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## Neurons Preferentially Respond to Self-MHC Class I Allele Products Regardless of Peptide Presented

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### Abstract

Studies of mice lacking MHC class I (MHC I)-associated proteins have demonstrated a role for MHC I in neurodevelopment. A central question arising from these observations is whether neuronal recognition of MHC I has specificity for the MHC I allele product and the peptide presented. Using a well-established embryonic retina explant system, we observed that picomolar levels of a recombinant self-MHC I molecule inhibited neurite outgrowth. We then assessed the neurobiological activity of a panel of recombinant soluble MHC Is, consisting of different MHC I heavy chains with a defined self- or nonself-peptide presented, on cultured embryonic retinas from mice with different MHC I haplotypes. We observed that self-MHC I allele products had greater inhibitory neuroactivity than nonself-MHC I molecules, regardless of the nature of the peptide presented, a pattern akin to MHC I recognition by some innate immune system receptors. However, self-MHC I molecules had no effect on retinas from MHC I-deficient mice. These observations suggest that neuronal recognition of MHC I may be coordinated with the inherited MHC I alleles, as occurs in the innate immune system. Consistent with this notion, we show that MHC I and MHC I receptors are coexpressed by precursor cells at the earliest stages of retina development, which could enable such coordination.

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It was long believed that neurons express little or no MHC class I (MHC I), except in response to functional impairment or traumatic injury (1-6). However, recent studies showed that MHC I is expressed by some CNS neurons, particularly those whose synapses undergo

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### Disclosures

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activity-dependent remodeling (7-9). Moreover, mutant mice that lack proteins involved in MHC I function ( $\beta 2$  microglobulin [ $\beta 2M$ ], TAP1, or CD3 $\zeta$ ) have ectopic clusters of retinal inputs in their thalamus, as well as enhanced hippocampal long-term potentiation and reduced long-term depression (8). In addition, MHC I seems to limit synaptic plasticity in the visual cortex (10). Thus, in addition to MHC I's central role in immune system function, MHC I is now believed to be involved in synaptic development.

The elucidation of the principles that govern the recognition of MHC I by immune cells has provided a cornerstone for understanding immune cell activity. In the adaptive immune system, CD8<sup>+</sup> T cells express TCRs that are generated by gene rearrangement. Each TCR recognizes a specific peptide presented by a particular MHC I allele product, and CD8<sup>+</sup> T cells use TCRs to survey cells for the presence of nonself-Ags (11-13). In contrast, the MHC I receptors of NK cells of the innate immune system (e.g., the Ly49 and KIR gene families) are germline encoded and recognize one to several MHC I allele products, usually without specificity for the presented peptide (14,15). NK cells monitor cells for MHC I expression and kill cells displaying insufficient MHC I (which is indicative of a viral infection or transformation). Thus, immune cells use different types of MHC I receptors for different surveillance functions, and the recognition properties of these MHC I receptors govern, in large part, the activity of the immune cell.

The principles that govern MHC I recognition by neurons are unknown. If neurons can distinguish between MHC I allele products and their displayed peptides, particular peptide/MHC I molecules on the neuronal cell surface could provide very specific ligands for neurodevelopment. Alternatively, changes in the expression levels of MHC I, regardless of the peptides presented, may be the most relevant for neurodevelopment. In this regard, it is notable that NK cells have the capacity to differentiate between cells that differ only slightly in their level of MHC I expression.

To begin to address this issue, we exposed retina explants from different strains of mice to defined recombinant MHC I molecules. We observed a new biological activity of MHC I: the ability to inhibit neurite outgrowth. Moreover, we found that neurons were especially sensitive to self-MHC I allele products, regardless of what peptide was presented. This suggests that mechanisms exist to coordinate neuronal sensitivity to MHC I with the inherited MHC I haplotype of the individual, paralleling observations in the innate immune system. Consistent with this possibility, we observed that MHC I and MHC I receptors were present at the earliest stages of retina development.

## Materials and Methods

### Mice

C57BL/6 mice and C57BL/6  $\beta 2M^{-/-}$  mice (catalog #002087) were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6  $K^bD^b^{-/-}$  mice were purchased from Taconic Farms (Germantown, NY; catalog #004215). After mating, mice were checked each morning for formation of a vaginal plug. The day of plug formation was designated day 0 of pregnancy.

### MHC I monomers

MHC I monomers presenting minor histocompatibility Ags (mHAgs) were prepared as previously described (16). All other MHC I monomers were provided by the National Institutes of Health (NIH) Tetramer Core facility. The peptide/MHC I molecules tested are described in Table II.

## Retinal explant cultures

All studies involving animals were approved by the University of California, Los Angeles Animal Research Committee. Retinas isolated from embryonic day (E)14 mice were placed into Matrigel (Collaborative Biomedical Products, Bedford, MA) containing the indicated concentration of MHC I monomer, in an eight-chamber slide on ice. The slides were incubated at 37°C for 30–60 min to solidify the gel. Neurobasal medium (0.2 ml, with *L*-glutamine, antibiotics, and 1× B27 supplement; Life Technologies [Gaithersburg, MD]) containing the indicated concentration of the MHC I monomer was added to each chamber. The cultures were incubated for ≈48 h (37°C, 5% CO<sub>2</sub>), after which the media was replaced with 4% paraformaldehyde (PFA) and the slides stored at 4°C. Images were captured using a digital camera connected to an inverted microscope (Zeiss Axiovert 200, Carl Zeiss, Germany). Explants were analyzed in a blinded fashion for the area covered by neurite projections and the length of the longest neurite using NIH Image software. Statistical significance was determined using Student *t* test. Group means were compared via random effects ANOVA controlling for dose and MHC group.

