Neural Stem/Progenitor Cells Express Costimulatory Molecules That Are Differentially Regulated by Inflammatory and Apoptotic Stimuli

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Increased expression of the costimulatory molecule CD80 (B7-1) was noted in the subventricular zone of the brain during the course of experimental autoimmune encephalomyelitis (EAE). This area of the brain is a neural stem cell (NSC) niche in the adult. We show that isolated NSCs from adult brain express CD80 and CD86 (B7-2) and this expression is increased after exposure to IFN- γ or TNF- α , the prototypical Th1 cytokines expressed during EAE. CD80 and CD86 expressed by NSCs are functional and can costimulate allogeneic cells in a mixed lymphocyte reaction. Furthermore, cross-linking of CD80 on the surface of NSCs results in apoptosis of NSCs. *In vitro*, we show that T cells can interact with NSCs and form conjugates with redistribution of CD3 on the surface of T cells to the area of contact. These data raise the possibility that during CNS inflammatory diseases such as EAE, NSCs may express immune molecules and interact with the inflammatory environment potentially resulting in injury to the NSCs, which may have implications for repair mechanisms in the central nervous system. (Am J Pathol 2004, 164:1615-1625)

Multiple sclerosis (MS) is an autoimmune demyelinating and degenerative disease of the central nervous system (CNS). The majority of MS patients experience relapsing-remitting symptoms followed by a secondary progressive phase that leads to permanent disability.^{1,2} Several studies have recently shown that axonal damage is present in both MS and its animal model, experimental autoimmune encephalomyelitis (EAE), and may contribute to fixed neurological deficits.¹ The adult mammalian CNS contains a population of immature, undifferentiated, multipotent cells, neural stem cells (NSCs), that may be called on

for repair in neurodegenerative and demyelinating diseases.³ NSCs may give rise to oligodendrocyte progenitor cells (OPCs) and other myelinating cells, as well as neural and glial precursors. In several models of experimental demyelination, it has been clearly demonstrated that progenitor cells of the adult mouse subventricular zone (SVZ) proliferate, migrate, and differentiate into oligodendrocytes.⁴ The current definition of a NSC relies on operational criteria rather than on a set of molecular markers alone. NSCs are cells from the CNS and peripheral nervous system that are multipotent and self-renewing.⁵ Multipotency is the ability of a single cell clone to give rise to the three major types of cells in the CNS, neurons, oligodendrocytes, and astrocytes, throughout the nervous system at all stages of life, as well as to repopulate those regions when depleted of cells. EAE is characterized by an infiltration in the CNS of autoreactive T cells and macrophages, accompanied by the production of pro-inflammatory Th1 cytokines⁶ and oxidative injury mediators.⁷ Studies of endogenous and transplanted NSCs suggest that during EAE NSCs may respond to the inflammatory milieu,^{8,9} although the molecular pathways are not known. We hypothesize that inflammatory molecules modulate the function of NSCs. Understanding the molecular pathways of this interaction is crucial to entertain repair therapy in neurodegenerative diseases with inflammatory component.¹⁰

CD80 is expressed on pluripotent embryonic stem cells¹¹ although the function of this molecule in these cells is unknown, we have recently demonstrated that CD80 expression is up-regulated on the surface of neurons as well as astrocytes during the course of EAE,⁶ and we have observed increased expression of CD80 in the SVZ during the course of EAE. In this report, we show that isolated NSCs express CD80 and CD86, and this expression is enhanced by exposure to pro-inflammatory cytokines IFN- γ and TNF- α . Furthermore, we demonstrate that CD80 and CD86 expressed by the NSCs are functional and that cross-linking of CD80 on the surface of

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NSCs *in vitro* enhances apoptosis of these cells. Our observations provide a molecular link between the inflammatory environment and neural stem cells, which may have implications for understanding the mechanisms for the paucity of repair observed in inflammatory neurodegenerative diseases such as MS.

