Influence of macrophages and lymphocytes on the survival and axon regeneration of injured retinal ganglion cells in rats from different autoimmune backgrounds

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Abstract
The immune response after neural injury influences the survival and regenerative capacity of neurons. In the primary visual pathway, previous studies have described beneficial effects of macrophages and T-cells in promoting neural survival and axonal regeneration in some rat strains. However, the contributions of specific cell populations to these responses have been unclear. In adult Fischer (F344) rats, we confirm prior reports that intravitreal macrophage activation promotes the survival of retinal ganglion cells (RGCs) and greatly enhances axonal regeneration through a peripheral nerve graft. Neonatal thymectomy that results in elimination of T-cell regeneration, thymectomy had only a small beneficial effect on RGC survival, and although Lewis lymphocytes reduced RGC viability in culture, they led to a 60% reduction in viable RGCs. Similar in vivo results were obtained in Sprague Dawley rats. By comparison, in adult Lewis rats, neither RGC survival nor axonal regeneration was increased after intravitreal macrophage activation. Neonatal thymectomy had only a small beneficial effect on RGC survival, and although Lewis lymphocytes reduced RGC viability in culture, they did so to a lesser extent. Thus, in addition to a complex role of lymphocytes, particularly T-cells, after central nervous system injury, the present results demonstrate that the impact of macrophages is also influenced by genetic background.

Introduction
Lymphocytes and macrophages are important cellular mediators of inflammation and immune responses. Various lines of evidence point to a deleterious effect of these cells after central nervous system (CNS) injury and in autoimmune diseases that affect the nervous system (Blight, 1992; Dijkstra et al., 1992; Hirschberg et al., 1994; Huitinga et al., 1995; Popovich et al., 1996; Newman et al., 2001; Kuhlmann et al., 2002; Neumann, 2003). However, other evidence also suggests potential neuroprotective actions of these cells (Prewitt et al., 1997; Rapalino et al., 1998; Leon et al., 2000; Kipnis et al., 2001; Yoles et al., 2001). For example, it was previously shown that zymosan-activated macrophages produce neuroprotective as well as detrimental factors (Yin et al., 2006). Macrophage activation in the eye promoted retinal ganglion cell (RGC) survival and axonal regeneration in crushed optic nerve (ON), a natural CNS environment that is hostile to axon regeneration, or in the more permissive environment of a peripheral nerve (PN) grafted onto the cut ON. Growth-promoting effects in Fischer (F344) rats were dependent on the timing of macrophage activation relative to ON injury (Yin et al., 2003).

There is now evidence in rodents that survival of traumatized CNS neurons is strain-dependent and influenced by polygenic loci that modulate resistance/susceptibility to experimental autoimmune encephalomyelitis (EAE) induced by active immunization with myelin basic protein. After ON crush injury, T-cell-related autoimmune protected RGCs from degenerative events in EAE-resistant Sprague Dawley (SPD) rats (Kipnis et al., 2001; Yoles et al., 2001) but not in EAE-susceptible Lewis rats (Kipnis et al., 2001). Better RGC survival was also observed in EAE-resistant strains as compared with EAE-susceptible strains in mice (Kipnis et al., 2001). Improved functional recovery was also observed after transfer of splenocytes in spinal cord-injured SPD rats (Yoles et al., 2001). In contrast, detrimental actions of CD4+CD25+ regulatory T-cells on RGC survival after ON crush and on locomotor activity after spinal cord contusion were also...
reported in mice (Kipnis et al., 2002). Recently, in EAE-resistant and EAE-susceptible mice, it was found that a predisposition to EAE predicted the magnitude of intraparenchymal inflammation but not lesion size/length or locomotor recovery following spinal contusion injury (Kigerl et al., 2006).

It is important to note that, in the above-cited studies (Kipnis et al., 2001; Yoles et al., 2001), what was measured in the different strains of rats and mice was the ability of RGCs to resist the impact of the partial ON crush and any subsequent secondary degenerative effects. Because retrograde labeling methods were used, only the population of RGCs whose axons were presumably intact after ON crush was counted; the total number of surviving RGCs whose axons were injured or transected was not assessed. On the other hand, survival of prelabeled RGCs, whose axons were either injured or not, were examined in the study of actions of CD4+CD25+ regulatory T-cells (Kipnis et al., 2002). Reported differences in the effects of different T-cell populations on RGC survival may in part be related to species differences but may also be a consequence of using different methods to assess viability of different RGC populations after injury.

