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Antigen Specific Tolerance Inhibits Autoimmune Uveitis in Pre-sensitized Animals via Deletion and CD4+CD25+ T-Regulatory Cells

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Abstract

The objective of the present 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 day 6, 7, 8 and 9 post-immunization induced tolerance and inhibited EAAU in all Lewis rats. 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 to non-tolerized animals. Additionally, FasL, 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 to the cells from non-tolerized animals. Level of IFN-γ and IL-2 decreased while TGF-β2 was elevated in the state of tolerance. Furthermore, the number of $CD4^+CD25^+FoxP3^+$ Tregs increased in the popliteal LNs of tolerized animals compared to non-tolerized animals. In conclusion, our results suggest that deletion of antigen specific T cells via apoptosis and active suppression mediated by Tregs plays an important role in the induction of antigen specific immune tolerance in animals with an established immune response against MAA.

Keywords

Apoptosis; Autoimmune Uveitis; CD4+CD25+ T regulatory Cells; Inflammation; Tolerance

INTRODUCTION

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.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

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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 (CB) and the anterior chamber. Human idiopathic AU has been considered an autoimmune disease where an immune response affects only the eye.3 Severe visual complications associated with recurrent and/or untreated AU lead to the 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 (CFA). We have shown that MAA is a 22 kDa fragment of type I collagen α2 chain.11 In this experimental model an immune response is initiated in the popliteal lymph node of Lewis rats after MAA is injected subcutaneously in the foot-pad.6–12 This immune response selectively targets the anterior segment of the eye and results in ocular pathology that is similar to that observed in humans with idiopathic AU.6–12 The disease in EAAU is characterized histologically by a lymphocytic infiltration in the iris and CB.6–12 Antigen specific $CD4^+$ T cells can adoptively transfer EAAU to naive 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 (i.e. prior to sensitization) lead to complete protection and suppression of EAAU.12 The purpose of the present study was to investigate if 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 our current 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 MAA Administration on EAAU in Pre-sensitized Animals

Antigen specific tolerance induced via the intravenous route in MAA sensitized Lewis resulted in the inhibition of EAAU and the degree of protection against EAAU was dependent on the dose of the antigen. A single intravenous injection of 100 and 200 μg MAA at day 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 PBS injected control animals (Table 1). In contrast, complete protection (tolerance) was observed when 400, 600 and 800μg of MAA were injected separately via the intravenous 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 ciliary body (CB) and the anterior segment of the eye (Figure 1a). Intravenous 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.

Intravenous MAA Administration causes Apoptosis of Popliteal Lymph Node Cells

First TUNEL assay was utilized to detect difference, if any between the number of apoptotic cells in the popliteal lymph nodes (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 intravenous administration of 400μg MAA as described above. The rats in group 2 received the same treatment with PBS (non-tolerized). Rats from both groups were sacrificed on day 5, 8 and 11 post-immunization, popliteal LNs were harvested and paraffin sections of popliteal LNs were used for TUNEL staining. Representative results shown in Figure 2a-f revealed that at day 8 and 11 post-immunization more cells were undergoing apoptosis in the popliteal LN of tolerized rats (Figure 2e and f) compared to non-tolerized animals (Figure 2band 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 as well as apostat separately. These cells were then analyzed by flow cytometry. As expected the percentage of total live lymphocytes that were undergoing apoptosis (annexin V^+PI^-) were similar (18.7± 1.9 %) on day 5 post-immunization in both tolerized and non-tolerized animals (Figure 2g) since 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^+ PI^-) were significantly higher ($p< 0.05$) in tolerized animals (27.15±0.21%) compared to non-tolerized animals (23.25 ± 0.91%). The percentage of apoptotic lymphocytes increased drastically in the popliteal LNs of tolerized animals (34.28 ± 2.48%) compared to 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 lymph nodes of tolerized and non-tolerized animals (Figure 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 tolerized animals at day 8 (21.8 ± 0.56%) and day 11 (29.13 ± 4.8 %) compared to non-tolerized animals at these time points (day 8 - 17.75 ± 0.21%; 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 to 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 (Figure 2j–l). In this method the cells were stained intracellularly by 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 to non tolerized animals at day 8 and 11 post-immunization (Figure 2j–l).