## In situ hybridization

In situ hybridization was performed as described (17) using D<sup>b</sup>-specific antisense and sense digoxigenin-labeled riboprobes (8).

## Immunohistochemical staining of MHC I

Unfixed frozen retina or brain sections (8–15 μm) from C57BL/6 mice were stained side-by-side with anti-D<sup>b</sup> mAb (HB-27, ATCC designation 28-14-8S, 10 μg/ml), control isotype-matched mouse anti-D<sup>k</sup> mAb (HB-16, ATCC designation 16-1-11N, 10 μg/ml), or PBS (alone) overnight at 4°C. After washing, the sections were fixed in 4% PFA for 15 min, washed, and incubated at room temperature (RT) for 3 h with Alexa 488 anti-mouse (1:500, Molecular Probes, Eugene, OR). Some brain sections were also stained with DAPI. In each study, all slides were processed simultaneously to ensure homogeneity in the staining procedure. Slides were mounted, and retinas were imaged using a confocal microscope (Leica TCS-SP MP, Leica, Deerfield, IL). Fluorescent images of brain sections were captured with a charge-coupled device camera at the same magnification and exposure time.

## MHC I tetramer staining of retina

Fresh frozen retina cryosections (8 μm) were incubated with an APC-labeled MHC tetramer (8 μg/ml) overnight at 4°C. After washing, the sections were fixed in 4% PFA for 15 min, washed, incubated at RT for 3 h with goat anti-APC Ab (1:1000, Biomedex, Foster City, CA) and rabbit anti-islet 1/2 (1/2000, a gift from Tom Jessell), and then washed and incubated with Alexa 594 chicken anti-goat IgG and Alexa 488 chicken anti-rabbit IgG (both at 1:500, Molecular Probes) for 3 h at RT. Sections were imaged using a confocal microscope (Leica TCS-SP MP).

## Results

### MHC I can inhibit neurite outgrowth

To define the principles that govern MHC I recognition by neurons, we exposed embryonic retina explants to different recombinant MHC I monomers. We chose the retinal explant model because the extent of neurite outgrowth from these explants has been widely used to assay neuroactive molecules *in vitro*, and because retina ganglion cells (RGCs) are expected to express MHC I receptors (8). Retinal explant cultures meet all of the requirements for organotypic cultures and preserve cell morphology and functions, including the generation of action potentials in response to stimulation (18,19). MHC I monomers, consisting of a

recombinantly produced MHC I heavy chain complexed with  $\beta 2M$  and a synthetic peptide, as well as tetramers of these molecules, are biologically active and activate cognate MHC I receptors on cells of the innate and adaptive immune systems (20-22).

Although much is known about the viral peptides presented by MHC I, there is little information regarding which peptides from neuronal Ags are presented by various MHC I allele products, with the exception of peptides from mHAgs. mHAgs can evoke immune responses to transplanted tissue (23). Therefore, we began by testing the effect of a self-MHC I ( $D^b$ ) loaded with a ubiquitously expressed mHAg peptide (H13a) (23) on neurite outgrowth from retinas isolated from embryonic day 14 (E14) C57BL/6 mice.

We observed that this MHC I molecule inhibited neurite outgrowth, as measured by the area covered by the neurites and the length of the longest neurite (Fig. 1A). In the presence of 100 pM  $D^b$ /H13a, neuronal outgrowth was reduced to approximately half of that in cultures with media alone (representative cultures are shown in Fig. 1B, 1C). Retina explants cultured with or without  $D^b$ /H13a had, on average, the same number of neurites, indicating that the exogenous MHC I did not inhibit their formation but rather inhibited their outgrowth. The inhibition of neurite outgrowth was mediated by properly folded MHC I, as evidenced by: 1) a mild heat treatment (65°C, 5 min) that disrupts MHC I monomers into free peptide, heavy chain, and  $\beta 2M$  abolished its inhibitory action (Fig. 1D); and 2) preabsorption of the  $D^b$ /H13a preparation with a conformation-dependent anti- $D^b$ , but not an anti- $K^k$ , mAb and protein A Sepharose removed the inhibitory neuroactivity (Fig. 1D). H13a peptide itself (100–500 pM) had no effect on neurite outgrowth. Soluble MHC class II molecules that were prepared in a similar fashion as the MHC I molecule had no neuroactivity (data not shown).

Recombinant MHC I's neuroinhibitory effect is unlikely to be mediated indirectly through infiltrating immune cells or glial cells in the E14 retinal explants because immune cells, such as dendritic cells, macrophages, and B cells, do not enter the retina until 1 wk after birth (24), and, as presented below, retinas from  $\beta 2M^{-/-}$  mice are unaffected by MHC I monomers, and these mice have functional glial cells, dendritic cells, macrophages, NK cells, and B cells. This inhibition of neurite outgrowth also was not due to the toxic factors in the recombinant MHC I preparations, because, as presented below, MHC I monomers had inhibitory activity on retinas with a matching MHC I allele, but had little or no activity on retinas lacking a matching MHC I allele. Thus, picomolar levels of MHC I can inhibit neuronal outgrowth, a previously unrecognized biological activity of MHC I.

