Original Article

Assessment of T Cell Activation in a Mouse Model of Traumatic Facial Nerve Injury

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Abstract
Objective To investigate T cell activation following facial nerve axotomization and latent neuroimmunologic mechanisms in traumatic facial paralysis. Methods A murine model of facial nerve transaction was used. Lymphocytes from cervical and mesenteric lymph nodes in BABL/c mice at specific times were collected and expression rates of CD69 on T cells were assessed by flow cytometry. Results Infiltrating T cells were detected around the facial neurons in the facial nerve nucleus in mice whose facial nerve was transected. Immunofluorescent staining showed recruitment of activated T cells. Three days post–facial nerve transection, the expression rate of CD69 on T cells from cervical draining lymphoid nodes(CDLNs) was significantly different from that on T cells from mesenteric lymph nodes(MLNs) (P=0.0457), whereas the latter was similar to that in animals undergoing sham surgeries and that in blank control animals(p= 0.2817 and 0.2724, respectively). Two weeks post–nerve transection, the T cell CD69 expression rate from CDLNs remained at a higher level and that in the sham–operation animals(p= 0.0007). At two weeks, CD69 expression rate on T cells from MLNs was also up–regulated and different compared with the sham–operation animals and with itself at three days postoperation(p= 0.0082 and 0.0133, respectively ). Conclusion T cells appear to be activated and up–regulated in CDLNs following facial nerve transection. There is even evidence of T cell activation in MLNs at 2 weeks post–nerve transection. This suggests an alteration of immune response from local to general immunity in the acute stage of facial nerve trauma, which may help coordinating and controlling the scales and orientation of the neuroimmune response during the pathogenesis and progression of facial nerve trauma.

Key words facial nerve; T cell; CD69; injury; Neuroimmunomodulation

1 Introduction
T cell activation plays an important role in triggering immune response in many diseases, including nerve injury1, 2. Interest in immune activation in facial nerve injury is increasing because studies have shown that irreversible axonal damage occurs earlier than previously thought3. The classic viewpoint of facial nerve injury pathology focuses on demyelinating lesions characterized by the presence of T cells, B cells and phagocytic macrophages4. Disease activity is probably initiated by systemic activation of myelin–reactive T cells, followed by activated T cell migration into the central nerve system.
(CNS), including facial nucleus, and the sequential recruitment of additional inflammatory cells to facial motoneurons. Cells primarily accumulate in the perivascular space and subsequently migrate into the CNS parenchyma. The recruitment and migration of leukocytes into the CNS are orchestrated by chemokines. And activated T cells that express specific combinations of adhesion molecules and chemokine receptors are preferentially attracted to CNS by chemokines produced within the target tissue. Hence, in patients with facial nerve injury, the expression of adhesion molecules and chemokines on the surface of activated T cells governs the recruitment of immune cells to the CNS. Moreover, the expression of other activation and differentiation molecules reflects the function of recruited T cells. However, the status of T cells and definitive molecular and cellular immunopathological mechanisms in facial nerve injury are unknown.

CD69 is the earliest cell surface marker expressed in activated immune cells. Generally speaking, in static state (without being stimulated), the immune cell does not express CD69, although it can be rapidly spurred to expressing by stimulating signals. Therefore, CD69 is the ideal index to appraise immune cell’s state of function. Its stimulation leads to IL-2 production and increases CD25 expression, finally causing T cell proliferation. The present study was consequently undertaken to detect the presence of activated T cells in a mouse model with facial nerve transection. To this point, T lymphocytes collected from cervical draining lymph nodes (CDLNs) and the mesenteric lymph nodes (MLNs) were used to represent local and “remote” (systemic) immune responses respectively in the current study. Early activation antigen CD69 on T cells was examined by flow cytometry so as to provide preliminary reference data on latent neuroimmunologic mechanisms of traumatic facial paralysis.

