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Phenotype of CD4⁺ T cell subsets that develop following mouse facial nerve axotomy

Junping Xin^{a,b,*}, Derek A. Wainwright^{a,b}, Craig J. Serpe^b, Virginia M. Sanders^{c,1}, and Kathryn J. Jones^{a,b,1}

^aDepartment of Cell Biology, Neurobiology, and Anatomy, Loyola University Medical Center, Maywood, IL 60153, USA

^bResearch and Development Service, Hines VA Hospital, Hines, IL 60141, USA

^cDepartment of Molecular Virology, Immunology, and Medical Genetics, The Ohio State University, Graves Hall, 333 W. 10th Avenue, Columbus, OH 43210, USA

Abstract

We have previously shown that CD4⁺ T helper (Th) 2 cells, but not Th1 cells, participate in the rescue of mouse facial motoneurons (FMN) from axotomy-induced cell death. Recently, a number of other CD4⁺ T cell subsets have been identified in addition to the Th1 and Th2 effector subsets, including Th17, inducible T regulatory type 1 (Tr1), and naturally thymus-born Foxp3⁺ regulatory (Foxp3⁺ Treg) cells. These subsets regulate the nature of a T cell-mediated immune response. Th1 and Th17 cells are pro-inflammatory subsets, while Th2, Tr1, and Foxp3⁺ Treg cells are anti-inflammatory subsets. Pro-inflammatory responses in the central nervous system are thought to be neurodestructive, while anti-inflammatory responses are considered neuroprotective. However, it remains to be determined if another CD4⁺ T cell subset, other than the Th2 cell, develops after peripheral nerve injury and participates in FMN survival. In the present study, we used FACS analysis to determine the temporal frequency of Th1, Th17, Th2, Tr1 and Foxp3⁺ Treg CD4⁺ T cell subset development in C57BL/6 wild type mice after facial nerve transection at the stylomastoid foramen in the mouse. The results indicate that all of the known CD4⁺ T cell subsets develop and expand in number within the draining lymph node, with a peak in number primarily at 7 days postoperative (dpo), followed by a decline at 9 dpo. In addition to the increase in subset frequency over time, FACS analysis of individual cells showed that the level of cytokine expressed per cell also increased for interferon- γ (IFN- γ), interleukin (IL)-10 and IL-17, but not IL-4. Additional control double-cytokine labeling experiments were done which indicate that, at 7 dpo, the majority of cells indeed have committed to a specific phenotype and express only 1 cytokine. Collectively, our findings indicate for the first time that there is no preferential activation and expansion of any single CD4⁺ T cell subset after peripheral nerve injury but, rather, that both pro-inflammatory and anti-inflammatory CD4⁺ T cells develop.

Keywords

T helper cells; Cytokine; Facial nerve axotomy; Motoneuron survival

*Corresponding author. Fax: +1 708 202 2327. E-mail address: jxin@lumc.edu (J. Xin).

¹These two authors share senior authorship.

1. Introduction

Autoreactive T cells that recognize self-antigens exist in the circulation (Genain et al., 1994; Villoslada et al., 2001; Burns et al., 1983), and are activated to proliferate when they encounter self-antigens released from damaged tissues after trauma (Purcell et al., 2006; Correale and Villa, 2004; Olsson et al., 1992). Unregulated and prolonged autoreactive T cell responses may be responsible for the pathogenesis of several neurological disorders, such as multiple sclerosis (MS), chronic inflammatory demyelinating polyneuropathy (CIDP), Guillain-Barre disease, and experimental autoimmune neuritis (McQualter and Bernard, 2007; Latov, 2002; Lewis, 2007). In contrast, autoreactive T cells also become activated by self-antigens to mediate neuroprotective effects (Stoll et al., 2002; Correale and Villa, 2004).