### Do neuronal MHC I receptors discriminate between the peptides presented by MHC I?

The mouse MHC I region (H-2) contains two main MHC I loci, K and D, which are highly polymorphic. C57BL/6 mice are H-2<sup>b</sup> (i.e.,  $K^b D^b$ , Table I). We loaded recombinant  $D^b$  and  $K^b$  MHC Is with different self- and nonself-peptides that bind to  $D^b$  or  $K^b$  (Table II) (25-35) and tested their neuroactivity on C57BL/6 retinas. We found that all of these H-2<sup>b</sup> MHC I molecules had similar levels of neurobiological activity, regardless of the self or nonself nature of the presented peptide (Fig. 2A). There was no apparent difference in neuroactivity between H-2K and H-2D proteins. Paralleling these results,  $D^d$  and  $K^d$  monomers loaded with a self- or nonself-peptide had similar neuroinhibitory activity on neurite outgrowth from retina explants from BALB/c mice (which are H-2<sup>d</sup> [ $K^d D^d$ ]) (Fig. 2B), as did various  $D^k$  and  $K^k$  monomers when tested on retinas from C3H mice (which are H-2<sup>k</sup> [ $K^k D^k$ ]) (Fig. 2C). Thus, retinal neurons from mice with different MHC I haplotypes do not discriminate between the peptides presented by self-MHC I allele products.

### Neurons are especially sensitive to self-MHC I allele products

To examine the allele specificity of neuronal MHC I receptors, we expanded the panel of recombinant MHC I/peptide molecules to include D<sup>d</sup>, K<sup>d</sup>, D<sup>k</sup>, and K<sup>k</sup> molecules with defined peptides from autoantigens or viral peptides known to bind to the particular MHC I allele products. The different peptide/MHC I molecules and their origins are detailed in Table II. We then tested the 11 MHC I/peptide molecules individually on retina explants from C57BL/6, BALB/c, and C3H mice, each of which possesses different MHC I haplotypes.

In control cultures without exogenous MHC I, retina explants from all three strains of mice had similar rates of neuronal outgrowth. Fig. 3A–C shows group data of the effects of different recombinant H-2 complexes on retina explants from three mouse strains. The MHC I molecules displayed neuroinhibitory activity in a distinctive pattern; self-MHC I allelic products generally exerted greater neuroinhibitory activity than nonself-MHC I allelic products. For example, when tested individually at 100 pM on C57BL/6 retinas, the different H-2<sup>b</sup> MHC I monomers reduced neurite outgrowth an average of ~63% compared with that in control cultures without exogenous MHC I ( $p < 0.001$ ; Fig. 3A). In contrast, at 100 pM, the various nonself-H-2<sup>d</sup> or -H-2<sup>k</sup> MHC I molecules had little effect on neurite outgrowth from C57BL/6 retinas (Fig. 3A). Similarly, H-2<sup>d</sup> complexes had the largest inhibitory effect on neurite outgrowth from BALB/c retinas (Fig. 3B). Likewise, H-2<sup>k</sup> complexes greatly inhibited neurite outgrowth from C3H retinas (Fig. 3C) but had little or no effect on C57BL/6 (Fig. 3A) or BALB/c retinas (Fig. 3B). Retinas from C57BL/6 mice had the greatest ability to discriminate between self- and allogenic recombinant MHC Is (at 100 pM), whereas C3H retinas were less discriminatory. The preferential sensitivity to self-MHC I allele products is not random, because the probability that the mean retinal outgrowth would be lowest in the presence of self-MHC I allelic products (at 100 and 500 pM) in all three mouse strains is  $(1/3)^6 = 0.0014$  (binomial-order statistic). Because each MHC I/peptide displayed neuroinhibitory activity on syngenic retina explants, it demonstrates that each had a conformationally correct structure, and the general reduction of their inhibitory effect on allogenic retina explants argues against the inhibition being due to a toxic contaminant.

These data indicate that by E14, wild-type retina cells somehow “know” their MHC I haplotype, well before their axon projections form synapses in the thalamus. Apparently, mechanisms exist that coordinate neuronal recognition of MHC I with the inherited MHC I alleles, as occurs in the innate immune system.

### Neurons from MHC I-deficient mice are unaffected by exogenous MHC I

Next, we tested the neuroactivity of D<sup>b</sup>/H13a on E14 retinas from C57BL/6 mice that lack  $\beta 2M$  ( $\beta 2M^{-/-}$  mice), which are functionally MHC I deficient. Although D<sup>b</sup>/H13a (100 and 500 pM) inhibited neurite outgrowth from wild-type C57BL/6 mice, it had no effect on neurite outgrowth from MHC I-deficient mice (Fig. 4A). Neurite outgrowth from  $\beta 2M^{-/-}$  retinas in the presence or absence of exogenous self-MHC I was the same as that of wild-type retinas in media alone or in media containing nonself-D<sup>k</sup> MHC I molecules. Because  $\beta 2M^{-/-}$  mice also have deficiencies in CD1, we also tested the effects of D<sup>b</sup>/H13a on C57BL/6 mice lacking K and D gene products ( $K^bD^b^{-/-}$  mice). As we observed with  $\beta 2M^{-/-}$  mice, exogenous self-MHC I had no inhibitory effect on neurite outgrowth from  $K^bD^b^{-/-}$  mice (Fig. 4B). These observations suggest that endogenous MHC I may be involved in sensitizing embryonic retina neurons to self-MHC I molecules

## MHC I receptors and MHC I are expressed by retinal precursor cells

For MHC I to play a role in sensitizing developing retina neurons to self-MHC I, MHC I and MHC I receptors should be present in the retina by E14, since our studies show that E14 retina explants display differential sensitivity to recombinant MHC I molecules. Therefore, we examined when and where MHC I and MHC I receptors were expressed in the developing eye.

