Antigen-specific tolerance inhibits autoimmune uveitis in pre-sensitized animals by deletion and CD4+CD25+ T-regulatory cells

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The objective of this study was to inhibit experimental autoimmune anterior uveitis (EAAU) by establishing antigen-specific immune tolerance in animals pre-sensitized with melanin-associated antigen (MAA). Intravenous administration of MAA on days 6, 7, 8 and 9 post-immunization induced tolerance and inhibited EAAU in all Lewis rats. The number of cells (total T cells, CD4+ T cells and CD8+ T cells) undergoing apoptosis dramatically increased in the popliteal lymph nodes (LNs) of the tolerized animals compared with non-tolerized animals. In addition, Fas ligand (FasL), TNF receptor 1 (TNFR1) and caspase-8 were upregulated in tolerized rats. Proliferation of total lymphocytes, CD4+T cells and CD8+ T cells (harvested from the popliteal LNs) in response to antigenic stimulation was drastically reduced in the state of tolerance compared with the cells from non-tolerized animals. The level of interferon (IFN)-γ and IL-2 decreased, whereas TGF-β was elevated in the state of tolerance. Furthermore, the number of CD4+CD25+FoxP3+ regulatory T cells (Tregs) increased in the popliteal LNs of tolerized animals compared with non-tolerized animals. In conclusion, our results suggest that deletion of antigen-specific T cells by apoptosis and active suppression mediated by Tregs has an important role in the induction of antigen specific immune tolerance in animals with an established immune response against MAA.

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Uveitis, a vision threatening ocular disease is responsible for over 2.8% of blindness with a higher disease rate for the older population in the United States of America.1,2 It can be classified anatomically as anterior, intermediate or posterior depending on the segment of the eye that is affected. Idiopathic anterior uveitis (AU) is the most common form of intraocular inflammation in humans and the inflammation in this disease is restricted to the anterior segment of the eye, which includes the iris, the ciliary body and the anterior chamber. Human idiopathic AU has been considered as an autoimmune disease, wherein an immune response affects only the eye.3 Severe visual complications associated with recurrent and/or untreated AU lead to permanent loss of vision.1,4 Unfortunately, treatment options available to uveitis patients have limitations because uveitis is treated symptomatically only and these therapies involve the use of steroids and/or immunosuppressive agents with adverse side effects.

Experimental autoimmune anterior uveitis (EAAU) is an organ-specific autoimmune disease of the eye and the disease in animals with EAAU mimics human idiopathic AU.5–12 EAAU is a CD4+ T-cell mediated autoimmune disease of the eye,6–12 that can be induced in Lewis rats by immunizing with bovine melanin-associated antigen (MAA) emulsified in complete Freund’s adjuvant. We have shown that MAA is a 22kDa fragment of type I collagen α2 chain.11 In this experimental model an immune response is initiated in the popliteal lymph node (LN) of Lewis rats after MAA is injected subcutaneously in the footpad.6–12 This immune response selectively targets the anterior segment of the eye and results in ocular pathology, which is similar to that observed in humans with idiopathic AU.6–12 The disease in EAAU is characterized histologically by a lymphohcytic infiltration in the iris and the ciliary body.6–12 Antigen specific CD4+ T cells can adoptively transfer EAAU to naïve syngenic recipients.8–11 Recently, we reported that the induction of antigen-specific immunologic tolerance against the uveitogenic antigen, MAA, in animals without an established immune response (that is, before sensitization) leads to complete protection and suppression of EAAU.12 The purpose of this study was to investigate whether EAAU can be inhibited by establishing antigen-specific immune tolerance in animals that have previously been sensitized with MAA and to explore the underlying mechanism(s). The results derived from this study may have important clinical implications because the experiments described here mimic the situation in patients with idiopathic AU during the period of remission; human idiopathic AU is a recurrent disease.1–4
RESULTS

Effect of intravenous (i.v.) MAA administration on EAAU in pre-sensitized animals

Antigen-specific tolerance induced by the i.v. route in MAA-sensitized Lewis rats resulted in the inhibition of EAAU and the degree of protection against EAAU was dependent on the dose of the antigen. A single i.v. injection of 100 and 200 μg MAA at days 6, 7, 8 and 9 post-immunization significantly (P<0.05) reduced the duration of the disease with significantly (P<0.05) delayed onset and severity compared with phosphate-buffered saline (PBS) injected control animals (Table 1). In contrast, complete protection (tolerance) was observed when 400, 600 and 800 μg of MAA were injected separately via the i.v. route (Table 1). Similar treatment with PBS (‘non-tolerized’, Table 1) did not induce tolerance and all animals developed the normal course of EAAU with massive cellular infiltration in the iris, the CB and the anterior segment of the eye (Figure 1a). I.v. injection of OVA had no effect on EAAU both clinically and histologically (Table 1). The minimum dose of MAA that resulted in i.v. tolerance against MAA and complete inhibition of EAAU as assessed clinically and histologically (Table 1 and Figure 1b) was 400 μg and was used in our subsequent experiments.