Effect of Intravenous MAA Administration on the Proliferation of LN Cells

Total lymphocytes harvested from popliteal LNs of tolerized and non-tolerized animals sacrificed at day 11 post-immunization were labeled with CFSE using the Cell Trace CFSE cell proliferation kit and were cultured with MAA (10 μg/ml) for 6 days. The cells were then 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 peak. (Figure 3a–h). Furthermore, the percentage of cells in each generation including the cells remaining in the parent generation as well as proliferation index can also be calculated using this program.12 Total lymphocytes from the LNs of the tolerized animal had a significantly ($p<0.05$) reduced ability to proliferate compared to total lymphocytes from non-tolerized animals in the presence of MAA (Figure 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 to the $CD4^+$ cells from non-tolerized animal in response to the antigen (Figure 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 to the $CD8^+$ cells from non-tolerized animals (Figure 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 (Figure 3g, h and j), $CD4^+$ (Figure 3j) 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 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 to 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 to 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 to non-tolerized animals.

$CD4^+CD25^+FoxP3^+$ Tregs in MAA-i.v. Tolerance

MAA-i.v. tolerized Lewis rats were sacrificed at day 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 to non-tolerized animals at these time points (Figure 5a).

Adoptive 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 us,8, 11 non-adherent cells (2×10^7 cells) were collected and were adoptively transferred to naive 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. 5×10^6 $CD4^+CD25^+$ T cells were adoptively transferred to naive Lewis rats via the tail vein. Total lymphocytes (2×10^7 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 CFA) 24 hrs 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 while the second phase which started by day 13–14 was due to the foot-pad 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. Interestingly, 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 (AICD) 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 (Figure 6a and b). Both tolerized and non-tolerized rats were sacrificed on day 11 post-immunization, popliteal LNs were harvested; total lymphocytes 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 lymph nodes of tolerized animals (29.3 ± 3.55%) compared to non-tolerized animals (23 ± 1.9%) at day 11 post-immunization (Figure 6a). Similarly, more $TNFR1^+$ lymphocytes were detected in the lymph nodes of tolerized animals (19.25 ± 2.73%) compared to 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 AICD was confirmed by determining the protein levels of activated caspase-8 in tolerized and non-tolerized rats using 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 to non-tolerized animals at day 11 post-immunization.

DISCUSSION

Disability associated with the loss of vision has long lasting impact on the quality of life. Idiopathic anterior uveitis (AU), the inflammation of the anterior uveal tract (i.e. the iris and the ciliary body) is one of the major causes of legal blindness in the United States. Currently non-specific therapies such as steroid and immunosuppressive agents are used to treat the patients with idiopathic AU and these treatment modalities are associated with adverse side effects and long term complications. Due to the lack of safe and effective therapeutic options available to the 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.13 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 lymph nodes; 14 clonal anergy 15 and active suppression by regulatory T cells16 and have been reported to act independently or in combination.17

Recently we reported that T cell non-responsiveness due to active suppression mediated by regulatory T cells (Tregs) facilitates the development of tolerance to MAA and inhibition of experimental autoimmune anterior uveitis (EAAU) in animals without an established immune response against the uveitogenic antigen.12 In the present study we demonstrate that the administration of MAA (400 μg, i.v.) on four consecutive days by iv route to Lewis rats that has previously received sensitization by subcutaneous injection of MAA (with CFA) 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.18, 19

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 lymph nodes (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 (AICD) 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 lymph nodes of tolerized rats after the i.v. administration of MAA on day 6, 7, 8 and 9 post-immunization. The interaction between death factors and death receptors leads to apoptosis in AICD.20–25 Binding of Fas with FasL or interaction of TNF with TNF receptor-1 (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 plays 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 to 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 suppression of effector T cells we investigated the role of $CD4^+CD25^+FoxP3^+$ cells. Our results demonstrated that the percentage of $CD4^+CD25^+FoxP3^+$ Tregs increased significantly in rats with EAAU that received i.v injection of MAA (tolerized) compared to 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-β expression. 26 In the present study our results demonstrated increased levels of TGF-β2 and decreased levels of IL-2 in the lymphocytes from popliteal LNs of tolerized rats compared to non-tolerized animals. This may possibly be responsible for the decreased proliferation of T cells in tolerized animals. Furthermore the levels of IFN-γ were reduced in the popliteal LNs of tolerized animals. This reduction in the level of IFN-γ may be due to the suppressive effects of $CD4^+CD25^+FoxP3^+$ Tregs or due to 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 EAAU8, 11, 12 to determine if MAA-iv 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 polpliteal 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 CFA and EAAU develops in two phases in the recipient rats.12 In the present 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, $CD4^+CD25^+$ Tregs can induce apoptosis of the lymphocytes in the LNs.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 AICD and active suppression mediated by $CD4^+CD25^+FoxP3^+$ T cell. Human idiopathic AU is a recurrent disease where patients experience repeated episodes of the disease separated by period of remission.1–4 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 our present study where the animals have an established immune response to the pathogenic antigen, but the uveitis has yet to develop. Our unpublished