F344 and Lewis rats are two inbred rat strains that are, respectively, resistant and susceptible to EAE. It is known that hypothalamic–pituitary–adrenal axis function modulates autoimmune response and vulnerability to EAE. Lewis rats, in contrast to F344 rats, have abnormalities in hypothalamic–pituitary–adrenal axis function, and genomic regions that regulate the different disease expression between the two strains of rats have been reported (Wildier et al., 2000), suggesting genetic control of the autoimmune response. In addition, the greater frequency of CD8+ regulatory T-cells that functionally inhibit myelin basic protein-reactive T-cells in F344 than in Lewis rats was found to be a factor that may contribute to the different susceptibility to EAE in the two strains (Sun et al., 1999). To further unravel the role of macrophages in F344 and Lewis rats, and how T-cells (lymphocytes) interact with macrophages in the survival and axonal regrowth of injured RGCs, a series of experiments was carried out in the two rat strains as well as another EAE-resistant strain (SPD), with or without thymus gland removal at birth. Neonatal thymectomy results in an absence of T-cells in adult rodents (Thorton & Shevach, 1998; Kipnis et al., 2001; Yoles et al., 2001). To better assess RGC axonal regrowth, we used the PN–ON graft approach, shown previously to allow quantitative analysis of the effect of macrophages and other agents on RGC viability and regeneration (Yin et al., 2003; Park et al., 2004; Luo et al., 2007). Finally, as an adjunct to these complex in vivo studies, the effects on RGC survival of lymphocytes from different rat strains were examined in tissue culture using adult retinal explants.

### Materials and methods

#### In vivo surgical procedures

F344, SPD and Lewis rats were obtained from Vital River, Beijing, China – the animals were originally derived from Charles River Laboratory. All experiments were in compliance with the guidelines of the Shantou University Medical College and the Chinese University of Hong Kong Animal Experimentation Ethics Committees and were approved by the Shantou University Medical College and Chinese University of Hong Kong Animal Experimentation Ethics Committees. All surgery was carried out under anesthesia with a 1 : 1 mixture (1.5 mL/kg) of ketamine (100 mg/mL) and xylazine (20 mg/mL).

The ON transection and PN–ON surgical procedures have been described previously (Vidal-Sanz et al., 1987; Cui et al., 2003). Briefly, after anesthesia with ketamine and xylazine the left ON was exposed through a posterior temporal intraorabital approach. The ON was completely transected about 1.5 mm behind the optic disc. Immediately after the transection, a 1.5-cm-long segment of autologous peroneal nerve was dissected out from the left leg and sutured with a 11/0 suture onto the proximal stump of the axotomized ON. The distal part of the PN was placed over the skull and tied with a 6/0 suture to connective tissue (Cui et al., 2003). For thymectomy, 1-day-old rats were anesthetized on ice. The chest was opened on the left side, and the whole thymus gland was gently pulled out and completely removed. The chest was closed with a 5/0 suture. Animals were kept on warm pads until recovery, and then returned to their mothers. The thymectomized animals were housed in air-filtered cages afterwards. Although thymus gland removal at birth results in an absence of T-cells in the adult (Thornton & Shevach, 1998; Kipnis et al., 2001; Yoles et al., 2001), recent evidence suggests that fetal thymus is colonized by progenitors that have made both T-cell and B-cell lineage choices (Jenkinson et al., 2006). Thus, B-cell production in the adult may also be affected by thymus gland removal.

#### In vivo experimental groups

Animals were allocated to different experimental groups (see Supplementary material, Table S1). All animals received a PN autograft at 8–10 weeks of age. Intravitreal injection of saline (3 μL) or zymosan (concentration of 1.25 μg/μL in 3 μL) was performed 3 days after the PN–ON procedure (Yin et al., 2003).

#### Visualization of regenerating RGCs

A survival time of 3 weeks after the PN–ON procedure was used for in vivo experiments. After anesthesia with ketamine and xylazine, the PN grafts lying on the skull were exposed. A small amount (0.2 μL) of 4% of fluorogold (FG; Fluorochrome Inc., Denver, USA) was slowly injected into the distal end of each PN graft. In our approach, injection of a small volume of FG was essential to avoid diffusion of the dye towards the optic disc and consequent staining of viable but non-axon-regenerating RGCs (Cui et al., 2003). Three days later, animals were killed by an overdose of the same anesthetics and perfused with cold 4% paraformaldehyde in phosphate-buffered saline (PBS). In order not to disturb macrophages on the retinal surface, care was taken to avoid touching the upper surface of the retina and no attempt was made to remove vitreous during retinal dissection and processing (Luo et al., 2007). The retinas were postfixed for 1 h, and then flat-mounted before being examined using fluorescent microscopy. To determine the total number of FG-labelled RGCs, a grid was randomly placed over the retinal image on computer and on each grid intersection the number of FG-labelled RGCs was counted under 25× magnification at that point. About 80 fields were sampled per retina (~8–10% of the retina) (Cui et al., 2003; Yin et al., 2003). The average number of FG-labelled RGCs per field was determined, and the total number was estimated by multiplying this figure by the retinal area.