I.v. MAA administration causes apoptosis of popliteal (LN) cells

First, terminal deoxynucleotidyl transferase (TdT)-mediated d-UTP-biotin nick end labeling assay was utilized to detect difference, if any, between the number of apoptotic cells in the popliteal LNs of tolerized and non-tolerized rats. EAAU was induced in Lewis rats by immunization with MAA (day 0) and the animals were divided into two groups (n=5 animals/group). Animals in group 1 were tolerized by the i.v. administration of 400 μg MAA as described above. The rats in group 2 received the same treatment with PBS (non-tolerized). Rats from both groups were sacrificed on days 5, 8 and 11 post-immunization, popliteal LNs were harvested and paraffin sections of popliteal LNs were used for terminal deoxynucleotidyl transferase (TdT)-mediated d-UTP-biotin nick end labeling staining. Representative results shown in Figures 2a–f revealed that at days 8 and 11 post-immunization, more cells were undergoing apoptosis in the popliteal LN of tolerized rats (Figures 2e and f) compared with non-tolerized animals (Figures 2b and c).

To determine that lymphocytes are undergoing apoptosis and to further identify the type of apoptotic lymphocytes, total lymphocytes were purified from the popliteal LNs of tolerized and non-tolerized animals and stained for CD4 and CD8 separately. CD4 and CD8 labeled cells were stained for Annexin V plus PI and apostat separately. These cells were then analyzed by flow cytometry. As expected, the percentage of total live lymphocytes that were undergoing apoptosis (annexin V<sup>+</sup>PI<sup>−</sup>) were similar (18.7 ± 1.9%) on day 5 post-immunization in both tolerized and non-tolerized animals (Figure 2g), as the i.v. treatment did not start till day 6 post-immunization. However, on day 8 post-immunization the percentage of total lymphocytes that were apoptotic (annexin V<sup>+</sup>PI<sup>−</sup>) were significantly higher (P<0.05) in tolerized animals (27.15 ± 0.21%) compared with non-tolerized animals (23.5 ± 0.91%). The percentage of apoptotic lymphocytes increased drastically in the popliteal LNs of tolerized animals.

Table 1 Effect of intravenous administration of MAA on EAAU

| MAA i.v. (μg) | OVA i.v. (μg) | PBS i.v. (μg) | Eyes with EAAU | Day of onset | Duration of disease (days) |
|--------------|--------------|--------------|----------------|--------------|--------------------------|
|              |              |              | Incidence | Mild | Severe |                     |                     |                     |
| 100          | 0            | 400          | 6/6       | 2/6  | 4/6    | 14.3 ± 0.5<sup>*</sup> | 10.7 ± 1.2<sup>**</sup> |                     |
| 200          | 0            | 400          | 6/6       | 4/6  | 2/6    | 15.7 ± 0.5<sup>*</sup> | 8 ± 1.4<sup>**</sup>   |                     |
| 400          | 0            | 400          | 0/24      |      |        |                     |                     |                     |
| 600          | 0            | 400          | 0/6       |      |        |                     |                     |                     |
| 800          | 0            | 400          | 0/6       |      |        |                     |                     |                     |
| 0            | 400          |              | 6/6       |      | 6/6    | 13.2 ± 0.6           | 12.5 ± 0.8          |                     |
| 0            | 0            | 400          | 24/24     |      |        | 13.2 ± 0.4           | 12.7 ± 0.5          |                     |

Abbreviations: EAAU, experimental autoimmune anterior uveitis; i.v., intravenous; MAA, melanin-associated antigen; OVA, ovalbumin; PBS, phosphate-buffered saline.

Incidence of EAAU given as positive/total eyes following clinical examination. Severity of inflammation on histopathological examination was grouped as normal (0), mild (1+ to 2+) or severe (3+ to 4+). *<sup>P</sup><0.05. **<sup>P</sup><0.01.