2 Materials and methods

2.1 Animals and surgical procedures

All BABL/c mice used in the present study were specific pathogen-free (SPF) animals. Seven-week-old male wild-type mice were obtained from Institute of Laboratory Animal Science, Chinese Academy of Medical Science (Beijing, China). All mice were provided autoclaved pellets and water ad libitum. Mice were permitted 1 week to acclimate to their environment before manipulation and used at 8 weeks of age in all experiments. All mice were housed under a 12-h light/dark cycle in microisoler cages contained within a laminar flow system to maintain a pathogen-free environment. All surgical procedures were completed in accordance with China Academic of Medical Science guidelines on the care and use of laboratory animals for research purposes. Mice were anesthetized with 10% Chloral Hydrate for all surgical procedures. Using aseptic techniques, the right facial nerve of animals in the facial nerve transection group was exposed at its exit from the stylomastoid foramen and completely transected. The proximal and the distal nerve stump were reset without suture. Behavioral observations were used to assess reconnection of the facial nerve (i.e., if the animal recovered from unilateral facial paralysis). For animals in the sham operation group, the right facial nerve was exposed, but not transected. For both groups, the left side was untreated and served as an internal control in comparison to the right side. A separate group of untreated BABL/c mice were included as blank control. Six animals from each group were examined and tested at 3 (DPO3) and 14 (DPO14) days post operation, respectively.

2.2 Light microscopic immunohistochemistry staining with CD3

2.2.1 Brain stem slice preparation

After the animals were killed with overdose
10% Chloral Hydrate, they were first perfused intracardially (30 ml/min) with 200 ml of phosphate buffer solution (PBS) (10 mM Na2HPO4, 0.84% NaCl, pH 7.4), followed by 200 ml of 4% formaldehyde (FA) in PBS (4% FA/PBS), and the brain stem was removed and post-fixed by 2 hr immersion in 1% FA/PBS at 4°C on a rotator (8 rpm). The tissue was cryoprotected by an overnight rotating immersion in sucrose (30% sucrose, 10 mM Na2HPO4, pH 7.4, 4°C), frozen on dry ice, and then cut in a cryostat at the level of the facial motor nucleus. Sections (30 μm) were collected on warm, 0.5% gelatin-dipped slides.

2.2.2 Immunohistochemistry staining with CD3

Tissue sections were incubated with the primary anti-mouse CD3 antibodies (PharMingen) overnight, then with biotinylated goat anti-rat secondary antibody and avidin–biotin peroxidase complex (ABC, Vector), completed with visualization with diaminobenzidine/H2O2 (DAB, Sigma). Sections were washed in PBS following each incubation step.

2.2.3 Light microscope examination of facial nucleus

Examination of the facial nerve nucleus was completed at the central lab of Peking Union Medical College Hospital with a Nikon microscope (Eclipse 80i). Sections of facial nucleus were examined for distribution and recruitment of T cells with positive CD3 staining, identified in Buffy color under the microscope.

2.3 Double-color immunofluorescent staining with antibodies and flow cytometry

Anti-CD3 mAb (monoclonal antibodies) –FITC, anti-CD69 mAb–PE and isotype-specific control antibodies were obtained from eBioscience (San Diego, CA) Lymphocytes harvesting and fluorescence antibody staining have been described previously in detail [11, 12]. In brief, the lymphocytes separated from CDLNs and MLNs at DPO3 and DPO14 were suspended in RPMI1640 with 10% fetal bovine serum, with the concentration adjusted to 10⁶ cell/L. After counting survival percentage with trypan blue, the suspension was washed twice with PBS and condensed to 50 μL. The suspension was incubated for 30 min in the dark at room temperature with the appropriate dilution of anti–CD3 mAb–FITC and anti–CD69 mAb–PE, washed twice with cold PBS, and finally fixed with 4% paraformaldehyde to ready for testing. Drops containing targeting cells from CDLNs were examined and photographed under fluorescence microscope with double-color immunofluorescent staining. A EPICS® XL flow cytometer (Beckman–Coulter) was used for flow cytometry analysis. Three samples were collected and tested on each set test day. Data are presented as mean±s, and differences between groups were compared using t–test. P value <0.05 is set as statistically significant.

3 Results

None of the animals in the present study showed signs of recovering from unilateral facial paralysis after facial nerve transection.

3.1 CD3 in facial nucleus

CD3 is one of the specific cell surface markers on T cells. Its expression on T cells in the facial nucleus in this study is depicted in Figure 1 as positive staining under the microscope.

3.2 Immunofluorescent staining of T cells from CDLNs in mice with facial nerve transection

Expression of T cell surface marker CD69 was visible under oil immersion lens following CD3–FITC, CD69–PE double–color immunofluorescent staining, indicating T cell activation (Fig 2).