#### Immuno reaction of the retinas for βIII-tubulin, ED1 and CD3 staining

After the numbers of regenerating RGCs were obtained, the retinas were then immunostained for βIII-tubulin using the TUJ1 monoclonal antibody (BabCO, Richmond, CA, USA). βIII-tubulin has been shown to be an RGC-specific marker in retinal wholenumounts (Cui et al., 2003; Yin et al., 2003; Fischer et al., 2004; Kopriwica et al., 2005). ED1 antibodies were used to identify macrophages (Yin et al., 2003). After PBS washes, nonspecific binding was blocked in PBS contain-
ing 10% normal goat serum, 1% bovine serum albumin and 0.2% Triton for 1 h, and then half of the retinas were incubated with mouse primary antibody TUJ1 (1 : 500; anti-III tubulin) and the other half were incubated with ED1 (1 : 200; Serotec, Kidlington, Oxford, UK) overnight at 4 °C. Retinas were rinsed with PBS and then incubated with fluorescein isothiocyanate (FITC)-conjugated (1 : 100; Sigma, Saint Louis, Missouri, USA) or cy3-conjugated (1 : 400; Jackson ImmunoResearch, West Grove, PA, USA) secondary antibody overnight at 4 °C. The retinas were eventually examined under the fluorescent microscope. Both III-tubulin-positive and ED1-positive cells were counted. The numbers of III-tubulin-positive surviving RGCs and ED1-positive macrophages were counted in the same way as above. Sections with omission of primary antibody were always used as negative controls.

To investigate whether T-cells were present and accumulated differently in F344 and Lewis retinas after zymosan treatment, retinas from both strains were obtained 3 days after zymosan treatment and immunoreacted with a general lymphocyte marker (CD3 antibody, 1 : 300; BD Biosciences, San Jose, CA, USA). Immunostaining procedures were as described above.

**Immunoreaction of blood and flow cytometry analysis of CD3+ T-cells**

To examine whether our thymectomy procedures were consistently successful, T-cell staining in the blood was carried out in adult rats. The blood obtained from the hearts of four adult rats of each strain that had been thymectomized at birth was immediately treated with heparin sodium (25 units/mL), and then histopaque-1083 (Sigma) was added for the isolation of the white blood cells. Blood from normal rats of each strain was used as control. The blood was centrifuged at 400 g for 20 min, the supernatant was removed, and the sediment was dropped onto a slide and air-dried. The cells were immunoreacted with mouse anti-rat CD3 monoclonal antibody (1 : 300) to identify T-cells of all populations. After washes, the slides were incubated with cy3-conjugated secondary antibody (1 : 400).

In a second approach, flow cytometry was used to examine the CD3+ T-cell population in the blood from these rats. Peripheral whole blood samples with EDTA (5 mm) as anticoagulant were obtained from adult F344 and Lewis rats with or without thymectomy at birth. Erythrocytes were lysed using buffered ammonium chloride (139.6 mM NH₄Cl, 16.96 mM Tris, pH 7.2) for 10 min, and then centrifuged at 400 g for 5 min and washed with buffer (PBS supplemented with 1% bovine serum albumin). Mouse anti-rat CD3-FITC/CD4-RPE (Serotec) or isotype antibody (10 µL) was used. The cells were eventually resuspended in 0.2 mL of 0.5% paraformaldehyde in PBS–bovine serum albumin and analysed by flow cytometry that allowed gating of leukocyte (monocyte) and FITC–CD3+ T-cells.

**Lymph-derived cell culture and retinal explant experiments**

To verify whether lymphocytes had a detrimental effect on RGC viability, RGCs were examined in vitro after the retinas were cocultured with congeneric or allogeneic lymphocytes. For lymphocyte culture, skin under the jaw was cut and lymph nodes were exposed. The nodes were then cut into 1 mm³ pieces and digested in 0.25% trypsin for 3 min at room temperature. Cell strainers of pore size 70 µm and 40 µm were used in sequence to filter the solution to remove undigested tissues. The solution was then centrifuged at 400 g for 5 min. The supernatant was discarded and the cells were resuspended with Dulbecco’s Modified Eagle’s Medium in 10% fetal bovine serum. Lymphocytes were then counted, and 1 × 10⁶ lymphocytes (derived from the three lymph nodes) were cultured in the incubator with 5% CO₂ at 37 °C for 1 day. The lymphocytes were then cocultured with half retina (n = 5–6 each group) for 3.5 days and then replenished with new lymphocytes (1 × 10⁶) and cultured for another 3.5 days (total incubation time 7 days).

To render an ON injury condition before obtaining the retinas for culture, the ON was cut 1.5 mm behind the eyeball after anesthesia with ketamine and xylazine. The retinas were dissected out in cold Hank’s Balanced Salt Solution after overdose with ketamine and xylazine 3 days after the procedure, and cut into halves. Retinas were mounted onto nitrocellulose filter paper with the RGC layer uppermost. The retinas were cultured in neurobasal A + B27 (Gibco) medium supplemented with glutamine and penicillin–streptomycin. Retinas were divided into control and lymphocyte treatment groups. Crossover examination (F344 retinas treated with Lewis lymphocytes and vice versa) of the lymphocyte effects was also carried out, and some retinas from intact animals were used for additional comparisons (supplementary Table S2). Cultured retinas were fixed with 4% paraformaldehyde for 1 h and immunoreacted with TUJ1 antibody (1 : 400) and then with cy3 secondary antibody (1 : 400) to identify viable RGCs in a protocol as described above. The number of viable RGCs was counted in the same way as for the in vivo experiments.