Peripheral facial nerve injury in the mouse results in an immune response marked by T cell infiltration both peripherally at the injury site and centrally within the facial nucleus (Olsson et al., 1992; Raivich et al., 1998; Ha et al., 2006). Evidence from our laboratory indicates that CD4⁺ T cells play an important role in facial motoneuron (FMN) survival after axotomy (Serpe et al., 1999, 2000, 2002, 2003). Further work established that the CD4⁺ T effector cell, T helper type 2 (Th2), is neuroprotective after facial nerve injury (Deboy et al., 2006), suggesting that CD4⁺ T cell-mediated neuroprotection might involve a specific T cell subset and be associated with an anti-inflammatory cytokine. Additionally, a dual compartment of T cell activation has shown to be associated with facial nerve injury, involving both a peripheral and central site (Byram et al., 2004). Historically, Th1 and Th2 cells have been characterized as two classic CD4⁺ T cell subsets that secrete pro-inflammatory cytokines, such as interferon- γ (IFN- γ), or anti-inflammatory cytokines, such as interleukin-4 (IL-4), IL-5, IL-10, and IL-13, respectively.

Recent studies have identified three new CD4⁺ T cell subsets in addition to the well-known Th1- and Th2-like cells. Two CD4⁺ subsets function as regulatory cells and include the inducible IL-10-secreting T regulatory type 1 (Tr1) and naturally thymus-born Foxp3⁺ T regulatory (Foxp3⁺ Treg) cells (Beissert et al., 2006; Sakaguchi et al., 1995; Asano et al., 1996). Tr1 and Foxp3⁺ Treg cells inhibit the initiation and/or development of certain immune cell responses, and are considered to be anti-inflammatory. The fifth additional CD4⁺ subset is the T helper 17 (Th17) cell that secretes IL-17 and promotes inflammation and autoimmunity (Weaver et al., 2006; Harrington et al., 2006). Because an inflammatory response in the central nervous system (CNS) can cause neuronal damage and may be involved in the pathogenesis of several neurodegenerative diseases (Gilgun-Sherki et al., 2006), it appears to be the Th1 and Th17 cells associated with experimental autoimmune encephalomyelitis (EAE), which is characterized by neurodegeneration and myelin degradation (Gocke et al., 2007). In contrast, a shift in the immune response toward the activation of a Th2 or Treg cell has been reported to delay neurodegenerative disease onset and/or inhibit disease progression (Town et al., 2002; Mei et al., 2006; Matejuk et al., 2003; Schwartz and Kipnis, 2005).

Therefore, in light of the discovery of additional CD4⁺ T cell subsets, it is important to determine if subsets other than the Th2 cell are involved with mediating neuroprotection after nerve injury, and whether the subsets function at the level of FMN survival or nerve regeneration. In this study, we identified the phenotype of CD4⁺ T cells that develop in lymphoid tissue after a facial nerve injury in the mouse model. The data show that all five CD4⁺ T cell subsets develop and expand in number within the draining lymph nodes after nerve injury, and that the level of cytokines expressed also increases, except for IL-4. Collectively, our findings indicate for the first time that there is no preferential activation and expansion of any single CD4⁺ T cell subset after peripheral nerve injury but, rather, that both pro-inflammatory and anti-inflammatory CD4⁺ T cells develop. The role played by each subset in promoting FMN survival and/or nerve regeneration remains to be determined.

2. Materials and methods

2.1. Animals and surgical procedures

All mice used in the present study were on a C57BL/6 background. Seven-week-old female wild-type mice were obtained from Taconic labs (Germantown, New York, NY). Mice were permitted 1 week to acclimate to their environment before manipulation and were used at 8 weeks of age in all experiments. All mice were housed under a 12-h light/dark cycle in microisolator cages contained within a laminar flow system to maintain a pathogen-free environment. All surgical procedures were completed in accordance with the National Institutes of Health Guidelines on the Care and Use of Laboratory Animals for Research Purposes. Mice were anesthetized with 3% halothane for all surgical procedures. Using aseptic techniques, the right facial nerve of each animal was exposed at its exit from the stylomastoid foramen and completely transected, as described in detail previously (Jones and LaVelle, 1985; Serpe et al., 1999). All experimental manipulations were performed approximately 4 h into the light cycle under aseptic conditions. Each experimental group had four animals/group. A total of 80 mice were used in this study.