3.3 Expression of CD69 on T cells three days after operation.

CD69 antigen was minimally or not expressed on CD3+ T cells from lymph nodes in blank control group mice in the state of quiescence. Three days after the operation, CD69 expression was seen on CD3+ T cells from CDLNs in mice that
had undergone facial nerve transection or sham-operation (Fig. 3A and B), indicating T cell activation. This was significantly different compared with T cells from MLNs (P=0.0457 and 0.0226, respectively). CD69 expression was positive on 0.81 ± 0.21% of T cells from CDLNs in mice with facial nerve transection, compared to 0.52 ± 0.16% in those in the sham-operation group (P=0.2759). CD69 expression rates in T cells from MLNs were similar in the nerve transection and sham-operation groups (P=0.4700) (Fig. 3C and 3D).

3.4 Expression of CD69 on T cells 14 days after operation.

Two weeks after facial nerve transection, CD69 expression on T cells from CDLNs remained positive (0.62±0.10) % and not significantly different from 3 days after operation (P=0.2294), indicating continuous T cell activation. However, CD69 expression on CDLN T cells in sham-operation mice had dropped to normal levels at 0.14 ± 0.01% (Fig. 3F), lower than that at DPO3 (P=0.0212). A significant difference in expression of CD69 on T cells from CDLNs was seen between mice with transected facial nerve and those in the sham-operation group (P=0.0007). At the same time, CD69 expression on T cells from MLNs increased in mice with facial nerve transection to 0.49±0.05% (Fig. 3G), higher than sham-operation mice and than at DPO3 (P=0.0082 and P=0.0133, respectively) (Fig. 3H).

4 Discussion

At present, Neuritis and demyelization are generally acknowledged as common pathophysiological foundation of peripheral facial paralysis (Bell’s palsy, traumatic, infectious and others) by scholars home and abroad[^4][^13]. Cellular immune response and humoral immune response play a key role in the outbreak and development of peripher-
al facial paralysis\(^{14,15}\). However, precise cellular and molecular immune mechanisms of peripheral facial paralysis have not been discovered so far, leading to a weak foundation of theories to the contemporary treatment. With currently available treatments (conservative medical treatments, surgeries, and complimentary treatments including traditional Chinese herbal medicines and acupuncture and moxibustion), a significant number of patients are left with such sequelae as facial paralysis and permanent facial deformity, causing great agony and psychological burden to them\(^{16}\). It can be inferred from our previous research that the representative immune cell activation and recruitment of T cell and monocyte/macrophage may be the cellular immunity foundations of peripheral facial nerve demyelization and facial motoneuron death\(^{17,18}\). The pathophysiological meanings of T cell activation and CD69 expression in a mouse model of traumatic peripheral facial paralysis were probed here with fluorescence-activated cell sorting.

T cell activation is a result of complicated action between multiple ligand–receptors, which are offered by T cells and antigen–presenting cells (APC) including the formation of immunological synapse(IS)\(^{19,20}\). The interactive integration triggers biochemistry events in T cells that lead to

### Figure 3
Expression of CD69 on T cells at DPO3 (A–D) and, DPO14(E–H). A, B, E and F: T cells from CDLN in the facial nerve transection (A and E) and sham–operation (B and F) groups, respectively. C, D, G and H: T cells from MLN in the facial nerve transection (C and G) and sham–operation (D and H) groups, respectively.

### Tab 1
Expression of CD69 on T cells ($\bar{x} \pm s$, %)

<table>
<thead>
<tr>
<th>Group</th>
<th>DPO3</th>
<th>DPO14</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>CDLN</td>
<td>MLN</td>
</tr>
<tr>
<td>Facial nerve transection</td>
<td>0.81 ± 0.21</td>
<td>0.18 ± 0.00</td>
</tr>
<tr>
<td>Sham–operation</td>
<td>0.52 ± 0.12</td>
<td>0.17 ± 0.08</td>
</tr>
<tr>
<td>P(^2)</td>
<td>0.2759</td>
<td>0.4700</td>
</tr>
</tbody>
</table>

**NOTE:**

1. P\(^1\), CDLN vs MLN; P\(^2\), Facial nerve transected group vs Sham–operation group
2. *P <0.05, (***)P <0.01
cellular immune response. Such effects as acceleration or inhibition on activation, proliferation, differentiation and effector action are carried out through cytokines secretion and cell–cell contact. T cell activation plays a key role either in normal or in pathological immune response. The degree and direction of immune response are determined by the upshot of T cell activation.