**Statistical analysis**

Data from all groups in vivo and in vitro were pooled, and statistically analysed using the Bonferroni test (Edwards & Berry, 1987) following one-way analysis of variance (ANOVA). We and others routinely use the Bonferroni test for intragroup comparison of mean values (Cui et al., 2003; Yin et al., 2003; Pearse et al., 2004; Cen et al., 2007).

**Results**

Detailed conditions and results of in vivo and in vitro experiments are shown in supplementary Tables S1 and S2, respectively. Representative fluorescence photomicrographs of FG-labeled axon-regenerating RGCs (left column) and III-tubulin-positive viable RGCs (middle column) under various conditions are shown in Fig. 1. The arrows in Fig. 1A and B point to some surviving RGCs that are also axon-regenerating in the same field. Typical views of ED1-positive macrophages in retinal wholemounts under different conditions are shown in Fig. 1 (right column). It is known that blood-borne macrophages start to invade the nerve fiber layer in the retina within 5 days after ON axotomy and reach a peak at 7 days (Garcia-Valenzuela & Sharma, 1999). In our studies, almost all the ED1-positive macrophages were seen in the nerve fiber layer of the retina and changed morphologically from a ramified form to a round activated shape devoid of obvious processes (right column in Fig. 1).

**Macrophage activation promoted RGC survival and axonal regeneration in F344 rats**

Following ON injury, RGCs die rapidly via apoptosis (Berkelaar et al., 1994; Isenmann & Bähr, 1997; Isenmann et al., 1999; Bähr, 2000). We first confirmed the outcome of a previous study in F344 rats in which zymosan, a yeast wall preparation, was used to activate macrophages in the eyes (Yin et al., 2003). Intravitreal injection of zymosan dramatically increased ocular macrophage numbers (P < 0.001; Fig. 2C), and this was accompanied by enhanced RGC survival.
The average numbers of FG-labeled axon-regenerating RGCs and ED1-positive macrophages in the retina were similar to each other in the PN–ON only and PN–ON plus saline groups (Fig. 2B and C). The number of βIII-tubulin-positive surviving RGCs was lower \((P < 0.05)\) in the PN–ON group than in the PN–ON plus saline group (Fig. 2A), suggesting a small detrimental effect of the eye injection procedure on RGC survival. Because an enlarged pool of surviving RGCs will probably lead to an increased number of axon-regenerating RGCs among all surviving RGCs under these conditions, in order to determine whether axon-regenerating ability was also affected. It was found that zymosan-induced macrophage activation did indeed significantly increase the proportion of viable RGCs that regrew an axon \((P < 0.001\) against PN–ON or saline group; Fig. 2B).

**Thymectomy results in enhanced RGC survival in F344 rats**

CD3+ T-cells were seen in normal blood (Fig. 3A), but no T-cells were detected in blood obtained from four F344 rats 11 weeks after thymectomy at birth (Fig. 3B). Accumulation of CD3+ T-cells was seen in retinal wholemounts (Fig. 3C) and retinal cross-sections (Fig. 3D) in zymosan-treated Lewis rats. No CD3+ T-cells were seen in F344 retinas after zymosan administration (data not shown). Moreover, flow cytometry showed that whereas there was a large number of CD3+ T-cells in normal blood, the T-cell population almost completely disappeared in blood from thymectomized rats (Fig. 3E). These two methods confirmed that the thymectomy approach was effective in preventing the production of T-cells in the adult. These results are also consistent with previous work in which thymectomy was used to prevent genesis of mature T-cells in rodents (Thornton & Shevach, 1998; Kipnis et al., 2001; Yoles et al., 2001).

After the PN–ON procedure, F344 rats with thymectomy showed a marked increase in the number of surviving RGCs in both the PN–ON and PN–ON plus saline groups as compared with their corresponding nonthymectomized counterparts \((P < 0.001\) in both cases; Fig. 2A). The data from the groups with and without thymectomy suggest that the autoimmune response of lymphocytes is detrimental to RGC survival after ON injury in EAE-resistant F344 rats. No further increase in the number of surviving RGCs was seen after zymosan treatment in the thymectomized F344 rats (Fig. 2A).

Despite the higher numbers of surviving βIII-tubulin-positive RGCs after thymectomy, the absolute numbers of RGCs that regenerated an axon into a PN graft in control and saline-injected rats were similar to that in nonthymectomized rats. Interestingly, the proportion of viable RGCs that regenerated an axon after thymectomy was similar in PN–ON animals but was about two-fold lower in the respective saline and zymosan treatment groups (Fig. 2B). Note that, after thymectomy, zymosan still stimulated some axonal regeneration (19.3% vs. 11.6% in saline group, \(P < 0.01\); Fig. 2B), but the extent of this increase was far less than that seen in nonthymectomized rats (47.8%).
together, these results demonstrate that thymectomy (T-cell removal) improved RGC viability but reduced the beneficial effect of macrophage activation on RGC axonal regeneration.