Materials and Methods

Cells Isolation and Culture

Two multipotent self-renewing progenitor cell clones were used: adult subventricular zone neural stem cells isolated from C57/BL6 mice by microdissection, protease digestion, cloned at a single cell per well as previously described^{12,13} and the neural stem cell clone C17.2, isolated from a newborn mouse cerebellum, propagated and maintained as previously described.¹⁴ These cells form uncommitted multipotent self-renewing clusters when cultured in DMEM/F12 (Life Technologies, Grand Island, NY) supplemented with N2 (Gibco, BRL), EGF and bFGF (20 ng/ml each) (Calbiochem, Novabiochem Corp., San Diego, CA) with heparin (8 μ g/ml) and 100 units each of penicillin, streptomycin and fungizone per ml (Gibco, Life Technologies) in 35-mm uncoated plates (Corning, Inc, Corning, NY).

Confocal Analysis of NSC-T Cell Conjugates

Primary NSCs (3×10^5) were cultured on poly-L-lysine-treated coverslips (2 mg/ml) for 24 hours with IFN- γ (100 U/ml). After this incubation the cultures were washed three times with phosphate-buffered saline (PBS), then allogeneic SJL/J T cells (5×10^5) were added to the NSCs monolayer and left at 37°C for 20 minutes. Cells were fixed with 3% paraformaldehyde in PBS for 10 minutes at room temperature. For immunohistochemistry, cells were incubated for 1 hour at 4°C with permeabilization buffer containing 0.1% Triton X-100 in PBS and incubated with primary antibodies overnight and then secondary antibodies for 2 hours. For analysis of polarization and conjugation, T cell-NSCs conjugates showing clear cell-cell contact were first identified by differential interference contrast imaging. Acquisition of immunofluorescent images was done with a Zeiss Laser-Scanning Microscope, and 3D-analysis software for multi-planar reconstruction (Zeiss, Thornwood, NY).

Cytokine Stimulation of NSCs

Nearly confluent NSCs cultured in EGF 20 ng/ml and FGF 20 ng/ml were stimulated with IFN- γ (100 U/ml) or TNF- α (0.5 ng/ml) for 12 to 72 hours at 37°C 5% CO₂. The NSCs were then harvested using Versene-EDTA for 10 minutes, centrifuged, and resuspended gently at the appropriate concentrations for flow cytometric analysis.

NSC-Stimulated Mixed Lymphocyte Culture

Primary NSCs were plated and stimulated with IFN- γ (100 U/ml) for 24 hours in EGF and FGF medium. The cells were detached with EDTA to preserve surface cell expression of B7 molecules then washed with HBSS/3% FBS to remove residual IFN- γ . Splenocytes were obtained from SJL/J, C57BL6, or CD28K0 mice and enriched for T cells by column separation (R&D Systems, Minneapolis, MN) (purity >90% CD3+). 1×10^5 NSCs were irradiated with 10000 rads (R) and incubated with 3×10^5 purified splenic T cells in 96-well plates (Costar, Cambridge, MA) and T cell medium HL-1 (Biowhitaker, Bakersville, MD). CTLA4Ig was added at a concentration of 1.0 to 5.0 μ g/ml. Blockade of major histocompatibility complex (MHC) class I was achieved by using anti-MHC-I antibody (Clone 28-14-8) at a concentration of 10 μ g/ml from Pharmingen (San Diego, CA). The cell cultures were incubated at 37°C and 5% CO₂, 48 to 72 hours later they were pulsed with ³H-thymidine 1 μ Ci/well (NEN, Boston, MA) added in 20 μ l of media to each well for another 16 hours. Cells were harvested with a Tomtec harvester (Tomtec, MA). These experiments were performed in quadruplicate. For experiments with non-irradiated NSCs, NSCs were prepared as above and incubated with T cells labeled with CFSE at a concentration of 1 μ mol/L for 86 hours and then flow cytometry was performed (Molecular Probes, Eugene, OR).

Antibodies and Reagents

The following monoclonal antibodies were obtained from Pharmingen (San Diego, CA): FITC-conjugated rat anti-mouse CD80 (IG10), FITC hamster anti-mouse CD80 (Clone 16-10A), FITC rat anti-mouse CD86 (GL1), FITC rat anti-mouse CD86 (PO3), mouse GFAP, mouse IgG1 nestin (clone Rat 401), PE rat anti-mouse CD28, FITC rat anti-mouse CD40, hamster anti-mouse ICAM-1, rat anti-mouse c-kit (2B8), FITC mouse IgG2a-H2D^b, FITC mouse IgG2a I-A^b, and FITC anti-mouse CD11b. The hybridomas for anti-CD80, anti-CD86 are a kind gift from Roche Pharmaceuticals (Nutley, NJ). The CD80 and CD86 F(ab) fragment were prepared by Bioexpress Inc. (West Lebanon, NH). The following antibodies were obtained from Caltag (Burlingame, CA): FITC rat anti-mouse CD80 clone RMMP-2, rat anti-mouse CD86 clone RMMP-1 FITC and rat IgG2a isotype control, and FITC-labeled goat anti-human Ig heavy and light chains. Recombinant murine IFN- γ and TNF- α were obtained from Pharmingen. CTLA4Ig, CTLA4IgY100F, and control Ig were obtained from Bristol-Myers-Squibb (Pennington, NJ).