2.2. Preparation of CD4⁺ T cells

To enrich for CD4⁺ T cells, right cervical lymph node (RCLN), mesenteric lymph node (MLN), and spleen cell suspensions were made from both uninjured and injured wild-type mice at 7, 9, and 14 days postoperative (dpo). Red blood cells were removed by treatment with ammonium chloride for four minutes at 37 °C and a cell separation media, Lympholyte-M (Cedarlane Lab, Ontario, Canada) was used to remove dead cells and enrich for viable lymphocytes. Cells were washed with phosphate buffered saline (PBS), pH 7.2, and resuspended in 90 µl of PBS + 5% BSA per 10⁷ total cells. To isolate CD4⁺ T cells, 10 µl of magnetic cell sorting (MACS) CD4 (L3T4) microbeads (Miltenyi Biotec, California) per 10⁷ total cells was added, mixed, and incubated for 15 min at 6–12 °C. Cells were washed with PBS + 5% BSA, centrifuged at 300g for 10 min, supernatant removed, and cell pellet resuspended in 500 µl PBS + 5% BSA per 10⁸ total cells. Cells were magnetically sorted using an automated cell sorter, autoMACS (Miltenyi Biotec, Bergisch-Gladbach, Germany).

2.3. Surface and intracellular staining, and flow cytometric analysis

For cell activation marker CD44 and CD62L staining, isolated CD4⁺ T cells were incubated with rat anti-mouse CD4-APC (clone: GK1.5; isotype: Rat IgG2b, κ, eBiosciences, San Diego, CA), plus rat anti-mouse CD62L-FITC (clone: MEL-14; isotype: rat IgG2a), and rat anti-mouse CD44-PE antibodies (clone: IM7; isotype: rat IgG2b), or rat IgG2a-FITC and rat IgG2b-PE isotype controls (BD Pharmingen, San Diego, CA). The stained cells were subjected to multi-color FACS analysis (Becton-Dickinson). For intracellular cytokine staining, isolated CD4⁺ T cells were first incubated with phorbol myristate acetate (PMA, 50 ng/ml, Sigma, St. Louis, MO) and ionomycin (500 ng/ml, P/I, Sigma, St. Louis, MO) for 6 h in the presence of brefeldin A (BFA, 10 µg/ml, Sigma, St. Louis, MO) during the final 2 h. The CD4⁺ T cells were then equally divided into five groups. Each group was permeabilized with saponin (0.1%, Sigma, St. Louis, MO) and doubly stained for surface CD4 and intracellular IFN-γ, IL-17, IL-4, IL-10 or Foxp3 with PE- or FITC-labeled corresponding antibodies. For the double staining of cytokines, cells were stained with three antibodies: anti-CD4-APC, one PE-labeled antibody (anti-IFN-γ, IL-17, IL-4 or IL-10) and one FITC PE-labeled antibody (anti-IL-17, IL-4 or IL-10). The frequency and expression levels of IFN-γ, IL-17, IL-4, IL-10, and Foxp3 positive cells were determined by a multi-color FACS Calibur flow cytometry device (Becton-Dickinson) and FlowJo analysis software (TreeStar, Cupertino, CA), with the splenocyte suspensions used for gate setting. The sources for antibodies and isotypes used in this study were as follows: anti-IFN-γ-PE (clone: XMG1.2; isotype: Rat IgG1), anti-IL-4-FITC/PE (clone: 11B11; isotype: Rat IgG2), and anti-Foxp3-PE (clone: FJK-16s; isotype: Rat IgG2a) were purchased

from eBiosciences (San Diego, CA). Anti-IL-10-PE (clone: JES3-9D7; isotype: Rat IgG1) was purchased from Abcam (Cambridge, MA). Purified anti-IL-10 (goat polyclonal IgG), anti-IL-17 (rabbit polyclonal IgG), anti-goat IgG-FITC (donkey IgG), and anti-rabbit IgG-FITC (donkey IgG) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