**Zymosan only transiently activates macrophages after thymectomy in F344 rats**

It is possible that the reduction in zymosan-induced axon regeneration in thymectomized rats (Fig. 2B) might in some way be related to a lack of macrophage activation or to an altered time-course of this activation. To test this, we quantified macrophage activation in the retina of thymectomized vs. nonthymectomized rats at different times after intravitreal zymosan injection. The number of macrophages in the retina of normal rats continued to increase over the examination period (2–18 days), whereas the same zymosan treatment only transiently activated macrophages in the eye after thymectomy (Fig. 4A). These data demonstrate that zymosan-induced macrophage activation and invasion into the retina is influenced by lymphocytes; in the absence of lymphocytes, the activation and recruitment of macrophages is not sustained. The smaller increase in axonal regeneration after zymosan treatment in thymectomized F344 rats (Fig. 2B) may therefore be a result of this reduced macrophage activation over time.

**Macrophage activation does not promote RGC survival or axon regeneration in Lewis rats**

In Lewis rats, the average number of βIII-tubulin-positive RGCs in the PN–ON plus saline group was not significantly different (P > 0.05) from that in the noninjected PN–ON group, and the average numbers of FG-labeled axon-regenerating RGCs were also similar between the two groups (Fig. 5A and B). In contrast to F344 rats, intravitreal injection of zymosan failed to promote RGC survival and axonal regeneration in Lewis rats (Fig. 5A and B), despite the fact that this agent also significantly activated macrophages in this strain (Fig. 5C). In fact, zymosan reduced the axon-regenerating ability of the injured RGCs in Lewis rats, because the proportion of axon-regenerating RGCs after zymosan treatment decreased significantly as compared with the PN–ON plus saline group (P < 0.01; Fig. 5B). Note that the number of ED1-positive macrophages was significantly (P < 0.001) lower in Lewis rats than in F344 rats in the PN–ON only and PN–ON plus saline groups (compare Figs 2C and 5C or see supplementary Table S1).

To investigate whether a reduced macrophage response to intravitreal zymosan might contribute to the low RGC survival and axonal regeneration in Lewis rats, we also investigated the time-course of macrophage activation after zymosan application in Lewis rats. It was found that macrophages were also activated soon after zymosan application and remained at a high level for the rest of the examination period (Fig. 4B). Thus, even though macrophages responded similarly to zymosan treatment in F344 and Lewis rats, they exerted very different actions on RGC survival and perhaps opposite actions on axonal regeneration. Interestingly, immunohistochemistry revealed that there were numerous CD3+ T-cells in Lewis retinas after zymosan treatment, primarily in the ganglion cell layer (Fig. 3C and D). In contrast, CD3+ T-cells were not seen in F344 rats after zymosan treatment (data not shown). Note here that, with the use of a different pan-T-cell antibody (R73), labeled cells were not visualized in either F344 or Lewis retinas after ON axotomy (Cui et al., 2007).

**Thymectomy also enhances RGC survival in Lewis rats, although to a lesser extent**

Similar to what was seen in F344 rats, no CD3+ T-cells were detected in the blood of the four Lewis rats 11 weeks after thymectomy at birth (data not shown). Although the protective effect of thymectomy on RGC survival was not as striking as in F344 rats, the trend was similar in Lewis rats. The average numbers of surviving RGCs in PN–ON, PN–ON plus saline and PN–ON plus zymosan rats were, respectively, 20%, 54% and 42% higher after thymectomy than in their respective counterparts without thymectomy (P < 0.05, P < 0.001 and P < 0.001, respectively; Fig. 5A). With regard to axonal regeneration, in all thymectomized Lewis treatment groups, there was no significant difference in the proportion of surviving RGCs that regenerated an axon, and no difference between the same treatment groups in rats with and without thymectomy (Fig. 5B).