RNA Extraction, RT-PCR and DNA Sequencing

Total cellular RNA was extracted from neural stem cells using Trizol (Life Technologies, Rockville, MD). RT-PCR was performed using 1 μ g of Dnase-treated RNA. Oligo (dT) primers were used for cDNA synthesis with superscript RT (Gibco BRL, Bethesda, MD). One microliter of cDNA was used as a template for PCR using AmpliTaq

Gold (Promega, Madison, WI). Primers for CD80, CD86, and PCR conditions have been previously described.¹⁵ The PCR products were separated in a 1.5% agarose gel with ethidium bromide and eluted using Wizard Mini Columns (Promega). The amplicons were sequenced (ABI Prism Automated DNA Sequencer, Core Facility Beth Israel Medical Center, Boston, MA), and compared with deposited GenBank sequences using BLAST algorithm.

Flow Cytometry

For surface expression, proliferating NSCs were detached using incubation with EDTA for 30 minutes at 37°C, the cells were washed, counted and resuspended in staining buffer, treated with FC block (Pharmingen), and stained with labeled antibodies. Cells were analyzed using a FACScan equipped with CellQuest Software (Becton Dickinson Immunohistochemistry, San Jose, CA); 10000 events were acquired. To ensure specificity for B7 staining, three clones of mAb were used: CD80 (1G10, 16–10A1, RMMP-2) and CD86 (RMMP-1, GI-1, PO3). For intracytoplasmic staining, NSCs cells were treated as above and antibodies to cell surface markers were added for 30 minutes. The cells were then fixed with 4% paraformaldehyde for 20 minutes at room temperature, washed and resuspended in permeabilization buffer (D-PBS without Mg²⁺ or Ca²⁺, 0.1% sodium azide, 1% heat-inactivated FCS, and 0.1% saponin) and incubated with antibodies against neural antigens or isotype controls at a concentration of 1 µg per 10⁶ cells for 30 minutes at 4°C, followed by incubation with secondary antibody for 15 minutes.

Blocking the Binding of CD80 and CD86 Antibodies

NSCs were incubated with 1.0 to 2.0 µg of CTLA4Ig, CTLA4IgY100F or unlabeled anti-CD80 and anti-CD86 for 30 minutes at 4°C and then stained with antibodies against CD80 and CD86 for flow cytometry. Isotype control was used for B7 antibodies and L6 peptide a molecule with no specific binding was used as a control for CTLA4Ig and CTLA4Y100F. Inhibition of fluorescence was calculated as previously described.¹⁶

Stress and Apoptosis Induction of NSCs

NSCs cultures were cultured in serum-deprived media with no growth factors for 24 to 72 hours. For oxidative stress, H₂O₂ (Alexis Biochemicals, San Diego, CA) at a concentration range of 10 to 100 µmol/L was added to the cultures (Alexis Biochemicals) for a 24 to 72 hour period. Trichostatin-A (TSA) (Wako Biomedical, NY), a specific inhibitor of histone deacetylase and an inducer of apoptosis, was used at concentrations between 50 to 500 ng/ml.¹⁷

Analysis of Apoptotic NSCs

Apoptosis was determined by the translocation of phosphatidylserine revealed with Annexin-V staining. NSCs undergoing apoptosis were distinguished from live and necrotic cells by the use of Annexin-V and 7-amino actinomycin (7-AAD) staining. NSCs were incubated with TSA for 12 to 24 hours. At several time points, aliquots of 10⁵ cells were incubated with Annexin and 7-AAD for 20 minutes at room temperature. The cells were then analyzed by flow cytometry, using a two-color FACS analysis; live cells were considered as being Annexin-V⁻ and 7-AAD⁻. Apoptotic cells were considered the sum of early and late apoptotic cells; early apoptotic cells are Annexin-V⁺ and 7-AAD⁻; late apoptotic cells as both Annexin⁺ and 7-AAD⁺; and necrotic cells are only 7-AAD⁺. A polyclonal antibody that recognizes the activated form of caspase-3 was also used. The cells were incubated for 30 minutes in caspase-3 antibody and used for flow cytometry.