results demonstrate that 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 wk old) were obtained from Harlan Sprague Dawley (Indianapolis, IN). This study was approved by the Institutional Animal Care and Use Committee, University of Arkansas for Medical Sciences, Little Rock, AR 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 (MAA) was purified from bovine iris and ciliary body as previously described by us.11 Male Lewis rats were immunized with 100 μl of stable emulsion containing 75 μg of MAA emulsified(1:1) in CFA (Sigma-Aldrich, St. Louis, MO) using a single-dose induction protocol in the hind footpad as previously described by us.6–12 Depending on the experimental design, animals were examined daily between days 3 and 30 post injection for the 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 to 6, as follows: 0 = normal; 1 = dilated iris vessels and thickened iris and ciliary body; exudates in the anterior chamber with protein, a few scattered inflammatory cells, or both; 2 = moderate infiltration of inflammatory cells in the iris, ciliary body, or both; moderate number of inflammatory cells within the anterior chamber; 3 = heavy infiltration of inflammatory cells within the iris and ciliary body and within the anterior chamber; 4 = heavy exudation of cells with dense protein aggregation in the anterior chamber; inflammatory cell deposits on the corneal endothelium; 5= 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; 6= 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.6, 7, 10–12

Induction of Immune Tolerance

To induce tolerance, MAA sensitized Lewis rat received a single intravenous (i.v.) injection of MAA on day 6, 7, 8 and 9 post-immunization. MAA was dissolved in 400 μl of PBS and different doses (100, 200, 400, 600 and 800μg) 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 lymph nodes (LNs) were fixed in neutral buffered 10% formalin solution (Sigma-Aldrich, St. Louis, MO) for 24 hours 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). Sections were examined using a light microscope (CarlZeiss Meditec, Inc., Thornwood, NY).

Cell Preparation from Popliteal Lymph Nodes

Popliteal LNs were harvested and a single cell suspension was prepared as described previously.12 Total lymphocytes were purified by using Histopaque gradient (Sigma Aldrich, St. Louis, MO) according to manufacturer's protocol. The cells were suspended in complete RPMI 1640 culture medium containing L-Glutamine (Mediatech, Herndon, VA) containing 1% (v/v) MEM (Life Technologies, Rockville, MD), NEAA (BioWhittekar, Allendale, NJ), mixture of antibiotics (penicillin 100 U/ml, streptomycin 100 U/ml and Amphotericin B 0.25 μg/ml) purchased from BioWhittekar, Allendale, NJ, and 10% (v/v)FCS (Mediatech, Herndon, VA).

Detection of Apoptosis

a) TUNEL 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 four-micron paraffin sections were prepared as described above. Terminal deoxynucleotidyl transferase (TdT)–mediated d-UTP-biotin nick end labeling (TUNEL) assay (Biovision Inc., Mountain View, CA) was used as follows. Deparaffinized sections of popliteal LNs were treated with proteinase K (20 μg/mL) in 10 mM Tris-HCl (pH 8.0) for 20 minutes at 37°C, followed by washing with PBS. The tissue was incubated with the TUNEL reaction mixture in a humidified chamber at 37°Cfor 1 hour. Negative control was treated similarly; however the enzyme solution was omitted. The tissue was then incubated with biotin-Texas red conjugate for 30 minutes at room temperature. The sections were covered by antifade reagent with DAPI (Invitrogen, Carlsbad, CA) and were examined under fluorescence microscopy (Carl Zeiss Meditec, Inc., Thornwood, NY).