**Zymosan temporarily activates macrophages after thymectomy in Lewis rats**

The numbers of macrophages in normal and thymectomized Lewis rats were low in the PN–ON and PN–ON plus saline groups 3 weeks...
after the PN–ON procedure (Fig. 5C). However, the number of macrophages in zymosan-treated Lewis rats was significantly lower \((P < 0.001)\) after thymectomy (Fig. 5C). This is a similar trend to that observed in F344 rats. To investigate whether macrophages reacted differently after zymosan treatment in Lewis rats with and without thymectomy, we also investigated the time-course of macrophage activation after zymosan application in thymectomized Lewis rats. We found that the number of macrophages increased soon after zymosan application, but to a far lower level than without thymectomy (Fig. 4B). This value then decreased slightly by 7 days, and to a very low level by 18 days after zymosan treatment (Fig. 4B). This time-course is similar to that seen in F344 rats with thymectomy. These data suggest that sustained macrophage activation and recruitment by zymosan into the retina is lymphocyte-dependent in Lewis rats. Despite the early macrophage activation, however, zymosan treatment failed to enhance neuronal survival and axonal regeneration after thymectomy (Fig. 5A and B) in this strain. These results thus demonstrate that macrophages of the two strains behave differently – whereas macrophages of F344 rats promote neuronal survival and axonal regeneration, those of Lewis rats do not exhibit any beneficial action. Indeed, relating the macrophage number (Fig. 5C) to the number of regenerating RGCs in Lewis rats (Fig. 5B), there is a trend suggesting that increased macrophage number is associated with reduced regenerative ability in the latter rat strain.

Retinal explant experiments confirm detrimental actions of lymphocytes of both strains

To further investigate whether lymphocytes of the two strains had a negative effect on RGC survival, the effects of these cells on the viability of RGCs were tested \textit{in vitro}. Lymphocytes and retinas of F344 and Lewis rats were cultured and the effects were examined. Crossover culture was also carried out to further verify the role of lymphocytes and the innate response of RGCs towards lymphocytes in each strain. Detailed retinal culture groups and results are shown in supplementary Table S2. The appearances of βIII-tubulin-positive viable RGCs in cultured retinas under different conditions are shown in Fig. 6A–D.

Consistent with the \textit{in vivo} observations, both F344 and Lewis lymphocytes exhibited detrimental effects on RGCs in culture. As compared with the control group, the numbers of surviving F344 and Lewis RGCs decreased significantly \((P < 0.001)\) after treatment with...
congeneic (from the same rat strain) or allogeneic (from the other rat strain) lymphocytes (Fig. 6E). F344 RGCs, however, appeared to be more vulnerable to congeneic than allogeneic lymphocytes, as the number of RGCs was significantly ($P < 0.001$) lower after congeneic lymphocyte treatment (Fig. 6E). Although lymphocytes of both strains appear to be cytotoxic, the reduced loss of F344 RGCs after treatment with Lewis vs. F344 lymphocytes suggests that lymphocytes of Lewis rats are less detrimental than F344 T-cells.

Lewis RGCs responded similarly to the cytotoxicity of congeneic and allogeneic lymphocytes, but to a lesser extent (Fig. 6E). Although lymphocytes of both strains appear to be cytotoxic, the reduced loss of F344 RGCs after treatment with Lewis vs. F344 lymphocytes suggests that lymphocytes of Lewis rats are less detrimental than F344 T-cells.

Lewis RGCs responded similarly to the cytotoxicity of congeneic and allogeneic lymphocytes, but to a lesser extent (Fig. 6E). Interestingly, a significant difference ($P < 0.001$) in RGC survival was seen after treatment with the same type of lymphocytes (F344) between F344 and Lewis rats (Fig. 6E). These data suggest that innate properties of rat RGCs with different genetic backgrounds may lead to different responses of RGCs to lymphocyte attack, with F344 RGCs being more vulnerable. Note that there was an apparent decline in the number of surviving RGCs in the cultured retinas obtained after ON injury in both strains (Fig. 6E), presumably as a result of RGC death initiated by the ON injury prior to retinal culture (Berkelaar et al., 1994; Bähr, 2000).

In vivo observations in SPD rats were similar to F344 data

To further confirm our observations, we also examined RGC survival and axonal regeneration after zymosan application or thymectomy in SPD rats, another EAE-resistant strain. We found that the numbers of surviving RGCs in saline control rats were slightly higher, but the numbers of axon-regenerating RGCs and macrophages in the retina were similar to those in F344 rats (Fig. 7). Importantly, as in F344 rats, zymosan significantly activated macrophages in the eye (Fig. 7C) and promoted RGC survival and axonal regeneration (Fig. 7A and B) in SPD rats.

Similar to what was seen in F344 rats, increased RGC survival was also seen in SPD rats that received thymectomy at birth ($P < 0.05$, saline vs. thymectomy plus saline). Again, zymosan further increased the numbers of both surviving and axon-regenerating RGCs in thymectomized SPD rats ($P < 0.001$; Fig. 7). In the absence of T-cells, the number of macrophages was also low after zymosan administration. Early macrophage activation may also contribute to enhanced RGC survival and axonal regeneration in zymosan-treated thymectomized SPD rats, as was the case in F344 rats. There were some

**Fig. 4.** Time-courses of macrophage activation after intravitreal injection of zymosan in F344 (A) and Lewis (B) rats with or without thymectomy. As compared with the peripheral nerve–optic nerve procedure only (Fig. 2C) in F344 rats (A), significant increases in the numbers of macrophages were seen in the F344 retinas of both nonthymectomized and thymectomized rats soon (2 days) after zymosan treatment. However, this early response of macrophages was different between the rats with or without thymectomy, because the number of macrophages in thymectomized rats was almost double that in nonthymectomized ones. Importantly, a significant increase in the number of macrophages continued in the nonthymectomized rats, whereas a continuous and significant reduction in the number of macrophages was seen following the examination period in the thymectomized F344 rats. In Lewis rats (B), whereas no significant change occurred in the number of macrophages over the examination period in Lewis rats without thymectomy, a substantial reduction in the number of macrophages was seen in the thymectomized Lewis rats, and the number of macrophages dropped to a very low level 18 days after zymosan application. *$P < 0.05$; ***$P < 0.001$. Error bars = SEM.

