Published in final edited form as:

Neuroscience. 2015 March 12; 288: 112–119. doi:10.1016/j.neuroscience.2014.12.037.

Recombinant T-cell Receptor Ligand RTL1000 Limits inflammation and Decreases Infarct Size after Experimental Ischemic Stroke in Middle-Aged Mice

W. Zhu^{a,*}, A. L. Dotson^{c,d,*}, N. L. Libal^a, A. S. Lapato^{c,d}, S. Bodhankar^{c,d}, H. Offner^{a,c,d}, and N. J. Alkayed^{a,b,c}

^aDept. Anesthesiology & Perioperative Medicine, Oregon Health & Science University, Portland, OR, 97239, USA

^bThe Knight Cardiovascular Institute, Oregon Health & Science University, Portland, OR, 97239, USA

^cDept. Neurology, Oregon Health & Science University, Portland, OR, 97239, USA

^dNeuroimmunology Research, Portland Veterans Affairs Medical Center, Portland, OR, 97239, USA

Abstract

We have previously demonstrated that recombinant T-cell receptor ligand 1000 (RTL1000) reduces infarct size and improves long-term functional recovery after experimental stroke in young transgenic mice expressing human leukocyte antigen DR2 (DR2-Tg). In this study, we determined the effect of RTL1000 on infarct size in 12-month old middle-aged DR2-Tg mice, and investigated its mechanism of action. Twelve months old male DR2-Tg mice underwent 60 min of intraluminal reversible middle cerebral artery occlusion (MCAO). Vehicle or RTL1000 was injected 4, 24, 48 and 72 h after MCAO. Cortical, striatal and total hemispheric infarcts were measured 96 h after stroke. The spleen and brain tissue were collected 96 h after stroke for immunological analysis. Our data showed that RTL1000 significantly reduced infarct size 96 h after MCAO in middle-aged male DR2-Tg mice. RTL1000 decreased the number of activated monocytes/microglia cells (CD11b⁺CD45^{hi}) and CD3⁺ T cells in the ischemic hemisphere.

© 2014 IBRO. Elsevier Ltd. All rights reserved.

Corresponding Author: Nabil J. Alkayed, MD, PhD, Department of Anesthesiology & Perioperative Medicine and The Knight Cardiovascular Institute, Oregon Health & Science University, Portland, OR, 97239, USA. Tel.: +1-503-418-5502; fax: +1 503-494-3092; alkayedn@ohsu.edu.

*Contributed equally to this work

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Conflict Of Interest

Dr. Offner, Dr. Alkayed 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.

Wenbin Zhu declares that he has no conflict of interest. Abby L. Dotson declares that she has no conflict of interest. Nicole L. Libal declares that she has no conflict of interest. Andrew S. Lapato declares that he has no conflict of interest. Sheetal Bodhankar declares that she has no conflict of interest.

RTL1000 also reduced the percentage of total T cells and inflammatory neutrophils in spleen. These findings suggest that RTL1000 protects against ischemic stroke in middle-aged male mice by limiting post-ischemic inflammation.

Keywords

Ischemic stroke; Immunotherapy; Recombinant T-cell receptor Ligand; HLA-DR2 transgenic mice

INTRODUCTION

Experimental stroke induces rapid activation of the peripheral immune system, which contributes to the brain's inflammatory response to stroke (Nilupu Perera et al., 2006; Dirnagl et al., 2007; Gee et al., 2007; Muir et al., 2007). The migration of monocytes, neutrophils and T cells into brain with the breakdown of blood-brain barrier contributes to the further activation of resident microglial cells and the expansion of brain tissue infarction. Among these cells, T cells are found in brain within hours after experimental stroke, which may play a significant role in exacerbating ischemic injury. T- and B-cell deficient mice sustain smaller lesion size and reduced inflammation after experimental stroke (Hurn et al., 2007), with CD4⁺ and CD8⁺ T lymphocytes playing a particularly important role in the inflammatory and thrombogenic response associated with experimental stroke by promoting an autoaggressive response to brain antigens (Yilmaz et al., 2006). It is believed that myelin-reactive antigens leak out of brain with the breakdown of blood-brain barrier, which is recognized by the immune system as a foreign antigen, leading to the recruitment of T cells into brain. These conclusions are supported by the findings of increased influx of myelin oligodendrocyte glycoprotein (MOG)-specific T cells into brain and of reduced infarct size after stroke by nasal vaccination with a MOG peptide (Frenkel et al., 2003).