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) for 15 minutes at 4°C to block Fc receptors. The cells were then stained with anti rat CD4-APC (BD Biosciences, San Jose, CA), anti–rat CD8-APC (Biolegend, San Diego, CA) separately in 100 μL stain buffer for 30 minutes in the dark at 4°C. After washing with cold PBS, the cells were stained with Annexin V-FITC and PI in binding buffer (Apoptosis Kit 1; BD Biosciences, San Jose, CA) according to manufacturer's instructions. In a separate set of experiments cells were stained for Apostat-FITC (R&D Systems, Minneapolis, MN) intracellularly using BD cytofix/cytoperm kit (BD Biosciences, San Jose, CA) according to

the manufacturer's instructions. Flow cytometric analysis was performed on FACS Calibur (BD Biosciences, San Jose, CA), 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 RNeasy kit (Qiagen, Valencia, CA). Quantitative real-time PCR (RT-qPCR) was performed using iQ™ SYBR® Green Supermix and iQ™5 real time PCR unit (both purchased from Bio-Rad, Hercules, CA). The primers were designed and ordered from Integrated DNA Technologies (Coralville, IA), using the criteria provided in the Bio-Rad users manual. Primer sequences including the predicted sizes of amplified cDNA are as follows: β-actin (103 bp) forward: 5′-AACCCTAAGGCCAACCGTGAAA-3′, reverse: 5′-AGGCATACAGGGACAACACA- 3′; IFN-γ (133 bp) forward: 5′-ACCAGGCCATCAGCAACAACAT -3′, reverse: 5′-TGCTGGATCTGTGGGTTGTTCA -3′; TGF β2 (115 bp) forward: 5′-CGTGAAGTGGCTGTTGATCT-3′, reverse: 5′-GTGTTTTCATCATGCTGGCT-3′; IL-2 (107 bp) forward: 5′-TGTGTTGCACTGACGCTTGT-3′, reverse: 5′-AGCACCTGTAAGTCCAGCAA-3′. Pilot 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 mRNA expression was normalized against β-actin expression. iQ™5 optical system software version 2.0 (Bio-Rad, Hercules, CA) was used to analyze real-time RT-qPCR data and derive threshold cycle (C_T) values according to the manufacturer's instructions. The DDC_T method was used to transform C_T 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.

Enzyme-Linked Immunosorbent Assay (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 hours. Supernatants were collected for quantitative ELISA for interferon (IFN)-γ, (BD Biosciences, San Diego, CA), and IL-2 (R&D Systems, Minneapolis, MN). ELISA was performed using paired mAbs 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 three days and non-adherent cells were collected. Non-adherent cells were labeled with CD4-APC and CD25-PE antibodies (BD Biosciences, San Jose, CA) and $CD4^+CD25^+$ cells were collected by cell sorting using FACSAria (BD Biosciences, San Jose, CA). 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) using BD cytofix/ cytoperm kit according to manufacturer's instructions (BD Biosciences, San Jose, CA). The stained cells were analyzed using Flow cytometer.

TNFR1 and FasL staining

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

In Vitro Cell Proliferation Assay

Total lymphocytes harvested from popliteal LNs were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) using Cell Trace CFSE Cell Proliferation Kit according to manufacturer's protocol (Molecular Probes, Invitrogen, Carlsbad, CA) and were cultured with MAA (10 μg/ml) for six 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 semidry electrophoretic transfer cell (Trans-Blot; Bio-Rad, Hercules, CA). Blots were stained at room temperature with 1:1000 dilution of one of the following antibodies: rabbit anti–caspase-8, (Biovision, Mountain View, CA), or anti–β-actin (Sigma, St Louis, MO). 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). Quantification of proteins was accomplished by analyzing the intensity of the bands (Quantity One 4.2.0; Bio-Rad, Hercules, CA). 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 ± SD. Data were analyzed and compared using Student's *t* test, and differences were considered statistically significant with $p < 0.05$.

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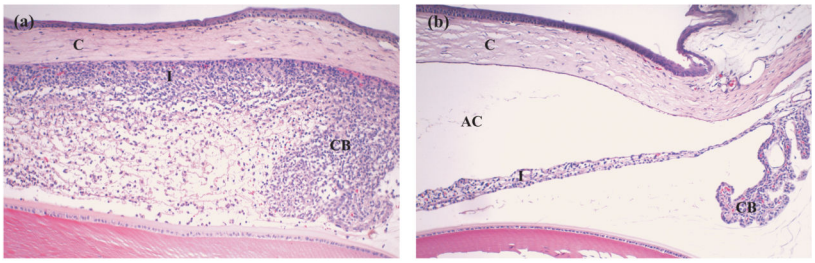

Figure 1.