Figure 1 Induction of tolerance to melanin-associated antigen (MAA) in experimental autoimmune anterior uveitis (EAAU) by intravenous (i.v.) injection of MAA (400 μg) in pre-sensitized animals. (a and b), Histopathological changes in the eyes of MAA sensitized non-tolerized (a) and tolerized (b) Lewis rats at the peak of EAAU (day 19 post-immunization). Hematoxylin and eosin (H&E) staining revealed heavy infiltration of inflammatory cells within the iris (I), the ciliary body (CB) and the anterior chamber (AC) of non-tolerized rats (a). No inflammation was observed in the eyes of tolerized rats (b). (Objective magnification, ×10).
Figure 2 Effect of intravenously injected melanin-associated antigen (MAA) on apoptosis of lymph node (LN) cells in MAA sensitized Lewis rats.  
(a–f) Terminal deoxynucleotidyl transferase (TdT)-mediated d-UTP-biotin nick end labeling (TUNEL) staining for apoptotic cells in the popliteal LNs of non-tolerized (a–c) and tolerized (d–f) animals. Green fluorescence represents number of apoptotic cells on day 5 (a and d), day 8 (b and e) and day 11 (c and f). (Objective magnification, ×20). (g–i), Differences in percentage of apoptotic cells in total lymphocytes stained with annexin V alone (g), annexin V+anti-CD4 (h) or annexin V+anti-CD8 (i) and analyzed by flow cytometry (■ tolerized; □ Non-tolerized). Total lymphocytes were also stained with apostat alone (j), apostat+anti-CD4 (k) or apostat+anti-CD8 (l) and analyzed by flow cytometry (■ tolerized; □ Non-tolerized). Data are representative of three separate experiments and cumulative data is shown as bar graph. *P<0.05.
(34.28 ± 2.48%) compared with non-tolerized animals (25.61 ± 4.34%) on day 11 post-immunization (Figure 2g). Next we compared the percentage of apoptotic (annexin V+ PI+) CD4+ T as well as CD8+ T cells in the LNs of tolerized and non-tolerized animals (Figures 2h and i). As shown in Figure 2h the percentage of annexin V+ PI- CD4+ T cells were significantly higher (P<0.05) in the popliteal LNs of tolerized animals at day 8 (21.8 ± 0.56%) and day 11 (29.13 ± 4.8%) compared with non-tolerized animals at these time points (day 8, 17.75 ± 0.21%; day 11, 23.03 ± 3.85%). Similarly, significantly more annexin V+ PI- CD8+ T cells (Figure 2i) were present in the LNs of tolerized animals (16.75 ± 0.35%) on day 8 and 21.9 ± 0.9% on day 11) compared with non-tolerized animals (13.75 ± 0.07% on day 8 and 14.83 ± 1.92% on day 11). These results were further confirmed by using intracellular caspase detection (Figures 2j–l). In this method the cells were stained intracellularly with FITC-conjugated pan-caspase inhibitor, Apostat. The fluorescence was then detected by flow cytometry. Our results demonstrated a significant (P<0.05) increase in apoptotic total lymphocytes, CD4+ T cells and CD8+ T cells in tolerized animals compared with non-tolerized animals at days 8 and 11 post-immunization (Figures 2j–l).

Effect of i.v. MAA administration on the proliferation of LN cells

Total lymphocytes harvested from popliteal LNs of tolerized and non-tolerized animals killed at day 11 post-immunization were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) using the Cell Trace CFSE cell proliferation kit and were cultured with MAA (10 μg/ml) for 6 days. The cells were collected and used for flow analysis. CFSE is an intracellular stain that is passed on to the next generation and ModFit Proliferation Wizard Program detects each generation in the form of individual peaks. (Figures 3a–h). Furthermore, the percentage of cells in each generation including the cells remaining in the parent generation as well as proliferation index can be calculated using this program.12 Total lymphocytes from the LNs of the tolerized animal had a significantly (P<0.05) increased ability to proliferate compared with total lymphocytes from non-tolerized animals in the presence of MAA (Figures 3a and d) with an average proliferation index of 1.477 ± 0.12 and 2.06 ± 0.27, respectively (Figure 3i). These in vitro cultured CFSE-labeled lymphocytes were next labeled with anti-rat CD4—APC and anti-rat CD8—APC (or anti-rat CD8—PerCP) separately and analyzed for in vitro proliferation. CD4+ cells from the LNs of the tolerized animals proliferated at a significantly (P<0.05) lower rate compared with the CD4+ cells from non-tolerized animals in response to the antigen (Figures 3b and e) with an average proliferation index of 1.28 ± 0.19 and 1.95 ± 0.39, respectively (Figure 3i). Similarly, CD8+ cells from the LNs of the tolerized animal proliferated at a significantly (P<0.05) lower rate compared with the CD8+ cells from non-tolerized animals (Figures 3c and f) with an average proliferation index of 1.26 ± 0.20 and 2.02 ± 0.38, respectively (Figure 3i). As expected, in the absence of MAA total lymphocytes (Figures 3g, h and j), CD4+ (Figure 3i) cells and CD8+ (Figure 3j) cells from both tolerized and non-tolerized animals proliferated at a low rate.

Effect of i.v. tolerance on cytokine expression

Differences in the expression of interferon (IFN)-γ, IL-2 and TGF-β2 between tolerized and non-tolerized animals were detected using quantitative real time PCR (IFN-γ, IL-2 and TGF-β2) and ELISA (IFN-γ and IL-2). Tolerance was induced in MAA sensitized rats (as described under the METHODS section) and the animals were sacrificed at day 11 post-immunization. Total lymphocytes purified from popliteal LNs cells were used for RNA extraction. The real time-PCR analysis revealed that mRNA expression of IFN-γ (Figure 4a) and IL-2 (Figure 4b) was significantly (P<0.05) decreased in the lymphocytes derived from popliteal LNs of tolerized animals compared with those harvested from non-tolerized animals. In contrast, the levels of TGF-β2 transcripts demonstrated a significant (P<0.05) increase in tolerized animals compared with non-tolerized rats (Figure 4c). Levels of IFN-γ (Figure 4d) and IL-2 (Figure 4e) proteins (as determined by ELISA) were significantly (P<0.05) reduced in tolerized rats compared with non-tolerized animals.