The vertebrate retina is made up of seven distinct cell types that are derived in a sequential order from precursor cells in the outer neuroblastic layer (ONBL) (36). RGCs are the first to differentiate (beginning around E11). By E14, the mouse retina contains precursor cells, RGCs, horizontal cells, cones, and amacrine cells. Subsequently, rods, bipolar cells, and Müller glia begin to differentiate (36). As the RGCs differentiate, they migrate inward, passing through the middle retina layers containing the other neuronal cell types, and form the innermost ganglion cell layer (GCL).

Using a D<sup>b</sup>-specific probe (8) and in situ hybridization, we found that D<sup>b</sup> transcripts were expressed throughout the E12 C57BL/6 retina (Fig. 5). By E15, MHC I transcripts were expressed at higher levels throughout the retina, especially in cells of the developing GCL (Fig. 5). We also stained E15 C57BL/6 retina sections with an anti-D<sup>b</sup> mAb. Heretofore, immunohistochemical analysis of MHC I expression in the CNS has been problematic because Abs that specifically recognize classic MHC I (Ia) molecules were developed to stain native MHC I for FACS analysis and do not recognize denatured MHC I in fixed brain sections, and the Abs that can stain MHC I in fixed mouse tissues do not distinguish between classical and nonclassical (Ib) MHC I (see review in Ref. 9). Therefore, we developed the ability to stain unfixed frozen retina sections with a conformation-dependent anti-D<sup>b</sup>-specific mAb or a control isotype-matched anti-D<sup>k</sup>-specific mAb. We found that anti-D<sup>b</sup>, but not anti-D<sup>k</sup>, stained many cells throughout the precursor and GCLs, matching the in situ hybridization results (Fig. 5). Thus, in situ hybridization and immunohistochemistry demonstrate that MHC I is expressed early in retina development.

We next assessed whether MHC I receptors were present in the developing retina. Because the molecular identity of the retinal MHC I receptor(s) is unknown, we used an MHC I tetramer as a pan-specific probe to stain embryonic retina cells that express classical MHC I receptors. We stained fresh-frozen E15 C57Bl6 retinas side-by-side with the D<sup>b</sup>/H13a tetramer or with control mouse MHC class II (loaded with OVA 323–39 or human CLIP), human MHC I (loaded with an EBV peptide), and human MHC class II (loaded with a glutamic acid decarboxylase 65 peptide) tetramers. After staining and fixation, the slides were stained with antinuclear homeodomain protein islet 1/2, which is an early RGC-specific marker. Although D<sup>b</sup>/H13a strongly stained cells in the precursor and GCLs, the other mouse class II and human MHC I and MHC II tetramers did not (Fig. 6A). No staining was observed in sections incubated with the detection Abs alone (anti-APC). Thus, MHC I tetramers seem to be a reliable pan-specific probe for neuronal MHC I receptors.

Using the D<sup>b</sup>/H13a tetramer to probe E12 C57BL/6 retina sections, we observed binding to the ONBL and the developing GCL (Fig. 6B). The tetramer-stained cells in the ONBL did not express islet 1/2, whereas the tetramer-stained cells in the developing GCL expressed this RGC marker (Fig. 6B). There were also occasional tetramer cells between the ONBL and GCL, all of which costained with anti-islet 1/2. These cells are likely to be differentiating RGCs that are migrating from the precursor layer to the GCL.

AtE15, the tetramer staining of GCL cells was more intense, and these cells costained with anti-islet 1/2 (Fig. 6B). Thus, MHC I receptors are highly expressed by the RGCs that will send projections to the thalamus.

## Discussion

A key to understanding MHC I's immunological activity has been to define the principles that govern its recognition by receptors in the adaptive and innate immune systems. To further understand MHC I's role in neurodevelopment, we sought to define the principles that govern MHC I recognition by neurons.

We found that picomolar levels of a self-MHC I molecule could inhibit retina neurite outgrowth in vitro. This neuroinhibitory activity was not due to toxic factors in the recombinant MHC I preparations because: 1) preabsorption with an anti-D<sup>b</sup>, but not an anti-K<sup>k</sup>, mAb removed the inhibitory factor; 2) recombinant MHC I inhibited neurite outgrowth from syngeneic retinas, but had little effect on retinas from an allogeneic mouse strain; and 3) retinas from  $\beta 2M^{-/-}$  and D<sup>b</sup>K<sup>b-/-</sup> mice were unaffected by recombinant MHC I. Recombinant MHC I's neuroinhibitory effect is unlikely to be mediated indirectly through infiltrating immune cells or glia cells in the E14 retinal explants because immune cells, such as dendritic cells, macrophages, and B cells, do not enter the retina until 1 wk after birth (24), and T cells are still developing in the thymus at E14, and retina explants from MHC I-deficient mice were unaffected by recombinant MHC I. Although MHC I-deficient mice have deficiencies in CD8<sup>+</sup> T cells, their other immune cells and retina glial cells are functional. This observation strongly argues against the notion that recombinant MHC I inhibited neurite outgrowth indirectly through infiltrating immune cells or glia. Finally, MHC I tetramer staining showed that MHC I receptor expression in the embryonic retina was limited to the RGCs and their precursor cells. The staining pattern was inconsistent with the staining of Müller glia, which arise well after E14 and have a distinctive laminar position, and microglia and astrocytes, which enter the eye after E16 and E19, respectively. Although we cannot completely rule out any contributions from immune cells or glia in our retina explant model, previous studies of immune cell development and our observations using retinas from two strains of MHC I-deficient mice, as well as MHC I tetramer staining, strongly suggest that the neuroinhibitory effects of recombinant MHC I were mediated through neuronal MHC I receptors.