3. Results

3.1. Activation of CD4⁺ T cells after facial nerve axotomy

When CD4⁺ T cells become activated, they express a high level of CD44 and a low level of CD62 ligand (CD44^{hi}CD62L^{low}). To determine the number of CD4⁺ T cells that became activated following a right facial nerve axotomy, CD4⁺ T cells were isolated from both the right cervical lymph node [RCLN, (draining LN)] and mesenteric lymph nodes [MLN, (non-draining LN)] from uninjured mice to establish a baseline number of activated cells, and from injured mice at 7, 9, and 14 days postoperative (dpo). As shown in Fig. 1, cells from the uninjured mice showed a higher basal number of activated CD4⁺ T cells in the RCLN (4.45%) than MLN (2.06%). At all time points tested after right facial nerve axotomy, there was an increase in the number of activated CD4⁺ T cells in the draining RCLN relative to the number in uninjured mice (6.15%, 7.49%, and 4.8% on dpo 7, 9, and 14, respectively), while there was essentially no change in the number of activated cells in the MLN (2.26%, 2.52%, and 2.24%). These results indicate that a right facial nerve axotomy induces the activation of CD4⁺ T cells in the draining RCLN, and that the CD4⁺ T cell response to facial nerve injury is a local, as opposed to systemic, response since the level of cell activation was unchanged in the non-draining MLN cells. The results also suggest that the increase in the number of activated CD4⁺ T cells before 9 dpo is likely due to activated cell expansion, and that the subsequent decline at 9 dpo may be activated cells undergoing apoptosis and/or exiting the draining lymph node and potentially trafficking to the sites where chemokines and cognate antigens are being released.

3.2. Development of pro-inflammatory CD4⁺ T cell subsets

It is well documented that following tissue injury, a local inflammatory response occurs to remove necrotic cells and damaged tissue (Lin et al., 2000; Li et al., 2001). Activated CD4⁺ T cells play an important role in promoting inflammation by secreting pro-inflammatory cytokines. For example, Th1 and Th17 cells secrete the pro-inflammatory cytokines IFN- γ and IL-17, respectively. To test whether IFN- γ -producing Th1 and/or IL-17-producing Th17 cells develop in response to facial nerve axotomy, CD4⁺ T cells were isolated from both RCLN and MLN from uninjured mice and injured mice on 7, 9, and 14 dpo. In the RCLN, the frequency of IFN- γ ⁺ cells increased 36% at 7, and returned to baseline at 9, dpo when compared to cells from uninjured mice (Fig. 2a), while the frequency of IL-17⁺ cells increased 235% and 119% above baseline at 7 and 9 dpo, respectively (Fig. 3a). By 14 dpo, the frequency of both IFN- γ ⁺ and IL-17⁺ cells in the RCLN were at or below baseline (Figs. 2 and 3a, respectively). In the non-draining MLN, no changes in the frequency of IFN- γ ⁺ or IL-17⁺ cells were found in injured compared to uninjured mice (Figs. 2 and 3a, respectively). IFN- γ and IL-17 expression levels, as determined by mean fluorescence intensity (MFI), increased in the CD4⁺ T cells within the draining RCLN when compared to basal levels in uninjured animals (Figs. 2 and 3b, respectively), with IFN- γ expression levels only increased 117% at 9 dpo, and IL-17 expression levels increased 84% and 100% at 7 and 9 dpo, respectively. At 14 dpo, expression levels of both cytokines began to decrease. No changes occurred in cytokine expression levels in cells collected from the non-draining MLN (data not shown). These data show that pro-inflammatory cells develop in the draining lymph nodes in response to facial nerve axotomy.