Induction of tolerance to MAA in EAAU by intravenous (i.v.) injection of MAA (400 μg) in pre-sensitized animals. **(a–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). 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, **10X)**.

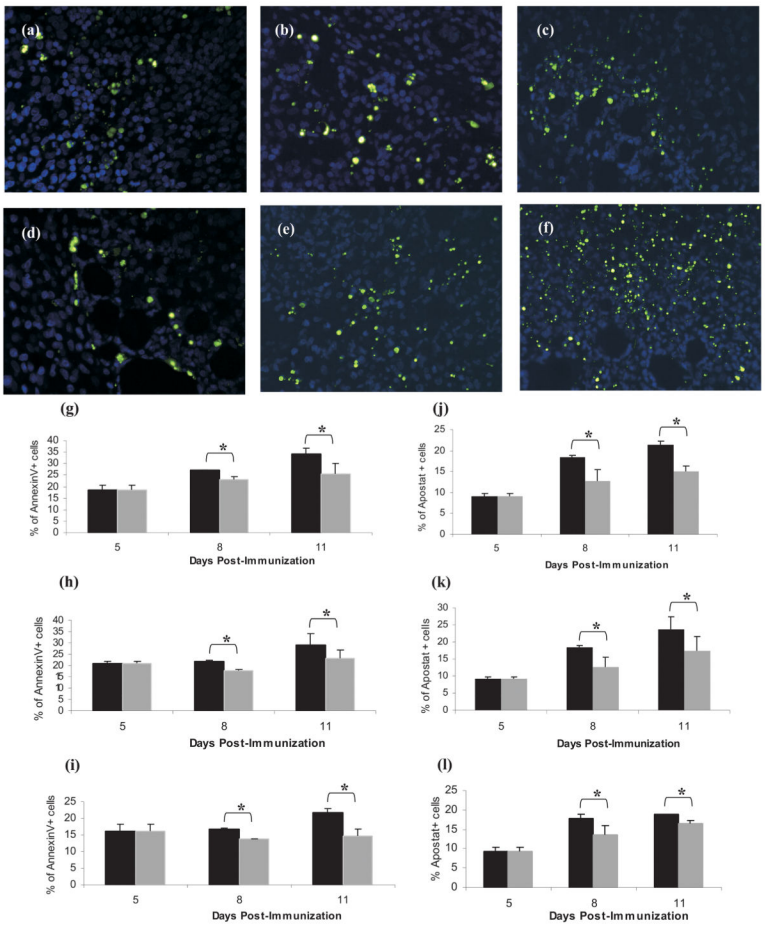

Figure 2.

Effect of i.v. injected MAA on apoptosis of LN cells in MAA sensitized Lewis rats. **(a–f)** 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, d**), day 8 (**b, e**) and day 11 (**c, f**). (Objective magnification, **20X**). (**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. *$p < 0.05$