**Fig. 5.** Average numbers of βIII-tubulin-positive surviving retinal ganglion cells (RGCs) (A), fluorogold-labeled axon-regenerating RGCs (B) and ED1-positive macrophages (C) 3 weeks after the peripheral nerve (PN)–optic nerve (ON) procedure under various experimental conditions in Lewis rats with and without thymectomy. The percentages (%) given in this figure represent the proportions of axon-regenerating RGCs (B) among surviving RGCs (A). *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; statistical analysis was done against the saline group. Error bars = SEM.
differences between F344 and SPD rats; unlike in F344 animals, the numbers of surviving and axon-regenerating RGCs were similar in zymosan-treated thymectomized and nonthymectomized SPD rats, and the extent of RGC protection and reduction of axon regeneration after thymectomy in SPD rats was less than in F344 rats. Importantly, however, the effects of zymosan on SPD rats were markedly and consistently different from those seen in Lewis rats (Fig. 5).

Discussion
We carried out a series of experiments in an attempt to discover the roles and relationship of macrophages and lymphocytes in RGC survival and axonal regeneration after ON injury. We found strikingly different effects of macrophages and lymphocytes on EAE-resistant F344 and SPD rats vs. EAE-susceptible Lewis rats.

The main in vivo findings include the following: (1) prolonged macrophage activation caused by intravitreal application of zymosan promoted RGC survival and axonal regeneration in F344 and SPD rats, whereas zymosan had no effect on RGC survival but had a small detrimental effect on axon regeneration in Lewis rats (Figs 2, 5 and 7); (2) thymectomy 1 day after birth resulted in better adult RGC survival in all strains of rat, although to a lesser extent in Lewis rats; (3) thymectomy reduced RGC axonal regrowth ability in F344 rats (Figs 2 and 4); and (4) after thymectomy, sustained macrophage activation by intravitreal zymosan administration was not achieved in all rat strains (Figs 4 and 7). Retinal culture studies confirmed a detrimental effect of both F344 and Lewis lymphocytes on RGCs, although F344 RGCs appeared to be more susceptible to lymphocytes, and Lewis lymphocytes were less damaging in their impact (Fig. 6).

Recruitment of T-cells into injury sites in the CNS has previously been shown (Popovich et al., 1996; Ling et al., 2006). T-cells have been widely implicated in inflammatory responses, leading to axonal damage and neuronal death, probably via the production of factors such as proinflammatory cytokines, chemokines and nitric oxide...
beneficial or detrimental to neuronal survival and axonal regeneration (Franzen et al., 1998; Flavin & Ho, 1999; Vincent et al., 2002; Yin et al., 2003; Correale & Villa, 2004; Mitrasinovic et al., 2005). The importance of recruiting and harnessing beneficial microglia, the CNS-resident macrophage-like immune cells, and avoiding their commitment to a destructive phenotype or even changing them from being detrimental to being beneficial has recently been pointed out (Butovsky et al., 2005; Schwartz et al., 2006). It is possible that macrophages from the three strains of rats received different signals and committed to different phenotypes at different stages after ON injury, leading to differential production of various molecules. In our previous studies, macrophages of F344 rats stimulated by zymosan soon after ON crush or axotomy injury committed to a beneficial phenotype, an effect not seen when activation was elicited either 1 week before or 1 week after ON injury (Yin et al., 2003).

A macrophage-derived factor, oncomodulin, has recently been identified as a potent factor in promoting axonal regeneration of RGCs (Yin et al., 2006). Because activated macrophages produce both beneficial and cytotoxic molecules (Yin et al., 2003), the timing of macrophage activation may lead to production of different levels of beneficial and detrimental molecules, resulting in different actions on RGC survival and axonal regeneration. Although we did not observe a damaging effect of macrophages on RGC survival in Lewis rats, a small macrophage-induced decline in axonal regenerative ability was seen (Fig. 5B). This is congruent with previous reports that infiltrated macrophages contributed to axonal loss in autoimmune disease EAE (Huitinga et al., 1990, 1995; Hendriks et al., 2006), to which Lewis rats are susceptible. Our zymosan treatment results in F344 rats with or without thymectomy (Figs 2 and 4A) showed that with lack of sustained macrophage activation in the thymectomized rats, RGC survival was slightly affected but axonal regeneration was significantly reduced. These data suggest that early macrophage activation is important in RGC protection, but prolonged macrophage activation is required for axonal regeneration. Similarly, differential effects of rho inhibition on RGC viability and axonal growth appear to depend on the time-course of this inhibition (Hu et al., 2007).