Anti-CD80 and CD86 in TSA-Treated NSCs

Confluent NSCs were exposed to TSA and anti-CD80 at a dose of 5 to 10 µg/ml. Cells were incubated for 12 to 18 hours and apoptosis was evaluated. High concentrations of anti-CD80 (50 µg/ml) were used alone to demonstrate the absence of apoptotic effect. Isotype control, CD80 Fab fragment, and anti-CD86 were used at a 5 to 10 µg/ml concentration.

EAE Induction

EAE was induced as previously described.¹⁸ Briefly, C57/BL6 mice are immunized subcutaneously with 200 ng of MOG peptide in PBS and CFA containing 0.4 mg *Mycobacterium tuberculosis* and intraperitoneally injected with 200 ng pertussis toxin on the day of immunization and 2 days later. For immunohistology, animals were sacrificed, perfused with 4% paraformaldehyde in PBS, tissues were harvested and post-fixed, dehydrated with sucrose 30% and placed in OCT, and stored at -80° until use.

Immunohistochemistry and Confocal Microscopy

The following antibodies and concentrations were purchased from Pharmingen: rat anti-mouse CD80 (IG10), FITC hamster anti-mouse CD80 (Clone 16–10A): (1:50–1:100), mouse IgG1 nestin (Clone rat 401)(1:100), mouse IgG1 GFAP (1:100), mouse IgG1 anti-Neu (1:100). Forty-µm sections of brain tissue from mice undergoing EAE and control mice were washed with PBS; blocked in PBS containing 4% goat serum, 0.3% BSA, and 0.3% triton; and then incubated with primary antibodies overnight and secondary antibodies for 2 hours in blocking solution. We used highly cross-adsorbed secondary antibodies to avoid cross-reactivity. Confocal microscopy was performed as described above. For quantitative analysis of CD80 expression in the SVZ, we used LSM 510 con-

Figure 1. Nestin-positive subventricular cells up-regulate CD80 during EAE. Sections of brain at the dorsolateral corner of the subventricular zone from naïve animals and animals with EAE were stained with antibodies against nestin and CD80. **A:** Naïve animals show very few nestin+ (red) cells that co-localize with CD80 (green) (arrows). **B:** EAE animals show an increase in the co-localization of nestin (red) and CD80 (green) (arrows) in cells from the subventricular zone and the ependymal layer, as shown in insert in B, bar, 20 μ m. **C:** Quantification of intensity profile of CD80 expression on nestin-positive cells from the subventricular zone. Nestin-positive cells were selected ($n = 34$) from four levels of sectioning of the SVZ for quantitative analysis. Nestin-positive cells in EAE animals shows a significant increase in the expression of CD80 $P < 0.0001$.

focal software. The mean fluorescent intensity (MFI) of pixels was determined, these values range between 0 (no signal) to 250 (maximum intensity). Using the same acquisition parameters, background values were determined from the negative control sections (55 ± 11 MFI), then values from controls and EAE animals were obtained. The MFI of nestin-positive cells from the SVZ was measured in two independent experiments. Each individual cell was analyzed examining the intensity of CD80 expression and the peak of intensity of each individual cell was annotated; cells with a minimum value of 125 MFI were considered positive.

Statistical Analysis

Statistical analysis was performed using paired Student's *t*-test. A *P* value of <0.05 was considered significant.

