Brain : a journal of neurology*. 2001; 124:2169–2176. [PubMed: 11673319]
- Cresswell P. Invariant chain structure and MHC class II function. *Cell*. 1996; 84:505–507. [PubMed: 8598037]
- Dahan R, et al. TCR-like antibodies distinguish conformational and functional differences in two-versus four-domain auto reactive MHC class II-peptide complexes. *Eur J Immunol*. 2011; 41:1465–1479. [PubMed: 21469129]
- Dixon AM, Stanley BJ, Matthews EE, Dawson JP, Engelman DM. Invariant chain transmembrane domain trimerization: a step in MHC class II assembly. *Biochemistry*. 2006; 45:5228–5234. [PubMed: 16618111]
- Fan H, et al. Macrophage migration inhibitory factor and CD74 regulate macrophage chemotactic responses via MAPK and Rho GTPase. *J Immunol*. 2011; 186:4915–4924. [PubMed: 21411731]
- Freedman MS, et al. Recommended standard of cerebrospinal fluid analysis in the diagnosis of multiple sclerosis: a consensus statement. *Archives of neurology*. 2005; 62:865–870. [PubMed: 15956157]
- Gonsette RE. Mitoxantrone in progressive multiple sclerosis: when and how to treat? *Journal of the neurological sciences*. 2003; 206:203–208. [PubMed: 12559512]
- Huan J, et al. Monomeric recombinant TCR ligand reduces relapse rate and severity of experimental autoimmune encephalomyelitis in SJL/J mice through cytokine switch. *J Immunol*. 2004; 172:4556–4566. [PubMed: 15034073]
- Huitinga I, van Rooijen N, de Groot CJ, Uitdehaag BM, Dijkstra CD. Suppression of experimental allergic encephalomyelitis in Lewis rats after elimination of macrophages. *J Exp Med*. 1990; 172:1025–1033. [PubMed: 2145387]
- Jasanoff A, Song S, Dinner AR, Wagner G, Wiley DC. One of two unstructured domains of Ii becomes ordered in complexes with MHC class II molecules. *Immunity*. 1999; 10:761–768. [PubMed: 10403651]
- King G, Dixon AM. Evidence for role of transmembrane helix-helix interactions in the assembly of the Class II major histocompatibility complex. *Molecular bioSystems*. 2010; 6:1650–1661. [PubMed: 20379596]
- Koch N, Zacharias M, König A, Temme S, Neumann J, Springer S. Stoichiometry of HLA class II-invariant chain oligomers. *PLoS One*. 2011; 6:e17257. [PubMed: 21364959]
- Kowarik MC, Grummel V, Wemlinger S, Buck D, Weber MS, Berthele A, Hemmer B. Immune cell subtyping in the cerebrospinal fluid of patients with neurological diseases. *Journal of neurology*. 2014; 261:130–143. [PubMed: 24162037]
- Lamb CA, Cresswell P. Assembly and transport properties of invariant chain trimers and HLA-DR-invariant chain complexes. *J Immunol*. 1992; 148:3478–3482. [PubMed: 1588042]
- Leng L, et al. MIF signal transduction initiated by binding to CD74. *J Exp Med*. 2003; 197:1467–1476. [PubMed: 12782713]
- Lennon VA, et al. A serum autoantibody marker of neuromyelitis optica: distinction from multiple sclerosis. *Lancet*. 2004; 364:2106–2112. [PubMed: 15589308]
- Liu L, et al. CXCR2-positive neutrophils are essential for cuprizone-induced demyelination: relevance to multiple sclerosis. *Nat Neurosci*. 2010; 13:319–326. [PubMed: 20154684]
- Meza-Romero R, et al. HLA-DR α 1 constructs block CD74 expression and MIF effects in experimental autoimmune encephalomyelitis. *J Immunol*. 2014; 192:4164–4173. [PubMed: 24683185]
- Neumann J, Koch N. Assembly of major histocompatibility complex class II subunits with invariant chain. *FEBS Lett*. 2005; 579:6055–6059. [PubMed: 16242130]
- Polman CH, et al. Recommendations for clinical use of data on neutralising antibodies to interferon-beta therapy in multiple sclerosis. *The Lancet Neurology*. 2010; 9:740–750. [PubMed: 20610349]
- Roche PA, Marks MS, Cresswell P. Formation of a nine-subunit complex by HLA class II glycoproteins and the invariant chain. *Nature*. 1991; 354:392–394. [PubMed: 1956401]
- Schwartz V, et al. A functional heteromeric MIF receptor formed by CD74 and CXCR4. *FEBS Lett*. 2009; 583:2749–2757. [PubMed: 19665027]