Three days post the operation, CD69 expression ascended in CD3+ T cells from CDLN in animals with facial nerves transection and sham–operation(Fig. 3 A and B) compared to T cells from MLNs, which reflected the systemic immune status, as in a “far site” local immune response. This suggest that the organism starts or increases local cellular immune response in response to a combined acute stage effect by trauma and nerve injury with activated T cells, although the systemic immune response is yet to start. The similarly high levels of CD69 expression in CD3+ T cells from the CDLN two weeks after facial nerve transection suggests continuous T cell activation. At this time, CD69 expression on CDLN T cells in animals with sham–operation had dropped to a normal level, similar to that at 3 days post operation, suggesting that the organism had recovered from unsophisticated traumatism (no–nerve–transected) with the normal level immunity response. Neural traumatism effect includes antigen exposure, continuous repair and generation based on low-level autoimmune response. A moderate upregulation of immune regulation network is needed to exert the function of immune elimination and immune surveillance. The status with appropriate T cell activation is a primary step to triggering the adjustment and control of the network.

The results of light microscopic immunohistochemistry and immunofluorescent staining indicate distribution and recruitment of T cells in the facial nucleus (Fig 1–2), confirming that, mediated by T cell’s antigen receptor, CD4+ T cells are activated by distinguishing peptides including autoantigens such as myelin basic protein (MBP) and proteolipid proteins (PLP), presented by antigen presenting cells (APC) such as stelliform glial cells, glial cell (GC) and Schwann cells. Then, activated CD4+ T cells overshoot blood brain barrier (BBB) and infiltrate facial nucleus [21]. Activated T cells, small GCs and stelliform GCs produce various kinds of chemokine, cytokines and adhesive molecules which recruit more T cells and monocytes / macrophages around the peripheral injury area and facial nucleus. Two effects are brought about: first, clearing and phagocytization of necrotic metamorphic motorneurons, myelin sheath and axon, etc; second, secondary allergy and experimental autoimmune encephalomyelitis(EAE) induced by conglomeration of inflammatory cells dominated by T cells and macrophages [22, 23]. There is a dynamic course for immune regulation in the immune system.

CD69 expression upregulation in T cells from MLNs is probably through the mechanism of systemic immune regulation networks started by local immune response. Two weeks after nerve injury, the upregulation of CD69 expression in T cells of lymph nodes is simply the result of the surveillance mechanisms of the immune regulation network, which is started by the key process immune response with appropriate T cell activation. Abnormal T cell activation, too high or too low, means nerve pathological emergence for the organism(autoimmunity/immune deficiency) [24].

The restorative effect in early stage of facial nerve injury (in two weeks) determines the result of final nerve repair. The change of micro–environmental in damaged nerve cells and axons, especially the expressions of various kinds of immune cells and molecules together with the dynamic equilibrium of their regulating networks, is the key incidents of nerve repair and regeneration. They impenetrable from the start to the end of rehabilitation and regeneration of nerve’s inju-
The conception of ‘immune microenvironment for repair and regeneration of facial nerve’, as we have previously introduced \cite{17,18,25}, mainly concerns with the change of expression and regulation network with various immune cells and immune molecules in the process of facial nerve renovation. It is a dynamic equilibrium for its maintenance of the normal immune microenvironment. It is the activated T cells that target to recruiting such immune activated cells as monocytes/macrophages, such cytokines as neurotrophi factors, which regulate Schwann cells proliferation and continuous axon regeneration \cite{26}. Studies have showed that specific T cell subgroups have a relationship with the survival of facial motoneurons\cite{18,27}. Many questions remain: what are the roles of dialogue mechanisms of T cell–axon / neuron– Schwann cells / glial cell? Which factors, and through which concrete signal pathways, play a key role for T cells and monocytes/macrophages system activation and recruitment? What are the roles of real molecular mechanisms of the action by specific T cells on facial motor neurons survival? We believe that they will be well explained with the progress of systematic studies on immune microenvironment for repair and regeneration of facial nerve.

Acknowledgements

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