Recombinant T cell ligands (RTLs) are a class of partial major histocompatibility complex (MHC) class II molecules comprised of covalently linked $\alpha 1$ and $\beta 1$ chains that are tethered to a MOG peptide (Burrows et al., 1999; Wang et al., 2003; Vandenbark et al., 2003). We have previously demonstrated that RTL551, a mouse MHC moiety (I-A^b) coupled to mouse myelin peptide (mMOG-35-55), reduces infarct size in 3-month old young adult C57BL/6 mice (Dziennis et al., 2011; Subramanian et al., 2009). The action mechanism involves in selective modulation of auto-aggressive CD4⁺ T cells by delivering partial agonist signals through the T cell receptor (TCR), and further inhibition of the accumulation of other inflammatory cells, particularly macrophages/activated microglial cells and dendritic cells, a kind of antigen presenting cells that assist with activation of T cells in brain.

We have previously found that RTL treatment is antigen-specific and MHC-specific. Our data show that RTL553, which has the same MHC moiety as RTL551 but is linked to a non-neuroantigen peptide (I-Ea-52-68), had no effect on infarct size in C57BL/6 mice. Similarly, RTL treatment with RTL342M, which has the same mMOG-35-55 peptide as RTL551 but a different MHC II moiety (HLA-DR2), failed to reduce infarct size (Dziennis et al., 2011). These findings indicate that RTL551 may not work in patients with stroke considering species-differences (murine vs. human) in antigens and MHC II molecules. To determine if

a RTL strategy would work against human stroke, we determined the efficacy of humanized RTL1000, which contains a human MHC moiety (HLA-DR2) covalently linked to a human myelin peptide (hMOG-35-55) in experimental stroke in humanized DR2-Tg mice which expresses human TCR (Subramanian et al., 2009; Zhu et al., 2014A). We found that RTL1000 indeed protects against ischemic injury in young male DR2-Tg mice. Behavioral testing showed that RTL1000 improves long-term cognitive function 28 days after stroke (Zhu et al., 2014A). A similar effect has also been demonstrated in young female DR2-Tg mice (Pan et al., 2014). We also confirmed that combining RTL1000 with t-PA does not alter its ability to reduce infarct in experimental ischemic stroke (Zhu et al., 2014B).

It's well known that ischemic stroke risk increases with age, and stroke is most common in the aging population. Unfortunately, most animal studies, including our own previous study using RTL1000 were conducted in healthy young mice. The Stroke Therapy Academic Industry Roundtable (STAIR) has identified age as an important factor to be considered in developing therapeutic agents for the treatment of stroke (STAIR, 1999). In order to meet the preclinical STAIR criteria (STAIR., 1999; Fisher et al., 2009), and to move our research findings closer to clinical practice, in the present study, we evaluated the efficacy of RTL1000 in protecting against ischemic in middle-aged (12 months old) humanized DR2-Tg mice. Furthermore, successful preclinical evaluation requires target validation to ensure outcomes are indeed linked to the purported mechanism of action of the compound (Feuerstein et al., 2008). Therefore, in the current study, we additionally validated that RTL1000 indeed reduces post-ischemic inflammation by limiting the infiltration of inflammatory cells into brain in middle-aged mice.

EXPERIMENTAL PROCEDURES

Ethics Statement

Animal experiments were conducted in accordance with National Institutes of Health guidelines for the use of experimental animals. The protocols were approved by the Animal Care and Use Committee at Oregon Health & Science University and the Portland Veteran Affairs Medical Center. All efforts were made to minimize the number of animals used and their suffering.

Animals and experimental groups

Studies were performed on male HLA-DRB1*1502 transgenic (DR2-Tg) mice (produced at the Portland VA Medical Center with foundation breeders provided by Dr. Chella David (Gonzalez-Gay et al., 1996)) aged 12 months (range from 52 to 54 weeks) and weighing 25.6 to 39.5 grams (a total of $n = 47$). Thirty three mice were used for infarct size analysis (16 in RTL1000 group and 17 in vehicle group) and splenocyte number counting and survival assay. Separate groups of 7 mice per group were used for analysis of cell populations in brain and spleen assay by flow cytometry. Mice were randomly assigned to either RTL1000 or vehicle groups, and investigators were blinded to treatment groups during surgery and tissue analysis.

RTL 1000 production and purification

RTL molecules consist of the $\alpha 1$ and $\beta 1$ domains of MHC II molecule expressed as a single polypeptide with or without antigenic amino terminal extensions (Burrows et al., 1999; Hurn et al., 2005). RTL1000 is a HLA-DRB1*1502 (DR2) molecule linked to human MOG⁻³⁵⁻⁵⁵ peptide (MEVGWYRPPFSRVVHLYRNGK) (Subramanian et al., 2009). RTL1000 was constructed de novo or by sequential site-directed mutagenesis of previous constructs. Protein purification was performed with a 30- to 40-mg yield of purified protein per liter of bacterial cell culture.

RTL1000 Treatment

Mice were randomized to injections of 100 μ L of either RTL1000 (1 μ g/ μ L) or vehicle (5% dextrose in Tris-HCl, pH 8.5) 4 h after stroke by subcutaneous (S.C.) injection, which was followed by 3 injections of the same volume and concentration at 24, 48, and 72 h after MCAO. The dose and injection protocol of RTL1000 was established in our previous studies (Subramanian et al., 2009; Zhu et al., 2014A; Zhu et al., 2014B).