The inhibition of neuronal outgrowth is a distinct biological activity from MHC I's previously described role in pruning, or stabilizing, synaptic connections (8,10). We found that this neurologic activity required conformationally correct MHC I, evidenced by: 1) a mild heat treatment (that dissociated the complex) abolished recombinant MHC I's neuroactivity; and 2) a conformation-dependent anti-MHC I mAb removed the inhibitory neuro-activity. The presented peptide by itself had no neuroactivity on retina explants. Thus, retinal MHC I receptors are unlike the receptors of vomeronasal neurons, which can respond to small peptides (alone) that possess MHC I-binding motifs (37).

Retina neurite outgrowth was inhibited by self-MHC I molecules, regardless of the peptide presented. The lack of specificity for the presented peptide may be a necessity; it is unlikely that a particular peptide would always be presented by different individuals' MHC I molecules, given that MHC I genes are highly polymorphic in outbred populations and each MHC I allele product binds different peptides. However, MHC I genes are differentially expressed temporally and spatially in the CNS (8,9). Accordingly, quantitative changes in MHC I expression, rather than changes in the particular presented peptides, are likely to provide information for neurodevelopment. Indeed, in the innate immune system, NK cells can exquisitely distinguish between cells that differ only in their expression levels of MHC I.

We observed that retina neurite outgrowth was especially sensitive to inhibition by self-MHC I allele products. For example, on average, at 100 pM, H-2<sup>b</sup> molecules (but not H-2<sup>d</sup>

or H-2<sup>k</sup> molecules) significantly inhibited neurite outgrowth from C57BL/6 retinas, and H-2<sup>d</sup> molecules (but not H-2<sup>b</sup> or H-2<sup>k</sup> molecules) significantly inhibited neurite outgrowth from BALB/c retinas. Interestingly, neurons from MHC I-deficient b2M<sup>-/-</sup> mice and K<sup>b</sup> D<sup>b</sup><sup>-/-</sup> mice were unresponsive to recombinant MHC I. These observations suggest that endogenous MHC I expression is required to sensitize retina neurons to self-MHC I.

How do neurons recognize MHC I given that MHC I is extremely polymorphic? Neurons could use a promiscuous MHC I receptor that is nonspecific for MHC I allele products. The PirB receptor in the visual cortex may represent such an indiscriminate neuronal MHC I receptor (10). However, PirB may not be the RGC MHC I receptor, because PirB-deficient mice have normal retinogeniculate connections (10). Alternately, neurons could express many MHC I receptors from a gene family, each with various MHC I allele specificities, such that at least one receptor can always recognize self-MHC I. Another possibility is that neurons undergo an “educational” process so that they express the correct MHC I receptors (out of a family of possible receptors), as occurs with Ly49 receptors on murine NK cells. Ly49 receptors are encoded by a large gene family encoding  $\geq 23$  different Ly49 receptors, each of which preferentially interacts with different MHC I types [e.g., Ly49A recognizes D<sup>b</sup>, D<sup>d</sup>, and D<sup>k</sup>; Ly49I recognizes D<sup>d</sup> and K<sup>d</sup>; and Ly49D recognizes D<sup>d</sup>, D<sup>f</sup>, and D<sup>SP</sup> (38,39)]; however, they have little or no specificity for the presented peptide (40). It is believed that individual developing NK cells are “educated” to recognize self-MHC I by sequentially and cumulatively expressing different members of the Ly49 gene family until the cell expresses at least one receptor that interacts with self-MHC I (14,15,41). In addition, it is believed that NK cells must interact with MHC I to be “licensed” to develop functional competence (42,43). Notably, Ly49 is expressed by mouse cortical neurons (44), but Ly49 is not expressed in humans. Hence, it is an open question whether mouse and human retinal neurons use an already known MHC I receptor(s), one or more of the many MHC I receptor-related genes whose function are not yet understood (45), or a completely novel gene(s).

For endogenous MHC I to play a role in coordinating RGC recognition of MHC I with the inherited MHC I haplotype, MHC I and MHC I receptors must be expressed very early in retina development. Because the molecular identity of the retina MHC I receptor(s) is unknown and may be a novel receptor, we used an MHC I tetramer as a pan-specific probe for classical MHC I receptors in retina tissue sections. This MHC I tetramer staining, along with in situ hybridization and immunohistochemistry to localize MHC I, showed that MHC I receptors and MHC I are expressed in the same regions, and likely on many of the same cells, very early in retina development. The tetramer staining was particularly dense on retinal precursor cells and developing RGCs. In the middle retina region wherein several neuronal types reside, we could discern individual tetramer-stained cells, all of which also stained for anti-islet, indicating that these cells were developing RGCs migrating from the precursor layer to the GCL and not some other neuronal or immune cell type. The expression of MHC I and MHC I receptors in E12 retinas suggests that MHC I–MHC I receptor interactions may play a role during the early stages of retinal development. These interactions occur well before the RGC projections reach the thalamus and may prepare the developing RGCs to recognize MHC I on their target neurons. In addition to sensitizing RGCs to self-MHC I, MHC I and MHC I receptors in the developing retina may be involved in directing axon projections toward the optic nerve and in intraretina remodeling.