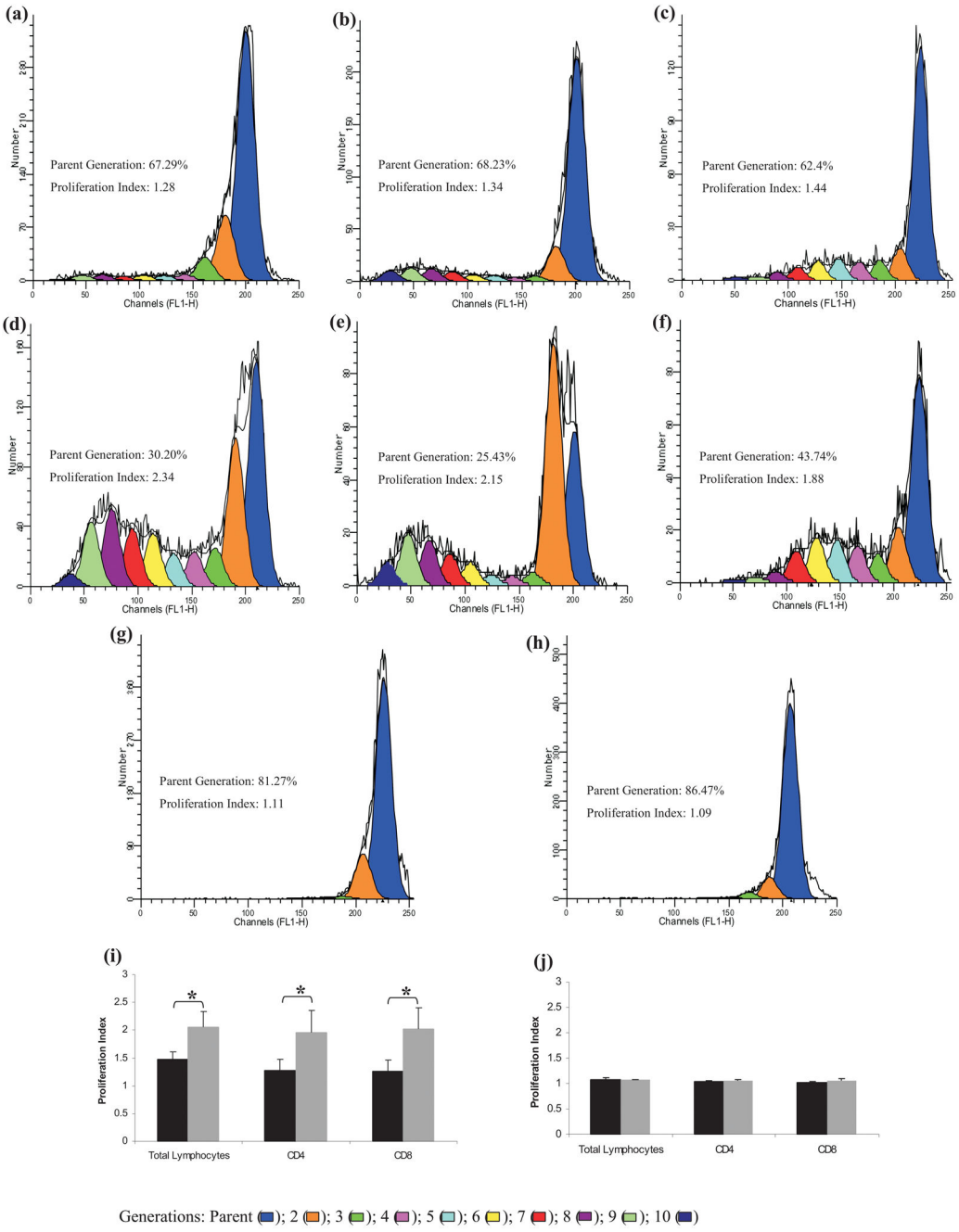

Figure 3.

Effect of i.v. injected MAA on the proliferation of cells harvested from the popliteal LNs. Representative raw data (obtained by FACS analysis) for *in vitro* proliferation of CFSE-labeled lymphocytes obtained from tolerized (**a–c, g**) and non-tolerized (**d–f, h**) rats in presence of MAA (**a–f**) or absence of MAA (**g–h**). *In vitro* proliferation of CFSE-labeled total lymphocytes (**a, d, g, h**), CD4 (**b, e**) and CD8 (**c, 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. *$p<0.05$.