Results

Expression of CD80 during the Course of EAE

EAE pathology is characterized by cellular infiltration in the CNS and the presence of CD4⁺ encephalitogenic T cells that secrete IFN- γ and TNF- α . In naïve animals we found a small subpopulation of nestin+ SVZ cells expressing CD80 (Figure 1A), as shown by dual immunofluorescence staining by confocal microscopy. Nestin+ SVZ cells include cells with NSCs capacity, ependymal stem cells, astrocytic stem cells, or type C multipotent neural precursors cells.^{12,19} On day 21 post-immunization, during the peak of acute disease, which is characterized by a proliferation and migration of neural progenitors,²⁰ we found a significant increase of nestin+ cells expressing CD80, immediately adjacent to the lateral ventricle, with the morphology of immature nestin+ ependymal cells

Figure 2. Expression of CD80 and CD86 mRNA and protein by neural stem cells. **A:** Isolation and characterization of neural stem cells: Phase contrast images showing culture behavior of freshly isolated SVZ cells. Single cell clones from SVZ divide and form large floating neurospheres clusters in serum-free medium. **B:** The neurospheres are positive for nestin (left, green) and can generate neurons (labeled with β -tubulin in green) and glial cells (labeled with GFAP in red), (right). **C:** Under non-differentiating conditions the cells uniformly express nestin, a marker of stem cells, and do not express markers for differentiated cell types such as astrocytes and microglia. **D:** Expression of costimulatory molecules by nestin+ neural stem cells. NSCs were stained with anti-CD80 and anti-nestin and analyzed using Becton Dickinson FACS scan flow cytometer. CD80 and CD86-FITC are on the x-axis and nestin-PE on the y-axis. **E:** Neural stem cell B7 mRNA expression: PCR products were visualized by ethidium bromide agarose (2%) gel electrophoresis. RT-PCR from C17.2 cells and subventricular zone progenitor cells, shows CD80 expression by both clones compared with plasmid containing CD80 full-length cDNA (bottom).

previously characterized as NSCs.^{12,13} There was also an increase in nestin+ cells from the dorsolateral corner of the subventricular zone (Figure 1B). Quantitative analysis by confocal mean fluorescent intensity (MFI) profile showed a significant increase in the expression of CD80 in nestin+ SVZ cells in EAE (172.1 ± 6.4) compared to normal controls (73 ± 6.2) $P < 0.0001$ (Figure 1C). Furthermore, the percentage of nestin+ cells that express CD80 increased from 8% in the control brains to 65% in EAE brains.

Expression of CD80 and CD86 mRNA and Protein by Neural Stem Cells

To confirm the expression of costimulatory molecules by NSCs, we isolated and characterized neural stem cells

from adult mouse subventricular zone. Clonally derived cells formed multipotent floating clusters in serum-free medium in culture (Figure 2A) and expressed nestin, an intermediate filament associated with immature, uncommitted neuroepithelial cells (Figure 2B, left).^{21,22} When cultured in differentiation medium, these cells differentiated into glia and neurons (Figure 2B, right). Under the conditions used in this study, the cells were negative for markers of neural differentiation, including glial fibrillary acidic protein (GFAP, characteristic of astrocytes) as well as for CD11b (characteristic of microglia) (Figure 2C). We examined the expression of CD80 and CD86 on these NSCs. Under non-stimulated conditions, CD80 and CD86 expression by NSCs was detectable by flow cytometry at low levels with a range of 4 to 10% positive cells. The expression of B7 molecules by the NSCs was confirmed

Figure 3. Expression of MHC Class I and CD40 by NSCs. NSCs were analyzed using Becton Dickinson FACS scan flow cytometer. The **thick line** represents staining under basal conditions, the **thin line** represents the staining after IFN- γ stimulation, and the **black profile** represents the isotype control.

both by immunocytochemistry (double staining for CD80 and CD86 along with an anti-nestin antibody) (Figure 2D) and by subsequent RT-PCR analysis. The RT-PCR revealed the expected 558-bp amplification product for *cd86* and the 458-bp amplification product for *cd80*. Total RNA from C57BL/6 mice splenocytes, and the BV-2 cell line (a microglial cell line that expresses both *cd80* and *cd86*²³) were used as positive controls for *cd80* and *cd86* expression, while negative controls consisted of mRNA samples processed in parallel in the absence of reverse transcriptase enzyme (RT minus) and DNase treatment (Figure 2E). The identity of *cd80* and *cd86* mRNA was confirmed by sequencing of the products with an automated sequencer. The sequences obtained matched the murine CD80 and CD86 cDNA sequences deposited in GenBank Accession No. X60958 (for *cd80*) and No. L25606 (for *cd86*).