3.3. Development of anti-inflammatory CD4⁺ T cell subsets

The major cytokine produced by Th2 and Tr1 cells are IL-4 and IL-10, respectively, which were used as marker for the two type cells in the present study. While IL-10 expression is the only available positive marker for Tr1 cells, Th2 cells and Foxp3⁺ Treg cells also produce IL-10. It is advised to take this into consideration when interpreting the data for IL-10⁺ cells. In the RCLN, the frequency of IL-4⁺ cells increased 213%, 123%, and 43% at 7, 9, and 14 dpo, respectively, when compared to cells from uninjured mice (Fig. 4a), while the frequency of IL-10⁺ cells increased 218% above baseline at 7, and returned to basal levels at 9 and 14 dpo, respectively (Fig. 5a). In the non-draining MLN, no changes in the frequency of IL-4⁺ or IL-10⁺ cells were found in injured mice compared to uninjured mice (Figs. 4 and 5a, respectively). IL-4 expression levels, as determined by MFI, did not change within the draining RCLN when compared to basal levels in uninjured animals (Fig. 4b), whereas, IL-10 expression levels only increased at 7 dpo (by 102%) in the draining RCLN (Fig. 5b). No changes occurred in cytokine expression levels in cells collected from the non-draining MLN (data not shown). These data demonstrate that anti-inflammatory cells develop in the draining lymph nodes in response to facial nerve axotomy.

Foxp3⁺ Treg cells also produce IL-10 and are a potent immunosuppressive CD4⁺ T subset. The frequency of Foxp3⁺ regulatory CD4⁺ T cells within both draining RCLN and non-draining MLN increased 148% and 115%, respectively, at 7 dpo (Fig. 6a), returned to baseline at 9 dpo, and then decreased below baseline at 14 dpo. IL-10 expression levels in Foxp3⁺ regulatory CD4⁺ T cells did not change after axotomy (Fig. 6b). These data demonstrate that T regulatory cells expand in the draining lymph nodes in response to facial nerve axotomy.

3.4. Co-expression of multiple cytokines in CD4⁺ T cell subsets

As an additional control experiment, CD4⁺ T cells isolated from the draining lymph node 7 days post-axotomy were double-stained for cytokine expression (Fig. 7). As indicated in Table 1, the percentage of CD4⁺ T cells co-expressing IFN- γ and either IL-4, IL-10 or IL-17 was 28.0, 23.7, and 19.5, respectively. The percentage of CD4⁺ T cells co-expressing IL-4 and either IFN- γ , IL-10 or IL-17 was 46.5, 27.7 and 13.3, respectively. The percentage of CD4⁺ T cells co-expressing IL-10 and either IFN- γ , IL-4 or IL-17 was 11.7, 8.8 and 5.7, respectively. The percentage of CD4⁺ T cells co-expressing IL-17 and either IFN- γ , IL-4 or IL-10 was 19.6, 26.0 and 20.8, respectively. These data demonstrate that the majority of CD4⁺ T cells in the draining lymph node activated by peripheral axotomy are already fully developed into a specific subset by 7 dpo and express a single cytokine associated with a particular T cell phenotype.

4. Discussion

Accumulating data support the hypothesis that an injury-induced immune response is necessary for nerve regeneration and remyelination in the peripheral nerve system (PNS), and neuronal survival in the CNS (Serpe et al., 1999; David and Ousman, 2002; Bieber et al., 2001). In particular, CD4⁺ T cells play a crucial role in motoneuron survival after peripheral axotomy (Serpe et al., 1999; DeBoy et al., 2006), through an antigen-dependent process involving both peripheral antigen presenting cells and CNS resident microglia (Byram et al., 2004). Because activation is the fundamental requirement for T cell proliferation, differentiation and homeostasis, we confirmed in the present study that CD4⁺ T cells are activated in draining lymph nodes after facial nerve axotomy. Furthermore, we determined that all known CD4⁺ T cell subsets developed after peripheral nerve injury. These data suggest that: (1) antigen(s) released from the injured nerve drains to cervical lymph nodes and is/are presented to naïve CD4⁺ T cells; (2) CD62L downregulates on the surface of CD4⁺ cells to facilitate their release from lymphoid tissue into the circulation; (3) CD4⁺ T cells increase their level of cytokine

expression, and (4) various roles exist for the CD4⁺ T cell subsets in regulating both the pro- and anti-inflammatory responses to nerve injury. Importantly, activation is also a requirement for T cells to cross the blood brain barrier (BBB), and the present results support the previous finding that CD4⁺ T cells become activated after peripheral axotomy (Olsson et al., 1992; Raivich et al., 1998; Ha et al., 2006).