Reversible Middle Cerebral Artery Occlusion

Reversible middle cerebral artery occlusion (MCAO) was induced via the intraluminal filament technique as described previously with slight modifications (Zhu et al., 2010). Mice were anesthetized with isoflurane (5% induction; 1.2% maintenance) using a mask connected with a vaporizer (Isotec 4; Cyprane, England) throughout surgery and during 60 min vascular occlusion until filament withdrawal and initiation of reperfusion. Rectal temperature was monitored and maintained at $36.5 \pm 0.5^{\circ}\text{C}$ throughout surgery with a warm water pad and a heating lamp. Cortical blood flow (CBF) was monitored by laser-Doppler flowmetry (LDF; Model DRT4, Moor Instruments Ltd., Oxford, England). The right lateral common carotid artery (CCA) was exposed and temporarily ligated. The right external carotid artery (ECA) was ligated and cauterized. Ipsilateral MCAO was accomplished by inserting a 6-0 nylon monofilament surgical suture (ETHICON, Inc., Somerville, NJ, USA) with a heat-rounded and silicone-coated (Xantopren comfort light, Heraeus, Germany) tip into the internal carotid artery (ICA) via the ECA stump till the tip of filament reach the beginning of middle cerebral artery (MCA). The successful occlusion of middle cerebral artery was confirmed by abrupt drop, followed by sustained CBF of less than 30% of baseline. The filament was withdrawn and the CCA was released to allow for reperfusion at 60 min occlusion. The mice were then allowed to recover from anesthesia and survived for 96 h following onset of ischemia. Animals were excluded if CBF failed to drop below 30% of baseline during MCAO or due to subarachnoid hemorrhage (SAH).

Determination of Infarct Volume

Animals were euthanized and brains were harvested 96 h after MCAO. Infarct volumes were measured after staining brain slices with 1.2% solution of 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, St. Louis, MO, USA). Four slices of 2-mm-thick coronal sections were incubated in 1.2% TTC for 15 min at 37°C , and then fixed in 10% formalin overnight. Both sides of each stained slice were photographed and evaluated by SigmaScan Pro 5.0 (Jandel, San G, Rarael, CA, USA). Infarct volume was expressed as a percentage of

contralateral structure (cortex, striatum, and hemisphere). To account for the effect of edema, infarct volume was calculated by subtracting the ipsilateral non-infarct region from the total contralateral structure volume, and dividing the difference by the contralateral volume (Zhang et al., 2013).

Leukocyte isolation from brain and spleen

Spleens from individual MCAO-treated mice were removed and a single-cell suspension was prepared by passing the tissue through a 100 μ m nylon mesh (BD Falcon, Bedford, MA). The cells were washed using RPMI 1640 and the red cells lysed using 1 $\times$ red cell lysis buffer (eBioscience, Inc., San Diego, CA) and incubated for 1 min. The cells were then washed with RPMI 1640 and counted. The brain was divided into the ischemic (right) and non-ischemic (left) hemispheres, digested for 60 min with 1 mg/mL Type IV collagenase (Sigma Aldrich, St. Louis, MO) and DNase I (50 mg/ml, Roche Diagnostics, Indianapolis, IN) in RPMI 1640 at 37°C with shaking at 200 rpm. Samples were mixed with a 1 mL pipette every 15 min. The suspension was washed once in RPMI 1640, resuspended in 80% Percoll/overlayed with 40% Percoll and centrifuged for 30 min at 1600 RPM. The cells were then washed with RPMI 1640, counted, and resuspended in staining medium.

Analysis of cell populations by flow cytometry

All antibodies were purchased from commercial vendors (BD Biosciences, San Jose, CA or eBioscience, Inc., San Diego, CA) as published (Schmid et al., 1992). Four-color (FITC, PE, PECy-5 and APC) fluorescence flow cytometry analyses were performed to determine the phenotypes of splenocytes and brain cells. 1×10^6 splenocytes or 2×10^5 brain cells were washed with staining medium (PBS containing 0.1% NaN₃ and 1% bovine serum albumin (Sigma, Illinois)) and incubated with combinations of the following monoclonal antibodies: CD11b (M1/70), CD45 (30-F11), CD11c (HL3), CD19 (1D3), CD3 (145-2C11), CD8 (53-6.7), CD4 (RM4-5), GR-1 (RB6-8C5), Ly6C (AL-21), CD44 (IM7), CCR5 (HM-CCR5), CD25 (7D4) and CD122 (TM- β 1) for 20 min at 4°C. CD11b is an integrin present on myeloid cells and microglia. CD45 is a protein tyrosine phosphatase expressed on all hematopoietic cells except mature erythrocytes and platelets. A high expression of CD45 along with CD11b designate activated monocytes/microglia in the brain. CD3 is expressed on T cells as a part of the T cell receptor complex. CD19 is a cell surface molecule on B cells that assists with antigen based B cell activation. CD11c is an integrin and transmembrane protein found at high levels on most dendritic cells. CD4 is a glycoprotein on a subset of T cells whose function is to help propagate an immune response. CD8 is a transmembrane glycoprotein on a T cell subset called cytotoxic T cells whose effector function is the killing of infected, cancerous or damaged cells.