CD4+CD25+FoxP3+ regulatory T cells (Tregs) in MAA-i.v. tolerance

Melanin-associated antigen intravenously tolerized Lewis rats were sacrificed at days 5, 8 and 11 post-immunization and total lymphocytes harvested from popliteal LNs were stained for CD4, CD25 and FoxP3. These cells were analyzed by flow cytometry to obtain the percentage of CD4+CD25+FoxP3+ T cells in the total CD4+ cell population. As expected, no difference in CD4+CD25+FoxP3+ Tregs was observed in LNs of tolerized animals on day 5 (Figure 5a). A significant increase (P<0.05) in CD4+CD25+FoxP3+ Tregs was observed in LNs of tolerized animals on days 8 and 11 post-immunization compared with non-tolerized animals at these time points (Figure 5a).

Adaptive transfer of tolerance

Total lymphocytes harvested from the popliteal LNs of tolerized animals at day 11 post-immunization were cultured in the presence of MAA (20 μg/ml) for 3 days, as previously described by the authors,8,11 non-adherent cells (2×106 cells) were collected and were adoptively transferred to naïve syngenic rats (n=3 rats/group) via the tail vein. In a separate set of experiment CD4+CD25+ T cells were sorted from non-adherent cells obtained from the popliteal LNs of tolerized animal that were cultured in the presence of MAA (20 μg/ml) for 3 days. A total of 5×106 CD4+CD25+ T cells were adoptively transferred to naïve Lewis rats via the tail vein. Total lymphocytes (2×107 cells) from non-tolerized animals cultured in the presence of MAA (20 μg/ml) for 3 days served as control. All animals were immunized with MAA (together with complete Freund's adjuvant) 24 h after the cell transfer. The animals were examined daily from day 3–30 for clinical signs of uveitis. In the animals that received total non-adherent lymphocytes from non-tolerized Lewis rats the disease developed in two phases. The first phase starting at day 5 was induced by adoptively transferred MAA-specific lymphocytes, whereas the second phase which started by day 13–14 was because of the footpad injection of MAA in the presence of adjuvant (Figure 5b). The clinical pattern of uveitis in both the phases was similar to our previous observations.12 Adoptive transfer of total lymphocytes from the tolerized animals did not induce anterior uveitis in the first phase. It is interesting to note that EAAU was not completely inhibited in the second phase; however, significantly (P<0.05) reduced severity of the inflammation was noted (Figure 5b). Similar results were obtained when CD4+CD25+ T cells purified from the popliteal LNs of tolerized animals were adoptively transferred (Figure 5b).

Mechanism of apoptosis induction in tolerized animals

We next investigated the role of activation-induced cell death in the induction of apoptosis in MAA-i.v. tolerized animals and determined the levels of Fas ligand (Fasl) and TNF receptor 1 (TNFR1). Using flow cytometric analysis we measured FasL+ and TNFR1+ lymphocytes in popliteal LNs of tolerized and non-tolerized animals (Figures 6a and b). Both tolerized and non-tolerized rats were killed on day 11 post-immunization, popliteal LNs were harvested; total lymphocytes
Figure 3 Effect of intravenously injected melanin-associated antigen (MAA) on the proliferation of cells harvested from the popliteal lymph nodes (LNs). Representative raw data (obtained by FACS analysis) for in vitro proliferation of carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled lymphocytes obtained from tolerized (a–c and g) and non-tolerized (d–f and h) rats in presence of MAA (a–f) or absence of MAA (g and h). In vitro proliferation of CFSE-labeled total lymphocytes (a, d, g and h), CD4 (b and e) and CD8 (c and f) from the popliteal LNs of tolerized rats (a–c) was markedly suppressed compared to those from non-tolerized rats (d–f) in the presence of MAA. Negligible proliferative response for total lymphocytes from tolerized (g) and non-tolerized (h) animals was observed in the absence of MAA. (i) and (j) represent the cumulative proliferation index data from three independent experiments for the proliferation of total lymphocytes, CD4⁺ and CD8⁺ cells harvested from the popliteal LNs of tolerized (■) and non-tolerized (□) animals and cultured in the presence (i) or the absence (j) of MAA. *P<0.05.
were purified and stained for FasL and TNFR1 separately. The results of flow cytometry demonstrated that the percentage of FasL+ lymphocytes increased drastically in the popliteal LNs of tolerized animals (29.3 ± 3.55%) compared with non-tolerized animals (23 ± 1.9%) at day 11 post-immunization (Figure 6a). Similarly, more TNFR1+ lymphocytes were detected in the LNs of tolerized animals (19.25 ± 2.73%) compared with non-tolerized animals (13.6 ± 1.47%) at this time point (Figure 6b) and these differences were statistically significant (P < 0.05). The involvement of activation-induced cell death was confirmed by determining the protein levels of activated caspase-8 in tolerized and non-tolerized rats using the western blot analysis (Figure 6c). Densitometric analysis of the western blots demonstrated that the levels of active (cleaved) caspase-8 (26 kDa) increased in tolerized rats compared with non-tolerized animals at day 11 post-immunization.