While CD4⁺ T cell subsets mutually inhibit development of each other, their functions also overlap. To understand how a nerve injury-induced immune response mediates neuroprotective effects rather than causing autoimmunity, it is critical to elucidate the development and balance of CD4⁺ T subsets. Here, we not only investigated the two classic CD4⁺ T subsets, i.e., pro-inflammatory Th1 cells and anti-inflammatory Th2 cells, previously implicated in neural injury and repair, but also examined the development of the recently identified Th17, Tr1, and Treg cells (Bettelli et al., 2006; Cottrez and Groux, 2004). The data indicate that facial nerve transection at the stylomastoid foramen increases the frequencies of all Th1, Th17, Th2, Tr1, and Foxp3⁺ Treg cell subsets in the cervical draining lymph nodes, with peak cellular expansion and cytokine expression by 1 week after nerve injury. Importantly, we tested each subset to determine that cells within that subset were expressing a single or multiple cytokines. The data indicate that the majority of cells had committed to a specific phenotype by 7 days, with some cells in each phenotype not yet fully differentiated. These results support the conclusions that multiple subsets do develop after axotomy, and are present in the draining lymph node 1 week after axotomy, as would be predicted based upon a peripheral immune response.

Th1 and Th17 cells are known as pro-inflammatory subsets. One of the major Th1 cell-derived cytokines is IFN- γ . IFN- γ is a potent pro-inflammatory cytokine reported to possess a dual role in neuro-inflammatory diseases, in that a high level of IFN- γ is associated with more severe symptoms, while neutralization or gene knockout of IFN- γ leads to an increase in morbidity, mortality, and susceptibility for the EAE (Billiau, 1996; Chu et al., 2000). The major cytokine secreted by Th17 cells is IL-17, which is involved in tissue inflammation and autoimmunity (Kolls and Linden, 2004; Langrish et al., 2005). IL-17 promotes angiogenesis and recruits multiple types of immune cells to the injury site (Laan et al., 1999; Starnes et al., 2001). Although both Th1 and Th17 cells are pro-inflammatory CD4⁺ T subsets, the kinetics of their development after facial nerve axotomy in the present study exhibited a different pattern, suggesting that these subsets may play different roles in regulating the immune response that is induced by axotomy. Based on their basic roles elucidated in the literature, our time course data in the present study suggest that Th1 cells might enhance the function of macrophages that participate in Wallerian degeneration by providing IFN- γ , and also support T cell expansion by secreting IL-2 in the early stages of inflammation. In contrast, Th17 cells may be responsible for the recruitment of neutrophils, macrophages, and T cells during both the early and later stages of a peripheral inflammatory response, as has been reported previously in other model systems (Laan et al., 1999; Starnes et al., 2001). Taken together, Th1 and Th17 cells may contribute synergistically to the maintenance of axotomy-induced inflammation, which is necessary for preventing foreign pathogen invasion, removal of damaged tissue, wound healing, Wallerian degeneration, and peripheral axonal regrowth (David and Ousman, 2002; Perrin et al., 2005; Stoll et al., 2002).

In comparison to pro-inflammatory subsets, a pronounced and prolonged IL-4- and IL-10-secreting cell response was observed following facial nerve axotomy. It has been shown that CNS delivery of IL-4 and IL-10 diminishes injury-induced degeneration and neurodegenerative diseases by inhibiting glial cell activation (Koeberle et al., 2004; Furlan et al., 2001; Cua et al., 1999, 2001). Although most of the cytokines produced in the periphery do not cross the BBB (Furlan et al., 2003), activated T cells readily cross the BBB and infiltrate the CNS parenchyma (Engelhardt and Ransohoff, 2005). When reactivated in the CNS, these infiltrating T cells, which were primed in the draining lymph node in the periphery, secrete

cytokine centrally. Since the present data demonstrate that all CD4⁺ T cell subsets develop in the draining lymph node after axotomy, we propose that the anti-inflammatory CD4⁺ T cell subsets are recruited preferentially into the facial nucleus in the CNS. Because T regulatory and Th2 cells share many of the same chemokine receptors, such as CCR4 and CCR8, (Lee et al., 2005; Soler et al., 2006), these anti-inflammatory cells might all be recruited preferentially to the CNS to counteract the release of pro-inflammatory mediators (Yang et al., 2002), and, subsequently, to reduce “bystander” damage to neurons. The remarkable increase of Th2, Tr1 and Foxp3⁺ Treg cells after facial nerve axotomy supports the idea that an inflammatory response in a healthy individual is well regulated by the parallel expansion of the anti-inflammatory arm of the immune response (O’Sullivan et al., 1995; Zang et al., 2004; Taylor et al., 2006).