To identify dead cells, 7-amino-actinomycin D (7-AAD) was added. Data were collected with BD AccuriTM C6 software on a BD AccuriTM C6 (BD Biosciences, San Jose, CA).

Statistical Analysis

Data are presented as mean $\pm$ SEM. Differences in cortical, striatal and total hemispheric infarct sizes between vehicle and RTL1000 groups were determined by 2-way ANOVA with Holm-Sidak analysis. Spleen and brain cell counts and percentages of cellular subtypes for

FACS analyses were analyzed by Student's t-test. Comparison of mortality was performed by Chi-squared test. Statistical analysis of infarct size and mortality was performed using SigmaStat3 statistical software (Systat Software, Inc., Chicago, IL, USA) and the data of cell counts and cellular subtypes was analyzed by GraphPad Prism software (GraphPad, La Jolla, CA). Statistical significance was set at $p < 0.05$.

RESULTS

Animal characteristics, mortality and exclusions

There were no differences in age or baseline body weight between vehicle and RTL1000 treated animals (Table 1). No differences in mortality were found between groups which was 36.4% (8 in 22) in vehicle group (Data not shown), compared to 27.3% (6 in 22) in RTL1000 group ($P = 0.746$). Two mice died at 48h, 4 at 72h and 2 at 96h after stroke in the vehicle group. In the RTL1000 group, 2 mice died at 48h, 3 at 72h and 1 at 96h after MCAO. No animals died within the first 24 h. According to autopsy, all deaths were attributed to massive infarction. Two animals in vehicle group were excluded because CBF was greater than 30% of baseline during MCAO and the other one mouse in RTL1000 group was excluded due to SAH.

RTL1000 reduced infarct size in middle-aged male DR2-Tg mice after experimental ischemic stroke

We tested the effect of RTL1000 on infarct size 96 h after MCAO. As shown in Figure 1A and 1B, male DR2-Tg mice treated with RTL1000 had smaller infarcts compared to vehicle treated mice. Quantitative analysis of TTC-stained brain slices 96 h after ischemia showed that RTL1000 significantly reduced infarct size 96 h after stroke which was $42.6\% \pm 2.3\%$, $73.5\% \pm 2.6\%$ and $30.4\% \pm 1.3\%$ respectively in cortex, striatum and total hemisphere in vehicle treated mice compared to $23.80\% \pm 1.9\%$, $51.8\% \pm 2.6\%$ and $18.1\% \pm 2.1\%$ in RTL1000 treated mice (Fig. 1B: $p < 0.01$ in all regions). There were no significant differences in CBF before, during MCAO or after reperfusion (Data not shown).

RTL1000 decreased inflammatory cells, particularly activated monocytes/microglia and T cells in the brain in middle-aged mice following MCAO

We reported previously that RTL551 reduced inflammatory cell infiltration into brain after MCAO in young C57BL/6 mice. In this study, we found that RTL1000 has a similar effect on experimental stroke in middle-aged DR2-Tg mice.

RTL1000 significantly reduced $CD11b^+CD45^+$ cells, which represent macrophages and activated microglia cells in the ischemic hemisphere of the brain (Fig. 2 A: 54.5% in vehicle treated mice versus 20.3% in RTL1000 treated mice). The absolute numbers of activated monocytes/microglia cells were 30838 ± 9887 in vehicle treated mice versus 5487 ± 768 in RTL1000 treated mice (Fig. 2 B and Table 2: $p < 0.05$). RTL1000 caused a reduction in absolute number of $CD3^+$ T cells in the ischemic hemisphere. Unexpectedly, we also observed a decrease in $CD3^+$ T cells in the non-ischemic hemisphere, which may be related to the specificity of RTL treatment to target T cells and cause a global decrease in that cell

type (Fig. 2 B and Table 2: $p<0.01$). There were no differences in absolute numbers of B cells and dendritic cells between vehicle and RTL1000 groups in ischemic hemisphere.

RTL1000 treatment altered peripheral immune subtypes in middle-aged mice 96 h after MCAO

Our data showed a trend of increased total splenocyte numbers and decreased cell death in RTL1000 treated mice, although the differences were not statistically significant (Figure 3 and table 3). Corresponding with the reduction of inflammatory cells in the brain, RTL1000 significantly reduced the percentage of CD3⁺ ($p<0.01$), CD4⁺ ($p<0.05$), CD8⁺ ($p<0.05$) T cells and neutrophils in the spleen (Figure 4), while no differences were found in the frequency of CD19⁺B cells, CD11C⁺ dendritic cells and monocytes/macrophages in the spleen between vehicle and RTL1000 treated mice. Further study indicated that RTL1000 decreased the frequency of CD8⁺CD122⁺ regulatory-like T cells (Table 4; $p<0.05$). There were no differences in CD4⁺CD44⁺, CD8⁺CD44⁺, CD3⁺CCR5⁺ and CD4⁺CD25⁺ activation and migration marker expression on T cells between vehicle and RTL1000 treated mice (Table 4).