Interestingly, whereas zymosan-activated macrophages were RGC-protective after ON injury in F344 rats (Yin et al., 2003), we recently observed a clear detrimental action of macrophages of Lewis but not F344 rats on RGC viability in a transient ocular hypertension and reperfusion injury model (Huang et al., 2007). Thus, the behaviors of macrophages are also influenced by the pathological conditions to which they are exposed.

Zymosan-induced macrophage activation was not sustained in the absence of T-cells (thymectomy) in all strains of rats examined. These data suggest that there is a close interaction between macrophages and T-cells. It is known that macrophage activation is influenced by cytokines such as interferon-γ, interleukin-10 and interleukin-13 (Gordon, 2003), and lymphocytes produce many different types of cytokine that may affect the status of macrophage activation. T-cell heterogeneity in macrophage activation has been widely shown, and T-cell-dependent differential macrophage activation 
in vivo is also well characterized in other systems (Bach et al., 1997; Gordon, 2003; Ghosemlou et al., 2007). Conversely, macrophage-dependent modulation of T-cell activity has also been widely shown, mostly in other systems (Gallina et al., 2006; Siciliano et al., 2006). In addition, macrophage-induced T-cell apoptosis has also been reported (Kusmartsev & Gabrilovich, 2005). It is thus probable that a variety of communications occur between T-cells and macrophages after CNS injury, and that these communications modulate the proliferation, migration and biological functions of these cells. In this regard, note that in our retinal explant studies, although the number of ED1-positive cells 
in vitro under all conditions was similar (unpub-
lished observations), it is possible that some of the observed cytotoxic T-cell effects could have been mediated or influenced by macrophages/microglia resident in the explants themselves.

It is likely that there are differences in production of detrimental or beneficial molecules between the lymphocytes from each rat strain, but it is also possible that innate properties of neurons between the three rat strains also result in different vulnerabilities towards lymphocyte damage after CNS injury. The differential loss of F344 and Lewis RGCs after treatment with the same F344 strain of lymphocytes in the retinal explant studies supports this notion. However, the observation that loss of RGCs was exacerbated by lymphocytes in EAE-resistant F344 rats is in apparent conflict with the previous studies in which T-cell-dependent RGC protection was reported (Kipnis et al., 2001). Part of the discrepancy may be explained by the fact that in earlier studies, the spontaneous beneficial response of T-cells in protecting uninjured RGC axons from secondary degeneration, thus maintaining the axons in the distal ON stump, was investigated (Kipnis et al., 2001), whereas the present study focused on the survival and axonal regeneration of directly injured RGCs. There were far fewer surviving RGCs with intact or spared axons in the earlier studies than in the whole surviving RGC population that we examined (about 2500 retina vs. 9400 retina in our control group).

Neuronal death and axonal degeneration are executed via different mechanisms (Dubois-Dauphin et al., 1994; Burne et al., 1996; Whitmore et al., 2003). The differential effects of T-cells on RGC viability seen in our other studies (Kipnis et al., 2001; Yoles et al., 2001) may also be explained by mechanisms of more efficient energy consumption and metabolism; some injured RGCs may have to die in order for uninjured RGCs or injured RGCs without axon retraction to obtain sufficient energy and maintain functional metabolism to survive the neurotrauma. In addition, it is possible that other factors, such as macrophages, also contribute to the difference in RGC survival between EAE-resistant and EAE-vulnerable animals. Of course, autoimmune profiles modulating the different vulnerabilities of these strains of rats to EAE may be different from those autoimmune responses underlying different susceptibilities of RGCs to lymphocytes or macrophages.

In conclusion, we demonstrate that macrophages and lymphocytes play important roles in neural damage and repair after CNS injury in rats with different genetic backgrounds. Our data show that, under different pathological conditions, the effect of the autoimmune system on neuronal viability and axonal regeneration is critically influenced by interactions between macrophages and lymphocytes.

Supplementary material

The following supplementary material may be found on http://www.blackwell-synergy.com

Table S1. In vivo experimental conditions and numbers of fluorogold-labeled axon-regenerating βIII-tubulin-positive viable RGCs and ED1-positive macrophages in retinal whole mounts of F344 and Lewis rats with and without thy Comeytosis.

Table S2. Numbers of βIII-tubulin-positive viable RGCs 7 days after various experimental conditions in vitro.

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Abbreviations

CNS, central nervous system; EAE, experimental autoimmune encephalomyelitis; FG, fluorogold; FITC, fluorescein isothiocyanate; ON, optic nerve; PBS, phosphate-buffered saline; PN, peripheral nerve; RGC, retinal ganglion cells; SPD, Sprague Dawley.

References