In addition, we have previously shown that CD4⁺ T cells, devoid of the CD4⁺CD25⁺ Treg subpopulation (former definition of Foxp3⁺ Treg cells), are able to support FMN survival, suggesting that CD4⁺CD25⁺ Treg cells may not be responsible for mediating the neuroprotective effect after facial nerve injury (Deboy et al., 2006). However, we do not know if the thymus-born Foxp3⁺ Treg cells might be involved in the regulation of axotomy-induced peripheral inflammation, as opposed to central neuronal survival. It has been shown that Foxp3⁺ Treg cells start to proliferate upon interaction with antigen-bearing antigen-presenting cells, especially, dendritic cells (Yamazaki et al., 2003, 2006; Nishimura et al., 2004). In the present study, we found that a systemic increase in Foxp3⁺ Treg cells occurred in injured mice, possibly responding to certain self-antigens that induced them to expand in number. While Foxp3⁺ Treg cells do not appear to be directly involved in rescuing FMN after axonal injury, they may play an important role in controlling the inflammatory Th cell responses that develop to facial nerve axotomy and, thus, prevent the development of autoimmune disease (Coutinho et al., 2001). In their absence, their immunosuppressive functions may be partly compensated for by Th2 and Tr1 cell functions, as we found that robust Th2 and Tr1 responses occurred after facial nerve axotomy. The results also suggest that Foxp3⁺ Treg cells account for less than one third of the IL-10-producing CD4⁺ T cells, with the remaining IL-10⁺ cells being Th2 and Tr1 cells (data not shown).

The antigens that activate CD4⁺ T cells in the draining nodes after facial nerve axotomy have not been identified thus far, though a few studies suggest myelin basic protein (MBP) is one possible candidate. Olsson et al. (1992) previously showed that facial nerve transection caused expansion of myelin-autoreactive T cells. Other groups also demonstrated that MBP-specific T cells conferred a neuro-protective effect in preventing secondary degeneration and mechanical injury-induced cell death (Hauben et al., 2000, 2003; Moalem et al., 1999; Hammarberg et al., 2000). In contrast, however, Ankeny and Popovich (2007) showed that MBP-specific T cells were involved in the exacerbation of axotomy-induced FMN loss.

In summary, the present data show that multiple CD4⁺ T subsets differentially develop in the draining lymph nodes after peripheral nerve injury, but may traffic to separate areas peripherally and centrally. These cells may then mediate both pro-inflammatory and host-defensive effects at the peripheral injury site, as well as anti-inflammatory and neuroprotective effects in the CNS. The results support our hypothesis that different roles exist for the various CD4⁺ T cell subsets in neural injury and repair (Byram et al., 2004), as well as the idea that a balance between pro- and anti-inflammatory immune actions exists in both CNS trauma and disease (Stoll et al., 2002; Correale and Villa, 2004).

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Fig. 1.

Expression of cell activation markers on CD4⁺ T cells in draining and non-draining lymph nodes after facial nerve axotomy. Lymph nodes were removed from uninjured mice for a baseline analysis and from the draining right cervical lymph node and mesenteric lymph nodes at 7, 9, and 14 days postoperative (dpo). Ten thousand events were collected for FACS analysis using a forward vs. side scatter threshold to gate for lymphocytes. The data were then gated on CD4⁺ cells and displayed as two-color dot plots as shown. The number of activated CD4⁺ T cells (CD62L^{low}CD44^{hi}) within the draining RCLN and non-draining MLN were calculated and the percentage is represented by the number within the lower right quadrant. The figure shows representative data of flow cytometry analysis from cells pooled from four animals per group from two independent experiments.