DISCUSSION

We have previously demonstrated that RTL1000 reduced infarct size and improved long-term functional recovery in young DR2-Tg mice. In the present study, we determined the effect of RTL1000 on ischemic stroke in middle-aged DR2-Tg mice and we investigated its mechanism of action. We found that RTL1000 is similarly effective in reducing infarct size by preventing the infiltration of inflammatory cells into brain in middle-aged mice.

RTL treatment was originally designed as a therapy for multiple sclerosis, an inflammatory demyelinating disease mediated by CD4⁺ T cells, which is followed by secondary infiltration of primarily macrophages in the central nervous system (CNS) (Burrows et al., 1999; Burrows et al., 2000; Burrows et al., 2001). RTLs reverse clinical and histological symptoms of experimental autoimmune encephalomyelitis by reducing secondary infiltration of immune cells including macrophages as well as the expression of chemokines and chemokine receptors required for entry into the CNS (Dziennis et al., 2011, Sinha et al., 2007). Since ischemic stroke induces activation of the peripheral immune system and recruitment of inflammatory cells into the brain, including neutrophils, macrophages and lymphocytes, which contribute to ischemic brain injury (Gelderblom et al., 2009; del Zoppo et al., 2001; Emsley et al., 2001), we tested if RTLs could be used as therapeutic agent in stroke. We found that RTL551 indeed reduced infarct size 96 h after MCAO (Dziennis et al., 2001; Subramanian et al., 2009). Considering species differences, we then tested the effect of another RTL construct, RTL1000, which contains a human MHC covalently linked to a human myelin peptide, in humanized DR2 transgenic mice which express human T cell receptor. Similar to RTL551 treatment in wild type mice, RTL1000 also reduced infarct size in young DR2-Tg mice 96 h after MCAO (Subramanian et al., 2009; Zhu et al., 2014).

However, it is uncertain if benefit in young, healthy animals can be extrapolated to ischemic stroke in humans which occurs mainly in middle-aged and aged people (Fisher et al., 2009). Therefore, in this project, we examined the effect of RTL1000 in middle-aged mice. In

agreement with our results in young mice, RTL1000 reduced infarct size 96 h after the stroke when it was administrated 4 h after occlusion. At the same time, RTL1000 decreased the number of activated monocytes/microglia cells and the absolute number of T cells in the ischemia hemisphere of the brain. This is similar to the effect of RTL551 in blocking the infiltration of inflammatory cells in young wild-type mice (Dziennis et al., 2011). We also observed a slightly increased total number of splenocytes, yet decreased CD3, CD4, CD8 and neutrophils in the spleen. We speculate that the mechanism by which RTL1000 reduced the frequency of T cells and neutrophils in the spleen was through inhibition of immune cell activation and expansion while also decreasing, although not significantly, splenic efflux to the periphery.

The molecular mechanism of action for RTL involves the modulation of T cell functional properties and blockade of immune cells filtrating into the brain. Myelin oligodendrocyte glycoprotein (MOG), a myelin antigen, leaks out of brain with the breakdown of blood-brain barrier after stroke (Dirnagl et al., 2007). MOG-specific CD 4⁺ T cells migrate into brain, being activated by MHC II molecule, mistaking brain antigens for foreign pathogens and attacking them. T cells also produce cytokine, which initiate an inflammatory cascade involving recruitment of other inflammatory cells, including neutrophils, macrophages and other lymphocytes (Jin et al., 2010). RTL1000 is a partial MHC II molecule tethered to a MOG peptide which targets myelin-specific T cells and profoundly changes their functional properties from pro-inflammation to anti-inflammation. Meanwhile, instead of recruitment of inflammatory cells, T cells modulated by RTL1000 could inhibit entry of other T cells and activated macrophages into the brain (Wang et al., 2003; Sinha et al., 2007), demonstrated in our present data. On the other hand, although we did not find any changes in activation and migration markers on T cells in the spleen, we did observe a significant decrease in CD8⁺CD122⁺ regulatory T cells. We speculate that the regulatory population migrated out of the spleen to the periphery and possibly the brain to reduce tissue specific inflammation and immune cell based neurodegeneration.

CONCLUSION

In summary, we demonstrated that RTL1000 protects against acute ischemic stroke in middle-aged mice. The mechanisms involve the modulation of RTL1000 on T cell functional properties and blockade of infiltration of inflammatory cells into the brain. Our results will move this new stroke therapy closer to clinical trials.

