Facial nerve injuries can cause significant psycho-social detriment to patients who experience short- or long-term paralysis. The innate and adaptive arms of the immune systems both participate in a complex interaction for neuroregeneration. Among factors critical to the viability of neurons after injury is the survival of the cell body and preservation of the electrical signal transmission pathway. An increasing body of evidence has shown a nonimmune role for the immune system both in development (e.g., regulating synaptic pruning) and in the response to injury, both centrally (stroke models) and peripherally (spinal cord injury models). Two areas of particular interest are the role of histocompatibility complex I and the classical complement pathway. MHC-1 represents a large, polymorphic family of genes. For example, MHC-1 has been shown to have a significant role in neuronal plasticity in the developing visual system. Knocking out just 2 of the more than 50 MHC-1 genes, H2-Kb (Kb) and K2-Db (Db), in KbDb−/− mice, enhances plasticity in the mouse visual cortex. Furthermore, KbDb−/− mice demonstrate decreased injury after stroke.

Three distinct paths activate the complement system: the classical pathway (activated by the binding of C1q to non-self-epitopes), the lectin pathway, and the alternative pathway. All 3 ultimately result in the formation of the membrane attack complex (MAC), leading to cell lysis and ultimately phagocytosis. The MAC has been shown to be important for rapid Wallerian degeneration and clearance of myelin, important steps in the process Development (e.g., regulating synaptic pruning) and in the response to injury, both centrally (stroke models) and peripherally (spinal cord injury models).

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of peripheral nerve regeneration. Alternatively, the complement cascade can alternatively facilitate proper neuronal development or accelerate chronic inflammatory response, depending on the developmental timing and local environment within the nervous system.

The facial nerve has also been examined for its dependence on immune regulatory mechanisms in the setting of injury. For example, MHC-1 has been shown to be upregulated in the facial motor nucleus (FMN) after axotomy of the nerve.

An age-dependent phenomena exist related to functional recovery. Peripheral nerve crush injury in juvenile mice results in loss of more than 50% of FMN cells, whereas minimal cell loss occurs in adult mice. Although the end-point activation of apoptosis can be blocked by overexpression of the antiapoptotic gene bcl-2, the mechanisms for this difference in sensitive upstream have yet to be elucidated. One study in our laboratory pointed to the possibility of a role for the immune system in this process.

The objective of our study was to investigate the complex interaction between the peripheral and central nervous system in providing a favorable microenvironment to promote regeneration. We also sought to elucidate the roles of these genes in synaptic refinement in the maturation process. An investigation into the role of MHC-I and C1q in cochlear maturation demonstrated hearing impairment alone with lack of expression of KbDb and not C1q. C1q represents a potential therapeutic intervention as a checkpoint to the complement cascade and role in the central nervous system in synapse regulation despite lack of findings in hearing impairment. Given these findings, and the recently uncovered role for the complement and MHC-1 systems in neuronal plasticity and injury response, we sought to examine 1 critical aspect of each of these pathways in a facial nerve injury paradigm. Specifically, we sought to determine the effect of MHC-1 by examining facial nerve injury and recovery in KbDb−/− mice. We further sought to determine the effect of inhibition of the classical complement activation pathway in C1q−/− mice.

METHODS

Animals

The Administrative Panel on Laboratory Animal Care at the Stanford University School of Medicine granted permission for experimentation. Study was performed per institutionally reviewed protocol.

Genotyping

KbDb−/− mice on a C57BL/6 genetic background were obtained from Dr. Carla Shatz (Stanford) and maintained as a homozygous breeding colony because both the targeted loci are now on the same chromosome. C57BL/6 controls were purchased from The Jackson Laboratory (Bar Harbor, Maine) and were bred and maintained in our facility. C1q−/− mice on a C57BL/6 background were generously provided by Drs. Ben Barres and Marina Botto, and littermates were used as controls.

Surgical Procedure

Mice underwent surgery at age P7 or P21. Mice were anesthetized with a mixture of ketamine and xylazine. Surgery was not started until the mice were areflexic, and this level of anesthesia was maintained throughout the procedure. A curvilinear infra-auricular incision was made and the facial nerve was identified. The nerve trunk was crushed distal to the auricular branch with the tips of jeweler’s forceps (Dumont forceps) for a 30-second interval. Forceps were custom-calibrated with a clamp to provide consistent force at time of injury. This resulted in an approximately 2-mm endoneurium gap at the crush site. Epineurium was noted to be intact at the completion of each crush injury. The skin incision was closed with cyanoacrylic glue. Crush injury was always on the left. All mice were recovered on a temperature-controlled heating pad until deemed ready to return to the litter. A single surgeon performed the surgical procedures to maintain consistency.