Fig. 2.

Th1 cell phenotype in draining and non-draining lymph nodes after facial nerve axotomy. Cells were prepared for FACS analysis as described in Fig. 1. (a) The number of IFN- γ^+ CD4 $^+$ T cells within the draining RCLN and non-draining MLN were calculated and the percentage is represented by the number within the lower right FACS plot. (b) Mean fluorescence intensity (MFI) indicates the level of IFN- γ expression in cells from the RCLN. The figure shows representative data of flow cytometry analysis from cells pooled from four animals per group from three independent experiments.

Fig. 3.

Th17 cell phenotype in draining and non-draining lymph nodes after facial nerve axotomy. Cells were prepared for FACS analysis as described in Fig. 1. (a) The number of IL17⁺ CD4⁺ T cells within the draining RCLN and non-draining MLN were calculated and the percentage is represented by the number within the lower right FACS plot. (b) MFI indicates the level of IL-17 expression in cells from the RCLN. The figure shows representative data of flow cytometry analysis from cells pooled from four animals per group from three independent experiments.

Fig. 4. Th2 cell phenotype in draining and non-draining lymph nodes after facial nerve axotomy. Cells were prepared for FACS analysis as described in Fig. 1. (a) The number of IL4⁺ CD4⁺ T cells within the draining RCLN and non-draining MLN were calculated and the percentage is represented by the number within the lower right FACS plot. (b) MFI indicates the level of IL-4 expression in cells from the RCLN. The figure shows representative data of flow cytometry analysis from cells pooled from four animals per group from three independent experiments.

Fig. 5. IL-10-producing T regulatory cell phenotype in draining and non-draining lymph nodes after facial nerve axotomy. Cells were prepared for FACS analysis as described in Fig. 1. (a) The number of IL-10⁺ CD4⁺ T cells within the draining RCLN and non-draining MLN were calculated and the percentage is represented by the number within the lower right FACS plot. (b) MFI indicates the level of IL-10 expression in cells from the RCLN. The figure shows representative data of flow cytometry analysis from cells pooled from four animals per group from three independent experiments.

Fig. 6. Foxp3⁺ T regulatory cell phenotype in draining and non-draining lymph nodes after facial nerve axotomy. Cells were prepared for FACS analysis as described in Fig. 1. (a) The number of Foxp3⁺CD4⁺ T cells within the draining RCLN and non-draining MLN were calculated and the percentage is represented by the number within the lower right FACS plot. (b) MFI indicates the level of IL-10 expression in the Foxp3⁺ T cells from the RCLN. The figure shows representative data of flow cytometry analysis from cells pooled from four animals per group from two independent experiments.

Fig. 7.

Percentage of CD4⁺ T cells co-expressing cytokines in draining lymph nodes after facial nerve axotomy. CD4⁺ T cells were isolated from the draining right cervical lymph node at 7 dpo. Cells were prepared for FACS analysis as described in Fig. 1. (a) Double staining for IFN- γ and IL-4, IL-10 or IL-17. (b) Double staining for IL-4 and IL-10 or IL-17. (c) Double staining for IL-10 and IL-17. The numbers of single and double cytokine positive CD4⁺ T cells were calculated and the percentages are represented by the number within corresponding quadrants. The figure shows representative data of flow cytometry analysis from cells pooled from four animals per group from two independent experiments.

Table 1Percentage^{*} of CD4⁺ T cells expressing two cytokines in the right cervical lymph nodes 7 days after axotomy

T-cell subset ^{**}	Cytokine			
	IFN- γ	IL-4	IL-10	IL-17
Th1 cells		28.0	23.7	19.5
Th2 cells	46.5		27.7	13.3
Tr1 cells	11.7	8.8		5.7
Th17 cells	19.5	26.0	20.8	

* Percentages are calculated by the frequency of double positive cells divided by the total frequency of the respective subset, as based upon the numbers presented in Fig. 7.

** Each T-cell subset is defined as follows: Th1 = IFN- γ positive CD4⁺ T cells, Th-2 = IL-4-positive, Tr1 = IL-10-positive, Th17 = IL-17-positive.