Our data suggest that inappropriate expression of MHC I on neurons could have deleterious consequences during neurodevelopment. It is believed that some neurodevelopmental diseases could arise from subtle abnormalities in axon path finding, axon number, synapse formation, and elimination. Many of the genes associated with human autism encode proteins involved in synaptic development (reviewed in Refs. 9 and 46). Viral infection during pregnancy increases the risk for autism in humans (reviewed in Ref. 47). Moreover,



the infection of pregnant mice with virus or treatment with other immune stimuli cause the offspring to have autism-like behaviors (48,49). These behavioral aberrations are believed to be due to maternal immune responses (e.g., inflammatory cytokines) that affected fetal neurodevelopment. Inflammatory cytokines, such as IFN- $\gamma$  and TNF- $\alpha$ , induce neuronal MHC I expression (1,4,5,50). Recently, the MHC region has been genetically implicated as a risk factor for schizophrenia (51). In other studies, we observed that a modest increase in neuronal MHC I expression (30–60% higher levels) in transgenic mice could alter hippocampal electrophysiology, the levels of synaptic markers in their hippocampus, and compensatory neuronal sprouting responses (Z.-P. Wu, manuscript in preparation). Thus, modest changes in neuronal MHC I expression can have neurobiological consequences in vivo.

MHC I has bifunctional activities in the immune system: it can help to activate or inhibit immune responses, depending on the context. Likewise, many molecules involved in axon guidance are bifunctional, providing attractive or repulsive signals, depending on cellular conditions. Accordingly, in a different context, MHC I may promote neuronal outgrowth.

We showed that MHC I could inhibit neurite outgrowth, which may explain, in part, why MHC I expression is tightly regulated in the CNS. We have also begun to decipher the principles governing neuronal recognition of MHC I. Our results suggest that inappropriate neuronal MHC I expression could be deleterious during neurodevelopment. A further understanding of the roles that MHC I and MHC I receptors can play in the nervous system may lead to new classes of treatments to promote recovery in some neuropathological conditions.

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## Abbreviations used in this paper

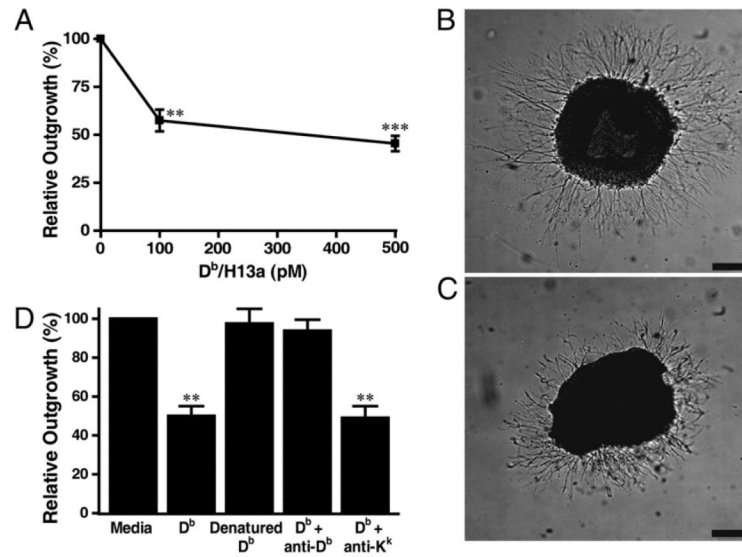
<b><math>\beta</math>2M</b>	$\beta$ 2 microglobulin
<b>E</b>	embryonic day
<b>GCL</b>	ganglion cell layer
<b>mHAg</b>	minor histocompatibility Ag
<b>MHC I</b>	MHC class I
<b>NIH</b>	National Institutes of Health
<b>ONBL</b>	outer neuroblastic layer
<b>PFA</b>	paraformaldehyde
<b>RGC</b>	retina ganglion cell
<b>RT</b>	room temperature