We also investigated the expression of MHC class I and class II molecules and other costimulatory molecules typically found in APCs or other stem cells. NSCs expressed class I MHC that was up-regulated after IFN- γ stimulation (Figure 3A), but they did not express class II MHC molecules (Figure 3B). NSCs expressed low levels of CD40 (2 to 3%), which was not modified by IFN- γ treatment (Figure 3C). NSCs did not express ICAM-1, CD45, CD3, CD4, CD8, or *c-kit*, the receptor for hematopoietic stem cell factor that is expressed by hematopoietic stem cells.

IFN- γ Up-Regulates the Surface Expression of CD80 and CD86

IFN- γ is expressed during the course of EAE and is the prototypic Th1 cytokine. IFN- γ regulates the expression of CD80 and CD86 molecules on astrocytes and microglia.^{6,15,23,24} We studied the kinetics of CD80 and CD86 expression on the surface of NSCs after culture with IFN- γ . When the NSCs were stimulated with IFN- γ , the expression of CD80 and CD86 was significantly up-regulated to 15 to 35% CD80+ and 15 to 25% CD86+ cells (Figure 4, A and C). NSCs were stimulated with 100 U/ml of IFN- γ for periods ranging from 0 to 72 hours. As can be seen in Figure 4E, an increase in percentage of CD80+ cells was noted at 48 hours, but the peak of CD80 expression occurred at 72 hours post-stimulation. In contrast, the expression of CD86 peaked at 24 hours and decreased thereafter (Figure 4F). In addition, TNF- α up-regulated the expression of CD80 but not CD86 at 72 hours post-stimulation (Figure 4, B and D), these results

Figure 4. Inducible expression of CD80 and CD86 on the surface of NSCs. NSCs were analyzed using Becton Dickinson FACS scan flow cytometer using anti-CD80 (clone 16-01A) and anti-CD86 (clone GL-1). The **thick line** represent staining after IFN- γ or TNF- α stimulation, the **thin line** represents staining under basal conditions, and the **black profile** represents the isotype control. The histograms show up-regulation of CD80 (A) and CD86 (C) expression after IFN- γ stimulation and TNF- α stimulation (B and D). E and F: Kinetics of expression after IFN- γ stimulation. NSCs were cultured with rIFN- γ (100 U/ml) alone for 12 to 72 hours. The cells were stained with anti-CD80 and anti-CD86. All staining reagents and FACSscan settings were kept constant for the duration of the time course.

were confirmed using at least two different clones of B7 antibodies. The specificity of B7 staining of NSCs was examined by competition assay with CTLA4Ig and CTLA4IgY100F, and splenocytes were used in parallel as positive controls. CTLA4Ig is a fusion protein that binds with high affinity to CD80 and CD86, while CTLA4IgY100F is a mutant form of CTLA4Ig that binds only CD80.²⁵ Concentrations of 0.5 to 1.0 mg/ml of CTLA4Ig blocked the binding of anti-CD80 and anti-CD86 $\times$ 90%. CTLA4Ig Y100F blocked the binding of CD80 mAbs by 95% (not shown).

CD80 and CD86 Molecules Expressed by NSCs Provide Functional Costimulation for T Cell Proliferation

First we established the NSCs capacity to interact with T cells, we studied the occurrence of T cell:NSC conju-

Figure 5. Primary neural stem cells are able to establish immunological synapses with T cells. **A:** Confocal phase microscopy shows a primary NSC-T cell conjugate and CD3 redistribution to the contact zone. NSC is stained green with nestin antibody and CD3 stained in red in a planar view. **B:** Three-dimensional reconstruction (left) and orthogonal view in the Z-plane (right) demonstrating the interaction between NSCs and T cells showing redistribution of CD3 to the contact zone (arrows). Quantitative profile analysis (bottom) by confocal microscopy shows intense expression of CD3 in the area of the contact zone (red peak). Observe the intensity profile vector in white dotted line used for measurements of intensity in contact zone (white arrows). **C:** Frequency of T cell-NSCs conjugates with or without pre-stimulation of NSCs with IFN- γ . NSCs were incubated with T cells for 20 minutes at 37°C and prepared for immunohistochemistry. The frequency of conjugates was measured by counting 100 NSCs in random fields per each well ($n = 3$) in a volume of 1 ml.