- Shi X, et al. CD44 is the signaling component of the macrophage migration inhibitory factor-CD74 receptor complex. *Immunity*. 2006; 25:595–606. [PubMed: 17045821]
- Sinha S, et al. 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. *J Neuroimmunol*. 2010a; 225:52–61. [PubMed: 20546940]
- Sinha S, et al. Recombinant TCR ligand reverses clinical signs and CNS damage of EAE induced by recombinant human MOG. *Journal of neuroimmune pharmacology : the official journal of the Society on Neuro Immune Pharmacology*. 2010b; 5:231–239.
- Sinha S, et al. Cytokine switch and bystander suppression of autoimmune responses to multiple antigens in experimental autoimmune encephalomyelitis by a single recombinant T-cell receptor ligand. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2009; 29:3816–3823. [PubMed: 19321778]
- Sinha S, et al. A promising therapeutic approach for multiple sclerosis: recombinant T-cell receptor ligands modulate experimental autoimmune encephalomyelitis by reducing interleukin-17 production and inhibiting migration of encephalitogenic cells into the CNS. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2007; 27:12531–12539. [PubMed: 18003831]
- Sospedra M, Martin R. Immunology of multiple sclerosis. *Annual review of immunology*. 2005; 23:683–747.
- Steinman L. Multiple sclerosis: a two-stage disease. *Nat Immunol*. 2001; 2:762–764. [PubMed: 11526378]
- Vandenbark AA, et al. A novel regulatory pathway for autoimmune disease: binding of partial MHC class II constructs to monocytes reduces CD74 expression and induces both specific and bystander T-cell tolerance. *J Autoimmun*. 2013; 40:96–110. [PubMed: 23026773]
- Vandenbark AA, et al. Recombinant TCR ligand induces tolerance to myelin oligodendrocyte glycoprotein 35–55 peptide and reverses clinical and histological signs of chronic experimental autoimmune encephalomyelitis in HLA-DR2 transgenic mice. *J Immunol*. 2003; 171:127–133. [PubMed: 12816990]
- Wang C, et al. Antigen-specific therapy promotes repair of myelin and axonal damage in established. EAE *J Neurochem*. 2006; 98:1817–1827.
- Yadav V, et al. Recombinant T-Cell Receptor Ligand (RTL) for Treatment of Multiple Sclerosis: A Double-Blind, Placebo-Controlled, Phase 1, Dose-Escalation. *Study Autoimmune Dis*. 2012; 2012:954739.

Figure 1. CD74 expression is up-regulated in EAE and MS subjects

A–B) DR*1501-Tg mice were immunized with mMOG-35–55/CFA+PTx. Blood macrophages (F4/80⁺CD11b⁺) and activated microglia and macrophages (CD11b⁺CD45^{high}) in spinal cord were analyzed for CD74 expression at different time points (n=3 at each time point). Error bars denote mean $\pm$ SD (# p<0.05, ## p<0.01, ### p<0.001 vs. control, * p<0.05, ** p<0.01, *** p<0.001). C) Human PBMC from healthy controls (n=8) and MS subjects (n=8) were analyzed for CD74 expression on CD11b⁺ monocytes. Error bars denote mean $\pm$ SD (** p<0.01).

Figure 2. Partial MHC class II constructs down regulate CD74 expression on monocytes

A) Binding of Alexa 488 labeled DRα1 construct to monocytes and down regulation of CD74 cell surface expression (left panel) and the inhibitory effect on CD74 expression of the specific G4 Fab against the DRα1 domain (right panel). B) linear regression analysis of dose-dependent binding of different RTL constructs *in vitro* with PBMC collected from naïve DR*1501-Tg mice. CD11b⁺ monocytes were evaluated for RTL binding and CD74 expression by FACS. C) PBMC from healthy controls (n=8) and MS subjects (n=8) were incubated with 10 μg of RTL1000 for 1h and analyzed for CD74 expression on CD11b⁺ monocytes. Error bars denote mean ± SD (** p<0.01, *** p<0.001). D) Isolated GFP⁺CD11b⁺ cells from DR*1501/GFP-Tg mice were treated with 10μg DRα1 Alexa546 for 60 min and evaluated by fluorescence microscopy.

Figure 3. Structure-activity relationship (SAR) of RTL modulation of CD74 and EAE severity CD74 levels were assessed on CNS-derived CD11b⁺ cells from naïve DR*1501-Tg mice (background levels of CD74 with no clinical EAE) and mMOG-35–55 immunized DR*1501-Tg mice treated with RTL342M (DR2/mMOG-35–55) “empty” RTL302-5D or Vehicle (maximal disease-induced levels of CD74 with no treatment effect) 15 days after induction of EAE and plotted versus the cumulative disease index of each group. CD74 expression levels were normalized to the Vehicle treated group.

Figure 4. Sample processing conditions that are affecting CD74 expression

CD74 levels were assessed on human CD11b⁺ cells from: A) Whole blood cells that were isolated by red blood cells lysis (black line) versus Ficoll isolated PBMC (grey line). B) Whole blood cells that were isolated by red blood cells lysis 1 hr (full black line), 24 hr (full gray line) or 48 hr (dotted black line) after blood draw. (samples were kept at room temperature).

Figure 5. Partial MHC class II mechanism of action in EAE