Acknowledgements

This work was supported by NIH Grants #NS076013 (STTR) and by the Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development, Biomedical Laboratory Research and Development.

References

- 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 Eng.* 1999; 12:771–778. [PubMed: 10506287]

- Burrows GG, Adlard KL, Bebo BF Jr, Chang JW, Tenditnyy K, Vandenbark AA, Offner H. Regulation of encephalitogenic T cells with recombinant TCR ligands. *J Immunol.* 2000; 164:6366–6371. [PubMed: 10843691]
- Burrows GG, Chou YK, Wang C, Chang JW, Finn TP, Culbertson NE, Kim J, Bourdette DN, Lewinsohn DA, Lewinsohn DM, Ikeda M, Yoshioka T, Allen CN, Offner H, Vandenbark AA. Rudimentary TCR signaling triggers default IL-10 secretion by human Th1 cells. *J Immunol.* 2001; 167:4386–4395. [PubMed: 11591763]
- del Zoppo GJ, Becker KJ, Hallenbeck JM. Inflammation after stroke: is it harmful? *Arch. Neurol.* 2001; 58:669–672. [PubMed: 11296002]
- Dirnagl U, Klehmet J, Braun JS, Harms H, Meisel C, Ziemssen T, Prass K, Meisel A. Stroke-induced immunodepression: experimental evidence and clinical relevance. *Stroke.* 2007; 38:770–773. [PubMed: 17261736]
- Dziennis S, Mader S, Akiyoshi K, Ren X, Ayala P, Burrows GG, Vandenbark AA, Herson PS, Hurn PD, Offner HA. Therapy with recombinant T-cell receptor ligand reduces infarct size and infiltrating inflammatory cells in brain after middle cerebral artery occlusion in mice. *Metab Brain Dis.* 2011; 26:123–133. [PubMed: 21472429]
- Emsley HC, Smith CJ, Gavin CM, Georgiou RF, Vail A, Barberan EM, Hallenbeck JM, del Zoppo GJ, Rothwell NJ, Tyrrell PJ, Hopkins SJ. An early and sustained peripheral inflammatory response in acute ischemic stroke: relationships with infection and atherosclerosis. *J Neuroimmunol.* 2001; 139:93–101. [PubMed: 12799026]
- Feuerstein GZ, Zaleska MM, Krams M, Wang X, Day M, Rutkowski JL, Finklestein SP, Pangalos MN, Poole M, Stiles GL, Ruffolo RR, Walsh FL. Missing steps in the STAIR case: a Translational Medicine perspective on the development of NXY-059 for treatment of acute ischemic stroke. *J Cereb Blood Flow Metab.* 2008; 28:217–219. [PubMed: 17579658]
- Fisher M, Feuerstein G, Howells DW, Hurn PD, Kent TA, Savitz SI, Lo EH. STAIR Group. Update of stroke therapy academic industry roundtable preclinical recommendations. *Stroke.* 2009; 40:2244–2250. [PubMed: 19246690]
- Frenkel D, Huang Z, Maron R, Koldzic DN, Hancock WW, Moskowitz MA, Weiner HL. Nasal vaccination with myelin oligodendrocyte glycoprotein reduces stroke size by inducing IL-10-producing CD4+ T cells. *J Immunol.* 2003; 171:6549–6555. [PubMed: 14662856]
- Gee JM, Kalil A, Shea C, Becker KJ. Lymphocytes: potential mediators of postischemic injury and neuroprotection. *Stroke.* 2007; 38(2 Suppl):783–788. [PubMed: 17261738]
- Gelderblom M, Leypoldt F, Steinbach K, Behrens D, Choe CU, Siler DA, Arumugam TV, Orthey E, Gerloff C, Tolosa E, Magnus T. Temporal and spatial dynamics of cerebral immune cell accumulation in stroke. *Stroke.* 2009; 40:1849–1857. [PubMed: 19265055]
- Gonzalez-Gay MA, Zanelli E, Khare SD, Krco CJ, Zhou P, Inoko H, Griffiths MM, Luthra HS, David CS. Human leukocyte antigen-DRB1*1502 (DR2-TGDW12) transgene reduces incidence and severity of arthritis in mice. *Hum Immunol.* 1996; 50:54–60. [PubMed: 8872175]
- Huan JY, Meza-Romero R, Mooney JL, Chou YK, Edwards DM, Rich C, Link JM, Vandenbark AA, Bourdette DN, Bachinger HP, Burrows GG. Rationally designed mutations convert complexes of human recombinant T cell receptor ligands into monomers that retain biological activity. *J Chem Technol Biotechnol.* 2005; 80:2–12. [PubMed: 22973070]
- Hurn PD, Subramanian S, Parker SM, Afentoulis ME, Kaler LJ, Vandenbark AA, Offner H. T- and B-cell deficient mice with experimental stroke has reduced lesion size and inflammation. *J Cereb Blood Flow Metab.* 2007; 27:1798–1805. [PubMed: 17392692]
- Jin R, Yang G, Li G. Inflammatory mechanisms in ischemic stroke: role of inflammatory cells. *J Leukoc Biol.* 2010; 87:779–789. [PubMed: 20130219]
- Muir KW, Tyrrell P, Sattar N, Warburton E. Inflammation and ischemic stroke. *Curr Opin Neurol.* 2007; 20:334–342. [PubMed: 17495630]
- Nilupul Perera M, Ma HK, Arakawa S, Howells DW, Markus R, Rowe CC, Donnan GA. Inflammation following stroke. *J Clin Neurosci.* 2006; 13:1–8.
- Pan J, Palmateer J, Schallert T, Hart M, Pandya A, Vandenbark AA, Offner H, Hurn PD. Novel humanized recombinant T cell receptor ligands protect the female brain after experimental stroke. *Transl Stroke Res.* 2014; 5:577–585. [PubMed: 24838614]