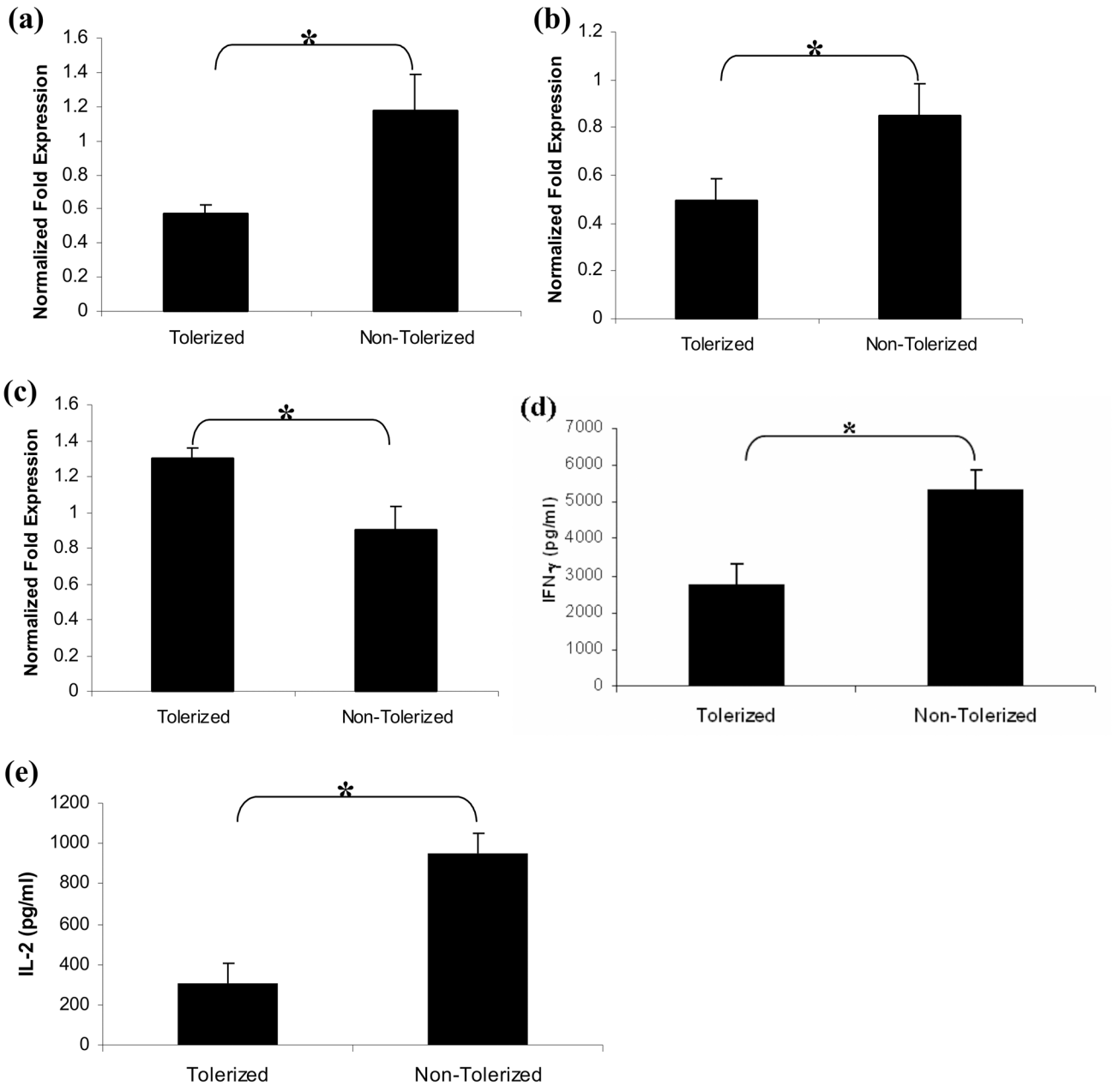

Figure 4.

Analysis of cytokine expression after intravenous MAA-induced tolerance in EAAU. Results of quantitative real time-PCR analysis for IFN-γ (**a**), IL-2 (**b**) and TGF-β2 (**c**) are shown. Protein levels of IFN-γ(**d**) and IL-2 (**e**) in culture supernatants were measured by ELISA. Data are reported as mean ± standard deviation for triplicate determinations. Results are representative of three independent experiments. *$p<0.05$.

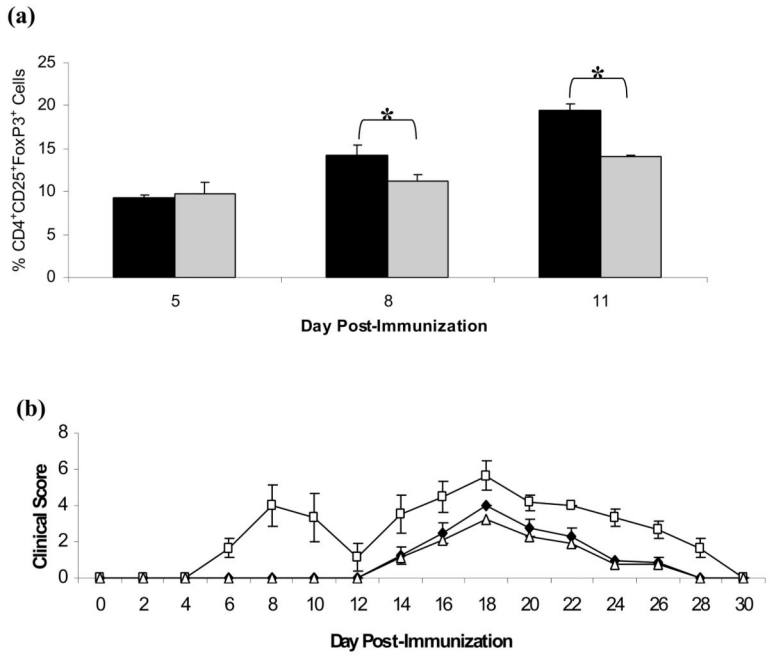

Figure 5.