## References

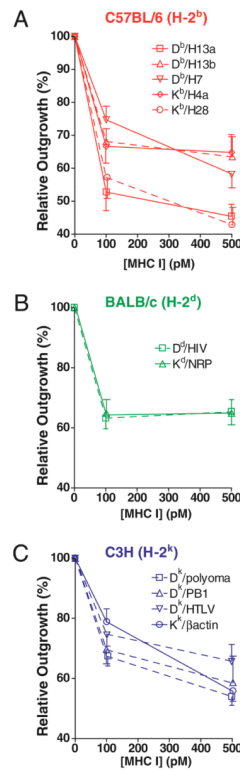
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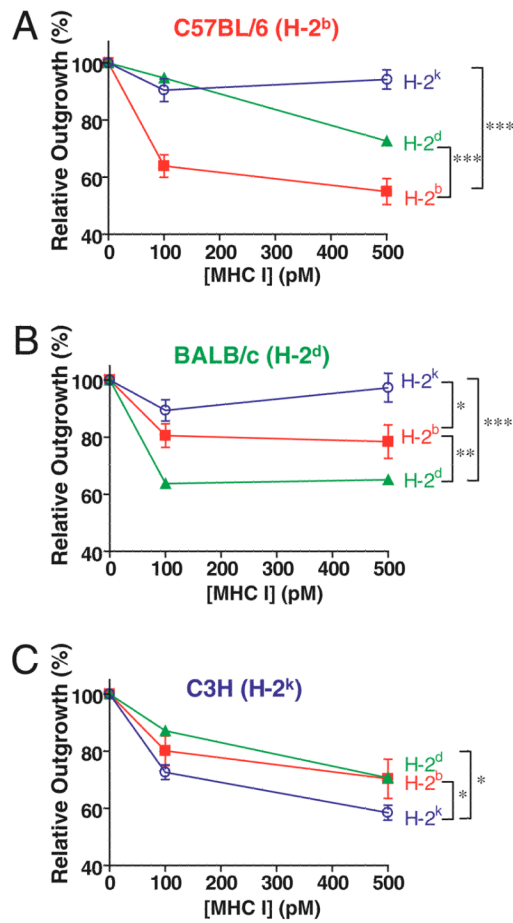
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**FIGURE 1.**

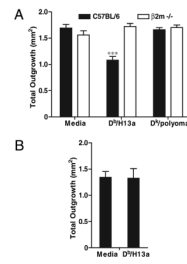
MHC I can inhibit retina neurite outgrowth. *A*, Isolated E14 C57BL/6 retinal tissue was cultured in Matrigel containing the indicated concentration of D<sup>b</sup>/H13a monomer. Data shown are the mean area  $\pm$  SEM covered by neurite projections relative to control cultures without exogenous MHC I. Similar results were obtained when measuring longest neurites (data not shown). Each MHC I dose was tested in at least three experiments with eight to 12 wells per group, and the results were analyzed using a one-tail Student *t* test. Representative cultures of retinas incubated in the absence (*B*) or presence (*C*) of 100 pM D<sup>b</sup>/H13a. Original magnification  $\times 5$ . Scale bar, 250  $\mu$ m. *D*, Neurite outgrowth in the presence of media alone, 100 pM D<sup>b</sup>/H13a, or D<sup>b</sup>/H13a that had been incubated at 65°C for 5 min or in a D<sup>b</sup>/H13a preparation that was preabsorbed with a conformation-dependent anti-D<sup>b</sup> mAb (B22-249) or an anti-K<sup>k</sup> mAb and protein A ( $n = 8-12$  retinal explants for each group in three separate experiments). \*\* $p < 0.001$ ; \*\*\* $p < 0.0001$ .

**FIGURE 2.**

Recombinant MHC I's neuroactivity does not depend on the nature of the peptide presented. Effects of D<sup>b</sup> and K<sup>b</sup> (A), D<sup>d</sup> and K<sup>d</sup> (B), and D<sup>k</sup> and K<sup>k</sup> (C) monomers loaded with different self- or nonself-peptides at different concentrations on C57BL/6 (A), BALB/c (B), and C3H (C) retinas. MHC I molecules presenting a self-peptide are indicated by a solid line, and those presenting a nonself-peptide are indicated by a dashed line. Data shown are the mean area  $\pm$  SEM covered by neurite projections relative to control cultures without exogenous MHC I. Each MHC I monomer was tested in at least three experiments with 12–16 wells per group. Details on the presented peptides are provided in Table II.

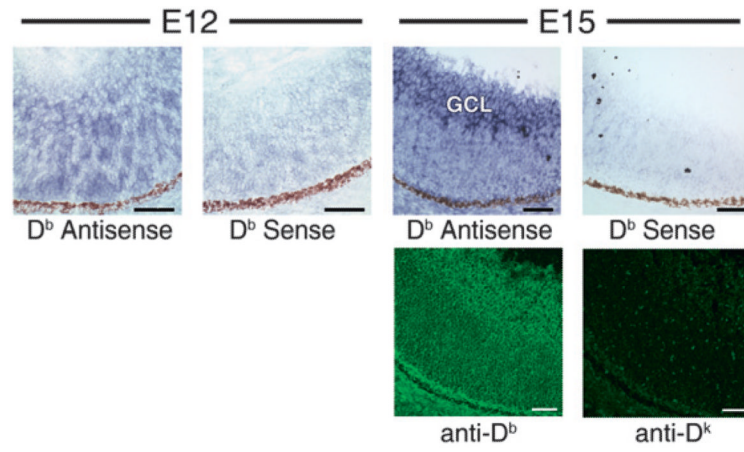
**FIGURE 3.**

Neurons preferentially recognize self-MHC I allele products. C57BL/6 (A), BALB/c (B), and C3H (C) retinas were exposed to the indicated concentrations of 11 different MHC I monomers made of D<sup>b</sup>, K<sup>b</sup>, K<sup>d</sup>, D<sup>k</sup>, and K<sup>k</sup> molecules loaded with self- and nonself-peptides. Each MHC I monomer was tested on all three mouse strains in at least three experiments with 12–16 wells per group. Data shown are the grouped average neurite outgrowth  $\pm$  SEM in the presence of H-2<sup>b</sup> (D<sup>b</sup> and K<sup>b</sup>), H-2<sup>d</sup> (D<sup>d</sup> and K<sup>d</sup>), or H-2<sup>k</sup> (D<sup>k</sup> and K<sup>k</sup>) monomers. The means were compared via random-effects ANOVA, controlling for dose and MHC group. For each mouse strain, the mean neurite outgrowth in the presence of H-2–matching MHC I monomers was always significantly less than that in the presence of the two H-2–mismatched MHC I monomer groups. The means of the two H-2–mismatched MHC I groups were not significantly different within each mouse strain, except for BALB/c. \* $p < 0.01$ ; \*\* $p < 0.001$ ; \*\*\* $p < 0.0001$ .