- Schmid I, Krall WJ, Uittenbogaart CH, Braun J, Giorgi JV. Dead cell discrimination with 7-amino-actinomycin D in combination with dual color immunofluorescence in single laser flow cytometry. *Cytometry*. 1992; 13:204–208. [PubMed: 1547670]
- Sinha S, Subramanian S, Proctor TM, Kaler LJ, Grafe M, Dahan R, Huan J, Vandenbark AA, Burrows GG, Offner H. 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. *J Neurosci*. 2007; 27:12531–12539. [PubMed: 18003831]
- Subramanian S, Zhang B, Kosaka Y, Burrows GG, Grafe MR, Offner H. Recombinant T cell receptor ligand treats experimental stroke. *Stroke*. 2009; 40:2539–2545. [PubMed: 19443805]
- Stroke therapy academic industry roundtable. Recommendations for standards regarding preclinical neuroprotective and restorative drug development. *Stroke*. 1999; 30:2752–2758. [PubMed: 10583007]
- Subramanian S, Zhang B, Kosaka Y, Burrows GG, Grafe MR, Vandenbark AA, Hurn PD, Offner H. Recombinant T cell receptor ligand treats experimental stroke. *Stroke*. 2009; 40:2539–2545. [PubMed: 19443805]
- Vandenbark AA, Rich C, Mooney J, Zamora A, Wang C, Huan J, Fugger L, Offner H, Jones R, Burrows GG. 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, Mooney JL, Meza-Romero R, Chou YK, Huan J, Vandenbark AA, Offner H, Burrows GG. Recombinant TCR ligand induces early TCT signaling and a unique pattern of downstream activation. *J Immunol*. 2003; 171:1934–1940. [PubMed: 12902496]
- Yilmaz G, Arumugam TV, Stokes KY, Granger DN. Role of T lymphocytes and interferon-gamma in ischemic stroke. *Circulation*. 2006; 113:2105–2112. [PubMed: 16636173]
- Zhang W, Davis CM, Edin ML, Lee CR, Zeldin DC, Alkayed NJ. Role of endothelial soluble epoxide hydrolase in cerebrovascular function and ischemic injury. *PLoS One*. 2013; 8:e61244. [PubMed: 23585883]
- Zhu W, Wang L, Zhang L, Palmateer JM, Libal NL, Hurn PD, Herson PS, Murphy SJ. Isoflurane preconditioning neuroprotection in experimental focal stroke is androgen-dependent in male mice. *Neuroscience*. 2010; 169:758–769. [PubMed: 20580788]
- Zhu W, Casper A, Libal NL, Murphy SJ, Bodhankar S, Offner H, Alkayed NJ. Preclinical evaluation of recombinant T cell receptor ligand RTL1000 as a therapeutic agent in ischemic stroke. *Transl Stroke Res*. 2014 A [Epub ahead of print] PMID: 25270354.
- Zhu W, Libal NL, Casper A, Bodhankar S, Offner H, Alkayed NJ. Recombinant T-cell receptor ligand treatment improves neurological outcome in the presence of tissue plasminogen activator in experimental ischemic stroke. *Transl Stroke Res*. 2014 B; 5:612–617. [PubMed: 24953050]

- The effect of immune modulator RTL1000 was tested in humanized transgenic DR2-Tg mice
- RTL1000 reduces infarct size after stroke in 12-month old DR2-Tg mice
- Neuroprotection by RTL1000 is linked to modulation of post-ischemic inflammation

Figure 1.

RTL1000 reduces infarct size 96 h after MCAO in HLA-DR2 middle-aged male mice.

Twelve-month old male mice were subjected to transient MCAO (60 min), then treated with 100 μ l vehicle or 100 μ g RTL1000 given S.C. at 4, 24, 48 and 72 h after MCAO. Brains were harvested 96 h after MCAO and brain slices were stained with 2,3,5-triphenyltetrazolium chloride (TTC) (A). Infarct size was expressed as a percentage of the contralateral structure. * indicates $p < 0.05$ compared to vehicle-treated group (B). CTX: cortex; CP: striatum; HMSPHR: hemisphere

Figure 2.