Tregs in iv-MAA 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. 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 (☆). Similar results were obtained when $CD4^+CD25^+$ cells from tolerized animals were adoptively transferred to naive Lewis rats (✦).

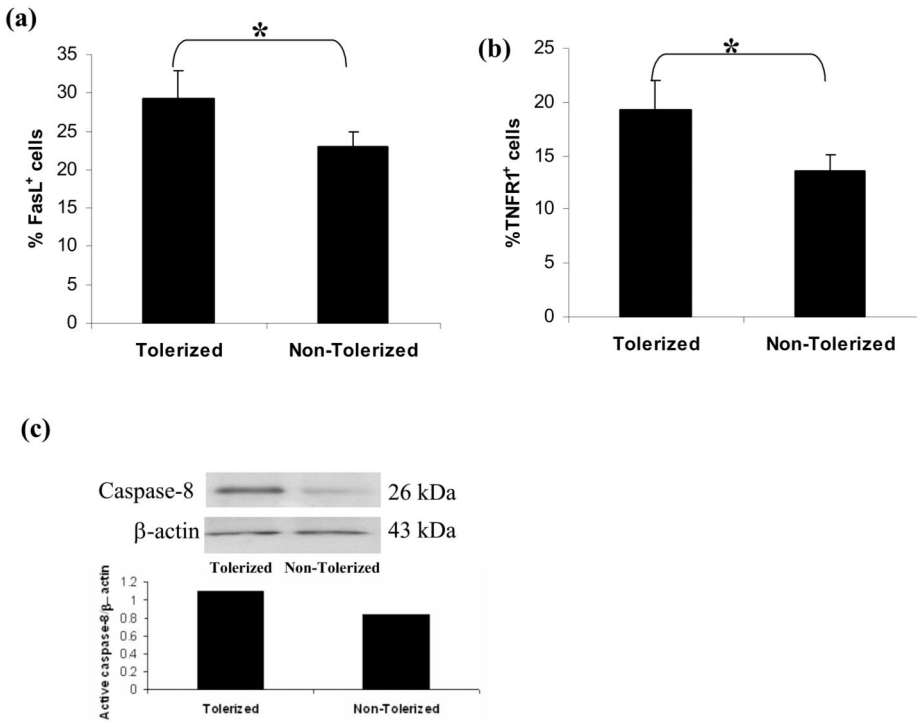

Figure 6.

Mechanism of apoptosis induction. Percentage of $FasL^+$ (**a**) and $TNFR1^+$ (**b**) cells in the popliteal 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.

TABLE 1

Effect of Intravenous Administration of MAA on EAAU

			Eyes with EAAU				
MAA i.v. (μg)	**OVA i.v. (μg)**	**PBS i.v. (μl)**	**Incidence**	**Mild**	**Severe**	**Day of Onset**	**Duration of Disease (days)**
100	0	400	6/6	2/6	4/6	14.3±.5*	10.7±1.2**
200	0	400	6/6	4/6	2/6	15.7±.5*	8±1.4**
400	0	400	0/24	-	-	-	-
600	0	400	0/6	-	-	-	-
800	0	400	0/6	-	-	-	-
0	400	400	6/6	-	6/6	13.2±.6	12.5±0.8
0	0	400	24/24	-	24/24	13.2±.4	12.7±0.5

EAAU = Experimental Autoimmune Anterior Uveitis. MAA = Melanin Associated Antigen, OVA = Ovalbumin, PBS = Phosphate buffered saline, i.v. = intravenous. Incidence of EAAU given as positive/total eyes following clinical examination. Severity of inflammation on histopathologic examination was grouped as normal (0), mild (1+ to 2+) or severe (3+ to 4+).

*, ** $p<0.05$.