DISCUSSION
Disability associated with the loss of vision has a long lasting impact on the quality of life. Idiopathic AU, the inflammation of the AU tract (that is, the iris and the ciliary body) is one of the major causes of legal blindness in the United States of America. At present, non-specific therapies, such as steroid and immunosuppressive agents, are being used to treat patients with idiopathic AU and these treatment modalities are associated with adverse side effects and long-term complications. Owing to the lack of safe and effective therapeutic options available to patients with idiopathic AU, further studies are required so that the strategies that specifically modulate the autoimmune response could be developed to treat and/or manage this vision threatening disease. Antigen-specific immune tolerance has been used in the past to treat autoimmune diseases. Tolerance mechanisms that protect the host from developing autoimmune diseases include clonal deletion of auto-reactive T and B cells in peripheral blood, the thymus and LNs; clonal anergy and active suppression by regulatory T cells and have been reported to act independently or in combination.

Recently, we reported that T cell non-responsiveness due to active suppression mediated by Tregs facilitates the development of tolerance to MAA and inhibition of EAAU in animals without an established immune response against the uveitogenic antigen. In this study we demonstrate that the administration of MAA (400 μg, i.v.) on four consecutive days by i.v. route to Lewis rats that has previously received sensitization by subcutaneous injection of MAA (with complete Freund’s adjuvant) in the footpad-induced tolerance and inhibited EAAU. Delivery of the antigen in the animals after immune response has been established, but before the clinical onset of disease is capable of inducing peripheral tolerance and protection.
Figure 5 Regulatory T cells (Tregs) in melanin-associated antigen (MAA)-intravenous (i.v.) tolerance. (a) CD4+CD25+ FoxP3+ Tregs in total lymphocytes harvested from the popliteal LNs of tolerized (■) and non-tolerized (□) animals at days 5, 8 and 11 post-immunization with MAA. Percentage CD4+CD25+ FoxP3+ Tregs was determined by flow cytometry and cumulative data from three independent experiments is shown as bar graph. *P<0.05. (b) Adoptive transfer of tolerance. Experimental autoimmune anterior uveitis (EAAU) developed in two phases in Lewis rats that received total non-adherent cells from the popliteal LN of non-tolerized rats (–−). Adoptive transfer of total non-adherent lymphocytes from tolerized Lewis rats did not induce inflammation in the first phase; the severity of inflammation was reduced in the second phase in the recipient animals (−Δ−). Similar results were obtained when CD4+CD25+ cells from tolerized animals were adoptively transferred to naive Lewis rats (−○−).

Figure 6 Mechanism of apoptosis induction. Percentage of Fas ligand (FasL+) (a) and TNF receptor 1 (TNFR1+) (b) cells in the popliteal lymph node (LN) of tolerized animals and non-tolerized animals analyzed by flow cytometry. *P<0.05. (c) Representative semi-quantitative western blot for cleaved caspase-8 proteins (inset) in the popliteal LNs of tolerized and non-tolerized Lewis rats at day 11 post-immunization and densitometric analysis of the intensity of active caspase-8 to those of β-actin protein bands.
Our results clearly established that the i.v. administration of MAA after animals have been immunized with same antigen resulted in apoptosis of T cells in the popliteal LNs. There are different mechanisms, which can contribute to this observation. One possibility is that in EAAU model, MAA specific T cells undergo activation-induced cell death in response to repeated challenge with MAA, which will lead to increased number of apoptotic cells in the popliteal LNs of tolerized animals. Consistent with this hypothesis are our results that the apoptosis of T cells increased dramatically in the popliteal LNs of tolerized rats after the i.v. administration of MAA on days 6, 7, 8 and 9 post-immunization. The interaction between death factors and death receptors leads to apoptosis in activation-induced cell death.\textsuperscript{20–25} Binding of Fas with Fasl or interaction of TNF with TNFR1 triggers a sequence of intracellular events, which initiate apoptosis of antigen specific T cells by activating caspase-8. Our results show that the TNFR1 and Fasl positive lymphocytes increased significantly in the popliteal LN of tolerized animals. Furthermore, we observed that the expression of caspase-8 was elevated in the state of tolerance.

Next we investigated if clonal anergy has any role in the induction of tolerance and observed that the lymphocytes from the popliteal LNs of tolerized animals proliferated at a significantly lower rate in response to antigenic stimulation compared with those from the non-tolerized animals. However, the proliferation of the lymphocytes from tolerized animals was not completely blocked. This suggested that these cells may not be completely non-responsive and that mechanism other than clonal anergy may be responsible for their low-proliferation rate. To investigate other possible mechanisms involved in the suppression of effector T cells, we investigated the role of CD4\textsuperscript{+}CD25\textsuperscript{+}FoxP3\textsuperscript{+} cells. Our results demonstrated that the percentage of CD4\textsuperscript{+}CD25\textsuperscript{+}FoxP3\textsuperscript{+} Tregs increased significantly in rats with EAAU that received i.v. injection of MAA (tolerized) compared with those that received PBS (non-tolerized). Tregs exert their suppressive activity by several mechanisms including IL-2 sequestration, inhibition of IL-2 production and increased TGF-\textbeta expression.\textsuperscript{26} In this study our results demonstrated increased levels of TGF-\textbeta2 and decreased levels of IL-2 in the lymphocytes from popliteal LNs of tolerized rats compared with non-tolerized animals. This may possibly be responsible for the decreased proliferation of T cells in tolerized animals. Furthermore, the levels of IFN-\gamma were reduced in the popliteal LNs of tolerized animals. This reduction in the level of IFN-\gamma may be because of the suppressive effects of CD4\textsuperscript{+}CD25\textsuperscript{+}FoxP3\textsuperscript{+} Tregs or because of decreased number of active effector T cells resulting from the increase in apoptosis.