Whisker Motion

To assess the whisker activity of mice, we filmed whisker movements of unrestrained mice over a period of 21 days postoperatively using a high-speed video camera at an acquisition rate of 500 frames per second. Whisker function monitoring is a validated measure for facial nerve recovery in a rodent model. A 2-step method was

Fig. 1. Orientation of the mouse head is estimated by performing linear regression on each side of the facial contour.
developed using MATLAB platform to track both the head movements and the movements of selected whiskers. The analysis was applied to multiple video recordings of the mice during whisking.

First, the head movement of a mouse was tracked by delineating the lower contour of the head in each image frame, while the head image was segmented from the background via thresholding and a morphological operation. Subsequently, the position of the tip of the snout was estimated by finding the furthest point from the centroid of the head, and the delineated head contour is divided into 2 sides corresponding to the right and left sides of the face. These steps were followed by a linear regression operation that was applied to each side of the head contour and used to determine the orientation of the head (Fig. 1).

Next, the whisker movement is tracked by estimating, on a frame-by-frame basis, the angular position of a selected whisker of the mouse during its whisking. In particular, a rectangular region of interest, which enclosed the selected whisker, was created and its location recorded with respect to the position of the tip of the snout—this operation was updated for each frame. The Sobel operator and the Hough transform were then applied within the region of interest to detect the whisker segment. The Sobel operator, well known for effective enhancement of edges, and the Hough transform, a feature extraction technique widely used for line detection, have been used to detect lines including whiskers in rats.20,21 These operations allowed us to obtain absolute angular position of the whisker on a frame-by-frame basis. To ensure that only the selected whisker is detected while other whiskers that may be present near the selected whisker are excluded, the orientation and the location of the detected whisker in each frame were used to generate an elliptical mask that was updated every frame and was applied to the subsequent frame. The Hough transform was exclusively applied to the elliptical mask for each frame in a sequence and used to detect the whisker of interest, and the absolute angular position of the whisker is recorded for each frame. Finally, by combining the tracking results obtained from movements of the head and whisker, we were able to eliminate the effect of any head movement and thereby report on actual movements of the selected whisker (Fig. 2).

**Tissue Processing**

All animals were euthanized with carbon-dioxide intoxication. The brains were immediately dissected free from the skull and placed in phosphate-buffered 4% paraformaldehyde. Tissue was kept on a mixer in fixative for 48 hours. Brains were then treated with 20% glycerol and 2% dimethylsulfoxide to prevent freeze artifacts and multiply embedded (19 mice brains per block) in a gelatin matrix using MultiBrain Technology (NeuroScience Associates; Knoxville, Tenn.). After curing, the block was rapidly frozen by immersion in isopentane chilled to −70°C with crushed dry ice and mounted on a freezing stage of an AO 860 sliding microtome. The MultiBrain block was sectioned coronally at 60 µm. All sections’ cuts were collected sequentially into a 4 × 5 array of containers filled with Antigen Preserve solution (50% phosphate-buffered saline, pH 7.0, 50% ethylene glycol, 1% polyvinylpyrrolidone) for sections to await Thionine Nissl staining.

**Neuronal Counting**

Thionine Nissl–positive nuclei and cell bodies were quantitatively and qualitatively evaluated for neuronal numbers. Neuronal counts were conducted manually on the FMN bilaterally. Brain areas were defined anatomically by atlas. The FMN is comprised of the nucleus proper, the dorsomedial subnucleus, dorso intermediate subnucleus, dorsolateral subnucleus, lateral subnucleus, ventral intermediate subnucleus, and the ventromedial subnucleus. The FMN is bordered anteriorly by the dorsal periolivary region, medially, laterally, and superiorly by the perifacial region.

![Fig. 2.](image-url)
zone, anteriorly/inferiorly by the caudal periolivary nucleus, and rostrally by the Botzinger complex and nucleus ambiguous. Facial motor nuclei were examined using Swift M10 microscope linked to a video camera.