**FIGURE 4.**

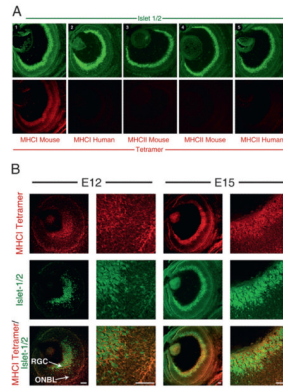
*A*, Retinas from  $\beta 2M^{-/-}$  C57BL/6 mice are insensitive to a self-MHC I monomer. Retinas from C57BL/6 and  $\beta 2M^{-/-}$  C57BL/6 mice were cultured with D<sup>b</sup>/H13a or a control D<sup>K</sup>/polyoma monomer. The D<sup>b</sup>/H13a monomer had no effect on neurite outgrowth from  $\beta 2M^{-/-}$  retinas at 100 pM or even 500 pM (shown). Data shown are from three experiments with 12–16 wells per group. \*\*\* $p < 0.0001$ . *B*, Retinas from C57BL/6 K<sup>b</sup>D<sup>b</sup><sup>-/-</sup> mice are insensitive to D<sup>b</sup>/H13a (100 pM).





**FIGURE 5.**

MHC I is expressed early in retina development. Top row: representative horizontal sections of E12 and E15 C57BL/6 retinas probed with antisense- or sense D<sup>b</sup>-specific riboprobes. Bottom row: E15 C57BL/6 retinas stained with anti-D<sup>b</sup> or an anti-D<sup>k</sup> isotype-matched mAb. Original magnification  $\times 10$ . Scale bar, 50  $\mu\text{m}$ .



**FIGURE 6.**

MHC I tetramer staining of classical MHC I receptors in retina. *A*, Fresh-frozen E15 C57B16 retina horizontal sections were stained side-by-side with MHC I D<sup>b</sup>/H13a tetramer (*panel 1*) or control APC-labeled tetramers consisting of human MHC I (loaded with an EBV peptide; *panel 2*), mouse MHC class II (loaded with OVA 323–39 or human CLIP; *panels 3 and 4*, respectively), or human MHC class II (loaded with a glutamic acid decarboxylase 65 peptide; *panel 5*). After staining and fixation, the slides were stained with anti-islet 1/2. Original magnification  $\times 2.5$ . *B*, MHC I receptors are expressed early in retina development. E12 and E15 C57BL/6 retina horizontal sections were stained with D<sup>b</sup>/H13a tetramer (red) and anti-islet 1/2 (green), an early RGC nuclear marker. No staining was observed in sections incubated with the detection Abs alone or an APC-labeled MHC class II tetramer (data not shown). Scale bar, 50  $\mu\text{m}$ .

**Table I**

MHC I alleles of tested mouse strains

Strain	H-2	Alleles
C57BL/6	H-2 <sup>b</sup>	D <sup>b</sup> , K <sup>b</sup>
BALB/C	H-2 <sup>d</sup>	D <sup>d</sup> , K <sup>d</sup> , L <sup>d</sup>
C3H	H-2 <sup>k</sup>	D <sup>k</sup> , K <sup>k</sup>

Table II

Tested peptide/MHC I molecules

H-2	Allele	Peptide	Sequence	Peptide Properties	Reference
H-2 <sup>b</sup>	D <sup>b</sup>	H13a	SSVVGWYWL	Ubiquitous self-mHAg in C57BL/6	(25)
	D <sup>b</sup>	H13b	SSVIGWYWL	H13 variant, nonself in C57BL/6	(25)
	D <sup>b</sup>	H7a	KAPDNRETL	Ubiquitous self-mHAg in C57BL/6	(26,27)
	K <sup>b</sup>	H4a	SGTVYIHL	Ubiquitous self-mHAg in C57BL/6	(28)
	K <sup>b</sup>	H28	I LENEPRL	mHAg not expressed by C57BL/6	(29)
H-2 <sup>d</sup>	D <sup>d</sup>	HIV 10-118	RGPGRFVVTI	HIV Ag, foreign	(30)
	K <sup>d</sup>	NRP	KYNKANAFI	Ubiquitous self-protein	(31)
H-2 <sup>k</sup>	D <sup>k</sup>	TAX (38-46)	ARLHRHALL	HTVL-1 Ag, foreign	(32)
	D <sup>k</sup>	PB1 (349-357)	ARLGKGYMF	Influenza virus Ag	(33)
	D <sup>k</sup>	MT (389-397)	RRLGRLLLL	Polyoma virus Ag	(34)
	K <sup>k</sup>	$\beta$ -actin	HETTENS I	Ubiquitous self-protein	(35)