We next used a combination of adoptive transfer and active immunization model of EAAU\textsuperscript{8,11,12} to determine if MAA-i.v. tolerance induced after the immune system has been activated can be adoptively transferred. In this model the recipient animals are first injected intravenously with the lymphocytes purified from the popliteal LNs of the donor Lewis rats with EAAU. Twenty-four hours after the cell transfer the recipient rats are challenged subcutaneously with MAA emulsified in complete Freund’s adjuvant and EAAU develops in two phases in the recipient rats.\textsuperscript{1,2} In this study the lymphocytes from the tolerized donor rats were able to completely block the first phase of the disease. However, these lymphocytes did not block the second phase of EAAU completely, instead reduced the severity of uveitis in the second phase. Similar results were obtained when purified Tregs from the tolerized donor animals were used in adoptive transfer experiments. These results suggest that in EAAU model although lymphocytes present in the popliteal LN of tolerized animals are suppressive, they alone are unable to protect the host completely from developing uveitis after an active immune response against MAA has been established. Alternatively, CD4\textsuperscript{+}CD25\textsuperscript{+} Tregs can induce apoptosis of the lymphocytes in the LNs.\textsuperscript{27} However, our results demonstrated that blocking CD25 on the lymphocytes from the popliteal LNs of tolerized animals had no effect on apoptosis, would argue against such a possibility (data not shown).

In conclusion, our results established that MAA when administered intravenously after the uveitogenic challenge, but before the onset of the clinical signs of the disease effectively prevented the development of EAAU by inducing the state of antigen-specific immune tolerance. The tolerance observed in this study was mediated by a combination of two mechanisms: deletion of MAA specific T cells through activation-induced cell death and active suppression mediated by CD4\textsuperscript{+}CD25\textsuperscript{+}FoxP3\textsuperscript{+} T cell. Human idiopathic AU is a recurrent disease, wherein patients experience repeated episodes of the disease separated by period of remission.\textsuperscript{3,4} Although no intra-ocular inflammation is detected clinically during remission, the immune system is active in these patients and potentially capable of mounting an inflammatory response upon re-challenge, which may lead to recurrent AU. This scenario is similar to the experiments described in this study, wherein the animals have an established immune response to the pathogenic antigen, but the uveitis has yet to develop. Our unpublished results demonstrate that the T-cell response to MAA is associated with idiopathic AU in humans, thus suggesting that T cells specific for MAA are present in the blood of patients with idiopathic AU. The results derived from our study may have implications for human idiopathic AU. We propose that antigen-specific immune tolerance can be used to prevent the recurrence of autoimmune idiopathic AU in humans.

**METHODS**

**Animals**

Pathogen-free male Lewis rats (5–6-weeks old) were obtained from Harlan Sprague Dawley (Indianapolis, IN, USA). This study was approved by the Institutional Animal Care and Use Committee, University of Arkansas for Medical Sciences, Little Rock, AR, USA and all animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Induction and evaluation of EAAU**

Melanin-associated antigen was purified from bovine iris and ciliary body as previously described by the authors.\textsuperscript{11} Male Lewis rats were immunized with 100 \mu l of stable emulsion containing 75 \mu g of MAA emulsified (1:1) in complete Freund’s adjuvant (Sigma-Aldrich, St Louis, MO, USA) using a single-dose induction protocol in the hind footpad as previously described by the authors.\textsuperscript{6–12} Depending on the experimental design, animals were examined daily between days 3 and 30 post-injection for clinical signs of uveitis using slit lamp biomicroscopy. EAAU was scored by an observer unaware of the experimental design. Intensity of uveitis was scored in a masked fashion on the arbitrary scale of 0–6, as follows: 0 = normal; 1 = dilated iris vessels and thickened iris and ciliary body; 2 = edema in the anterior chamber with protein, a few scattered inflammatory cells or both; 3 = moderate infiltration of inflammatory cells in the iris, ciliary body or both; 4 = moderate number of inflammatory cells within the anterior chamber; 5 = heavy infiltration of inflammatory cells within the iris and ciliary body and within the anterior chamber; 6 = extreme hemorrhage with extremely heavy infiltration of inflammatory cells within the iris, the ciliary body and the anterior chamber, as well as dense inflammatory cell deposits on the corneal endothelium; 7 = presence of hemorrhage with extremely heavy infiltration of inflammatory cells within the iris, the ciliary body and the anterior chamber, as well as dense inflammatory cell deposits on the corneal endothelium; 8 = extreme hemorrhage with very dense deposits on corneal endothelium. Eyes were also harvested at various time points for histological analysis to assess the course and severity of inflammation using the criteria previously reported.\textsuperscript{6,7,10–12}
Induction of immune tolerance
To induce tolerance, MAA sensitized Lewis rats received a single i.v. injection of MAA on days 6, 7, 8 and 9 post-immunization. MAA was dissolved in 400 μl of PBS and different doses (100, 200, 400, 600 and 800 μg) of MAA were injected separately at these time points. Lewis rats injected similarly with 400 μg of ovalbumin (Sigma-Aldrich) or PBS (400 μl) were used as control. Animals were monitored for the development and severity of EAAU as described above.