Effect of RTL1000 on brain mononuclear cells in middle-aged mice after MCAO.

Representative flow cytometry dot plots of activated monocytes/microglia cells (CD11b+CD45^{hi}) from the non-ischemic and ischemic hemispheres of mice treated with vehicle or RTL1000 following MCAO (A). Absolute number of activated monocytes/microglia cells, T cells, B cells and dendritic cells in the ischemic hemisphere of mice treated with vehicle (open bars) or RTL1000 (grey bars) following MCAO (B). Absolute number refers to the total number of each cell type in the ischemic hemisphere. Absolute number of each cell phenotype was calculated using phenotype percent multiplied by the total cell number per ischemic hemisphere. * indicates $p < 0.05$ and ** indicates $p < 0.01$ by t-test. $n = 4$ vehicle, 6 RTL1000.

Figure 3.

The effect of RTL1000 on splenocyte viability following MCAO in middle-aged mice. Total viable splenocyte number (determined by Trypan Blue) in middle-aged mice treated with vehicle (open bars, n=12) or RTL1000 (grey bars, n=16) following MCAO (A). Percent splenocyte death (7-AAD+) in middle-aged mice treated with vehicle (open bars, n=5) or RTL1000 (grey bars, n=6) following MCAO (B).

A.

B.

Figure 4.

Spleen immune subset analysis with RTL1000 treatment after MCAO in middle-aged mice. Total CD3⁺ T cell or CD4⁺ and CD8⁺ T cell subsets in the spleen of mice treated with vehicle or RTL1000 following MCAO (A). Percent of B cell, dendritic cell, neutrophil and monocyte/macrophage populations in the spleen of middle-aged mice treated with vehicle or RTL1000 following MCAO (B). * indicates $p < 0.05$ and ** indicates $p < 0.01$ by t-test. Sample size was $n = 8$ for vehicle and $n = 10$ for RTL1000 for the analysis of CD3, CD4, CD8, CD19 and CD11b⁺Ly6C⁺. For CD11c, $n = 10$ vehicle and $n = 10$ RTL1000, and for CD11b⁺Gr-1⁺, $n = 4$ vehicle and $n = 5$ RTL1000.

Table 1

Age and body weight of vehicle (n=8) and RTL1000 (n=10) mice

	Vehicle	RTL1000
Body Weight (g)	31.1 $\pm$ 1.6	34.6 $\pm$ 1.2
Age (d)	365.8 $\pm$ 0.5	365.3 $\pm$ 0.5

Table 2

Absolute numbers of infiltrating cells in the non-ischemic (left) hemisphere and ischemic (right) hemisphere of vehicle (n=4) and RTL1000 (n=6) treated mice 96 hours after MCAO.

	Nonischemic hemisphere		Ischemic hemisphere	
	Vehicle	RTL1000	Vehicle	RTL1000
CD45 ^{hi} CD11b+	1928 ± 289.8	1202 ± 233.7	30838 ± 9887	5487 ± 767.6*
CD3+	1921 ± 292.5	745.3 ± 142.1**	4114 ± 746.1	1485 ± 350.7**
CD19+	931.3 ± 218.0	758.7 ± 51.09	2116 ± 737.1	1547 ± 227.1
CD11c+	911.3 ± 356.2	670.8 ± 90.02	6110 ± 2433	1944 ± 477.2

* indicates $p < 0.05$ and

** indicates $p < 0.01$ between vehicle and RTL mice.

Table 3

Absolute splenocyte number of vehicle (n=12) and RTL1000 (n=16) treated mice and percent splenocyte death of vehicle (n=5) and RTL1000 (n=6) mice 96 hours after MCAO.

	Vehicle	RTL1000
Splenocyte count	$2.91 \times 10^7 \pm 5.16 \times 10^6$	$4.33 \times 10^7 \pm 6.71 \times 10^6$
Cell death	11.6 ± 3.73	5.43 ± 1.01

Table 4

Distribution of activation (CD44), migration (CCR5) and regulatory-like (CD4⁺CD25⁺, CD8⁺CD122⁺) markers on T cell subsets in the spleen of vehicle (n=8) and RTL1000 (n=10) mice 96 hours after MCAO.

	Vehicle	RTL1000
CD4+CD44+	39.7 ± 3.52	36.69 ± 2.24
CD8+CD44+	50 ± 3.13	43.7 ± 2.86
CD3+CCR5+	8.09 ± 1.06	10.97 ± 1.06
CD4+CD25+	13.59 ± 1.12	12.33 ± 0.72
CD8+CD122+	43.1 ± 3.71	32.23 ± 2.45*

* indicates $p < 0.05$ between vehicle and RTL mice.