**Statistical Analysis**

Statistical comparisons of the functional and neuronal survival data were made by means of paired and unpaired t tests.

**RESULTS**

All mice were examined every 1 to 3 days for 21 days after unilateral crush injury. Observations began the day after surgery. Whisker function was scored according to the system detailed in the Methods section. At the beginning of the observation period, there was no detectable whisking noted on the crushed side in all mice. Juvenile mice demonstrate an impaired level of functional recovery. Whisker functional recovery is seen starting at postinjury day 9 in adult mice. The relationship between functional outcome and neuronal survival after crush injury was also evaluated.

**Functional Recovery and Facial Motor Neuron Survival after Facial Nerve Crush Injury in KbDb−/− Mice**

Recovery rate reached about 40% compared with normal whisker function in juvenile mice. A 2-tailed t test did not demonstrate any statistically significant difference in whisker functional recovery between the KbDb−/− pups and control group (P < 0.45). Adult KbDb−/− mice demonstrated a statistically significant impairment in recovery beginning on postinjury day 9 (P < 0.01). By postinjury day 21, only 50% of whisker function had been regained compared with control group (Fig. 3).

Stereographic analysis of the FMN survival correlated with functional outcomes. KbDb−/− pups and adults both had a statistically significant decreased FMN survival (P < 0.05) (Fig. 4).

**DISCUSSION**

Facial nerve injuries are a significant cause of morbidity within the realm of otolaryngology and plastic surgery. Elucidating the mechanism for central neuronal plasticity...
and peripheral nerve recovery in the facial nerve are vital steps in the process for developing therapeutic interventions in the acute phase of injury. Herein, we have examined both the MHC-1 and classical complement pathways for their role in recovery after facial nerve crush injury. To our knowledge, this is the first such study to simultaneously examine facial motor neuron survival, concomitant functional recovery, and age-dependent neuronal plasticity with regard to these immune pathways.

Presently, we have demonstrated that deletion of KbDb, 2 of many MHC-1 genes, is sufficient to cause significant decrement in facial nerve recovery after crush injury in adult mice rendering them similar to juvenile mice in terms of return of function. In addition, both adult and juvenile mice demonstrated decreased neuron number after injury. The juvenile knockout group demonstrated similar functional recovery to the wild-type group despite statistically significant difference in FMN survival. This lends credence to the model of developmental switch or immature repair mechanism present in juvenile compared with adults. Our data suggest that MHC-1 plays a role in promoting peripheral nerve regeneration in the adult FMN after injury, with functional consequences when only one pair of the MHC-1 genes is deleted.

We previously demonstrated a dose-dependent response to cell-body survival and functional recovery in relation to corticosteroid use for treatment of facial nerve injury in the murine model. Several studies have illuminated that a subset of primarily CD4+ T cells compared with CD8+ T cells is responsible for migrating from periphery into the central nervous system to promote FMN survival by neurotropic growth factors and modulation of microglial activity. The interaction of adaptive immune system with the innate component during Wallerian degeneration is important for axonal survival after injury. Ramaglia et al showed previously that the classical complement pathway activation was primarily involved in acute nerve damage. A subsequent study with complement inhibition of all pathways correlated to accelerated recovery of sensory and motor function in rat sciatic nerve injury model and correlated this to histological measures of cell-body survival and periphery axonal repair.

We have found that blockade of activation of the classical pathway does not affect facial motor neuron survival or functional recovery in the facial nerve crush injury model. Although this may seem initially to contradict the findings of Ramaglia et al, this may not necessarily be the case. They did denote that other pathways of complement activation may have later contribution in acute nerve trauma. However, the alternative and lectin pathways for complement activation may very well play a role in this model, something that we have not examined. Nevertheless, the finding that the classical pathway alone is at the least not of prime importance in the injury/recovery of facial motor axons is an important finding.

In summary, our study supports the concept that a complex degree of interaction exists between the immune, central, and peripheral nervous systems in mechanisms of repair after injury. This study also provides evidence that MHC-1 may play a role in central neuronal sensitivity to peripheral injury during maturation. Further studies may aim at specific inhibition of the final common end point of the complement pathway, MAC, to determine its role.
and potential for development of novel therapeutics. We hope that future studies will help further elucidate the components, interaction, and timing of immune mechanisms involved in neuroprotection to provide therapeutic options with minimum morbidity.

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