Histology
Freshly enucleated rat eyes and popliteal LNs were fixed in neutral buffered 10% formalin solution (Sigma-Aldrich) for 24 h at room temperature, dehydrated in ethanol through ascending series of ethanol concentrations and embedded in paraffin. Four-micron sections were stained with hematoxylin and eosin (H&E) purchased from Fisher (Fair Lawn, NJ, USA). Sections were examined using a light microscope (Carl Zeiss Meditec, Thornwood, NY, USA).

Cell preparation from popliteal LNs
Popliteal LNs were harvested and a single-cell suspension was prepared as described previously. Total lymphocytes were purified by using Histopaque gradient (Sigma-Aldrich) according to the manufacturer’s protocol. The cells were suspended in complete RPMI 1640 culture medium containing L-Glutamine (Mediatech, Herndon, VA, USA) containing 1% (v/v) MEM (Life Technologies, Rockville, MD, USA), NEAA (BioWhittaker, Allendale, NJ, USA), mixture of antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml and Amphotericin B 0.25 μg/ml) purchased from BioWhittaker (Allendale, NJ, USA) and 10% (v/v) FCS (Mediatech, Herndon, VA, USA).

Detection of apoptosis
(a) Terminal deoxynucleotidyl transferase (TdT)-mediated d-UTP-biotin nick end labeling assay. Lewis rats immunized with MAA were sacrificed at different time points (three rats per time point) after immunization. Popliteal LNs were harvested from tolerized and non-tolerized animals and 4-μm paraffin sections were prepared as described above. Terminal deoxynucleotidyl transferase (TdT)-mediated d-UTP-biotin nick end labeling assay (Biovision, Mountain View, CA, USA) was used as follows. Deparaffinized sections of popliteal LNs were treated with proteinase K (20 μg/ml) in 10 mins Tris-HCl (pH 8.0) for 20 min at 37°C followed by washing with PBS. The tissue was incubated with the terminal deoxynucleotidyl transferase (TdT)-mediated d-UTP-biotin nick end labeling reaction mixture in a humidified chamber at 37°C for 1 h. Negative control was treated similarly; however, the enzyme solution was omitted. The tissue was then incubated with biotin-Texas red conjugate for 30 min at room temperature. The sections were covered by antifade reagent with DAPI (Invitrogen, Carlsbad, CA, USA) and were examined under fluorescence microscopy (Carl Zeiss Meditec).

(b) Flow cytometry. Lewis rats immunized with MAA were sacrificed at different time points (three rats per time point) after immunization. Popliteal LNs were harvested from tolerized and non-tolerized animals and total lymphocytes were purified as described above. Cells (1×10^6) were treated with anti-rat CD32 antibody (BD Biosciences, San Jose, CA, USA) for 15 min at 4°C to block Fc receptors. The cells were then stained with anti-rat CD4–APC (BD Biosciences), anti-rat CD8–APC (Biologend, San Diego, CA, USA) separately in 100 μl stain buffer for 30 min in the dark at 4°C. After washing with cold PBS, the cells were stained with Annexin V-FITC and PI in binding buffer (ApoTox Kit 1; BD Biosciences) according to the manufacturer’s instructions. In a separate set of experiments cells were stained for Annexin V-FITC and PI in binding buffer (ApoTox Kit 1; BD Biosciences) according to the manufacturer’s instructions. Flow cytometric analysis was performed on FACS Calibur (BD Biosciences), and data were analyzed in cytometry software (Win MDI 2.8; Windows Multiple Document Interface for Flow Cytometry).

Real time quantitative RT-PCR
Total RNA was extracted using the RNaseasy kit (Qiagen, Valencia, CA, USA). Quantitative real-time PCR (RT-qPCR) was performed using Q SYBR Green Supermix and iQ 5 real time PCR unit (both purchased from Bio-Rad, Hercules, CA, USA). The primers were designed and ordered from Integrated DNA Technologies (Corvalis, LA, USA), using the criteria provided in the Bio-Rad user manual. Primer sequences including the predicted sizes of amplified cDNA are as follows: β-actin (103 bp) forward: 5'-AACCTTAAAGCAGCAACGG-3', reverse: 5'-AGGACTACAGGAGCACAAC-3'; IFN-γ (133 bp) forward: 5'-AAGGAGATACGAGAAACACT-3', reverse: 5'-TGCTGATCCTTGTTGGTTC-3'; TGF-β2 (115 bp) forward: 5'-CCTGAATGCGTGTTGTT-3', reverse: 5'-AGACCTTGATGTCAGAAGC-3'. Real-time RT-qPCR experiments were performed to determine optimal condition for each primer. All real-time RT-qPCR experiments were performed in duplicate. The primer specificity of the amplification product was confirmed by melting-curve analysis of the reaction products using SYBR Green as well as by visualization on ethidium bromide stained agarose (1.5%) gels. The housekeeping gene β-actin was used as an internal control, and gene-specific miRNA expression was normalized against β-actin expression. IQ 5 optical system software version 2.0 (Bio-Rad) was used to analyze real-time RT-qPCR data and derive threshold cycle (Ct) values according to the manufacturer’s instructions. The DDCt method was used to transform Ct values into relative quantities with standard deviations. Same software was used to calculate the normalized expression of the gene of interest, using β-actin as reference gene and the results were expressed as normalized fold expression.

ELISA
Total lymphocytes were harvested from popliteal LNs of tolerized and non-tolerized animals. Equal number of cells were cultured in the presence of MAA (10 μg/ml) for 24 h. Supernatants were collected for quantitative ELISA for IFN-γ (BD Biosciences) and IL-2 (R&D Systems). ELISA was performed using paired Abs according to the manufacturer’s recommendations. The concentration of each cytokine was calculated by computer software using the standard curves obtained from known concentrations.

Purification of Tregs
Total lymphocytes harvested from the popliteal LNs of tolerized and non-tolerized animals were plated with MAA (20 μg/ml) for 3 days and non-adherent cells were collected. Non-adherent cells were labeled with CD4-APC and CD25-PE antibodies (BD Biosciences) and CD4+CD25+ cells were collected by cell sorting using FACSaria (BD Biosciences). Approximately 90% purification was achieved by this method.

Staining for Tregs
For FoxP3 intracellular staining, total lymphocyte cell suspension was prepared from the popliteal LNs harvested at different time points post-immunization. The Fc receptor was blocked and stained for CD4-APC and CD25-PE as described above. Cells were stained with FITC labeled FoxP3 antibody (eBiosciences, San Diego, CA, USA) using BD cytofix/cytperm kit according to manufacturer’s instructions (BD Biosciences). The stained cells were analyzed using flow cytometer.

TNFR1 and FasL staining
One million cells were treated with anti-rat CD32 antibody (BD Biosciences) for 15 min at 4°C to block Fc receptors. Appropriately diluted primary antibody (anti-rat FasL and anti-rat TNFR1 from Abcam, Cambridge, MA, USA) was added separately and incubated for 30 min at 4°C in dark. The cells stained with anti-Fasl were washed and stained with biotinylated anti-hamster Ab (Vector Lab, Burlingame, CA, USA) followed by APC conjugated strepta-vidin (BD Biosciences) and the cells stained with anti-TNFFR1 were stained with Cy3 conjugated anti- rabbit secondary antibody (Sigma) for 30 min at 4°C. All the cells were washed and resuspended in 500 μl of stain buffer (BD Biosciences) and were then subjected to flow analysis on BD FACS Calibur (BD Biosciences). The data was analyzed on WinMDI 2.8.

In vitro cell-proliferation assay
Total lymphocytes harvested from popliteal LNs were labeled with CFSE using Cell Trace CFSE Cell Proliferation Kit according to manufacturer’s protocol (Molecular Probes, Invitrogen) and were cultured with MAA (10 μg/ml) for 6
days. CFSE-labeled total lymphocytes from tolerized and non-tolerized rats cultured in the absence of MAA served as control. The cells were labeled for surface markers and were used for flow analysis. The raw data from FACSCalibur was analyzed for percentage of parent generation and proliferation index using ModFit Proliferation Wizard Program.

Western blot analysis
The samples were subjected to SDS-PAGE. After SDS-PAGE on a 12% linear slab gel, separated proteins were transferred to a polyvinylidene difluoride membrane using a semidy electrodephoretic transfer cell (Trans-Blot; Bio-Rad). Blots were stained at room temperature with 1:1000 dilution of one of the following antibodies: rabbit anti-caspase-8, (Biovision, Mountain View, CA, USA), or anti–β-actin (Sigma). After washing and incubation with a horse radish peroxidase (HRP)-conjugated secondary antibody, blots were developed using the enhanced chemiluminescence western blotting detection system (ECL Plus; Amersham Biosciences, Piscataway, NJ, USA). Quantification of proteins was accomplished by analyzing the intensity of the bands (Quantity One 4.2.0; Bio-Rad). These experiments were repeated three times with similar results.

Statistical analysis
All experiments were repeated three times with similar results. The data are expressed as the mean ± s.d. Data were analyzed and compared using Student’s t-test, and differences were considered statistically significant with P<0.05.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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