Figure 6. NSC can stimulate T proliferation in a B7-dependent fashion. **A:** Primary NSCs can elicit an allogeneic T cell response. NSC (2×10^5) were stimulated with IFN- γ (100 U/ml) and incubated at 37°C for 24 hours, detached with Versene-EDTA then washed with HBSS 3% FBS and irradiated with 10000 R. Splenic-purified T cells (3×10^5) from SJL/J mice were used as responders. Cultures were pulsed with ^3H (1 $\mu\text{Ci}/\text{well}$) after 72 hours and harvested at 96 hours. There was a significant increase in proliferation between NSC+T cells versus NSCs alone. ($P < 0.05$ Student's paired t -test). **B:** T cell response to primary NSC is blocked by CTLA4Ig. NSCs (2×10^5) and T cells were prepared as in **C**. There was a significant increase in proliferation between p-NSC+T cells versus T cells alone, ($P < 0.01$ Student's paired t -test, SI: 39). When CTLA4Ig was added to the cultures there was a significant decrease in proliferation ($P < 0.05$ by Student's paired t -test, SI: 10). **C:** C17-2 NSCs elicit an allogeneic T cell responses that is blocked by CTLA4Ig. C17.2 cells (2×10^5) were stimulated with IFN- γ (100 U/ml) and incubated at 37°C for 24 hours, detached with Versene-EDTA then washed with HBSS 3% FBS and irradiated with 10000 R. Splenic-purified T cells (3×10^5) from SJL/J mice were used as responders. Cultures were pulsed with ^3H (1 $\mu\text{Ci}/\text{well}$) after 72 hours and harvested at 96 hours. Experiments were performed in quadruplicate in at least in three independent experiments. CTLA4Ig was used at a concentration of 5 $\mu\text{g}/\text{ml}$. There was a significant increase in proliferation between NSC+T cells versus T cells alone, ($P < 0.02$ Student's paired t -test stimulation index (SI): 11.3). When CTLA4Ig was added to the cultures there was a significant decrease in proliferation ($P < 0.05$ by Student's paired t -test SI: 3.6). **D:** Allogeneic T cell response with CD28-/- T cells as responders. Wild-type T cells stimulated with NSCs proliferate significantly compared to wild-type T cells alone ($P < 0.001$ by Student's paired t -test). When CTLA4Ig was added to wild-type cultures there was a significant decrease in proliferation ($P < 0.02$ by Student's paired t -test). CD28-/- T cells show a significant decrease in the proliferative response compared with wild-type cells ($P < 0.002$ by Student's paired t -test). The results are representative of three independent experiments.

gates in culture. We found that activated primary NSCs are able to establish conjugates with purified CD3+ cells within 20 minutes of co-culture. The frequency of conjugates was increased from 4% to 18% after pre-incubation of primary NSCs with IFN- γ (Figure 5C). Furthermore, the

Figure 7. NSC can stimulate CD8+ T cell proliferation and is dependent on CD80 and MHC class I expression. **A:** Primary NSCs cells (2×10^5) were stimulated with IFN- γ (100 U/ml) and incubated at 37°C for 24 hours, detached with Versene-EDTA then washed with HBSS 3% FBS and irradiated with 10000 R. Splenic purified CD8+ T cells (3×10^5) from SJL/J mice were used as responders. Cultures were pulsed with ^3H (1 μCi /well) after 72 hours and harvested at 96 hours. Experiments were performed in quadruplicate. CTLA4Ig was used at a concentration of 5 μg /ml and anti-MHC-I was used at a concentration of 10 μg /ml. There was a significant increase in proliferation in NSC+ CD8+ T cells *versus* CD8+ T cells alone, ($P < 0.01$). When CTLA4Ig was added to the cultures there was a significant decrease in proliferation ($P < 0.05$), and anti-MHC-I also caused a significant decrease in T cell proliferation ($P < 0.05$). **B:** Primary NSCs stimulated with IFN- γ 100 U/ml for 24 hours or control unstimulated were incubated without irradiation with CD8+ CFSE-labeled T cells and allowed to proliferate for 86 to 90 hours and analyzed by FACS. Stimulated but not unstimulated NSCs induces CD8+ T cell decrease of CFSE staining indicative of proliferation.

conjugates remain stable despite several washes with PBS, and their formation was accompanied by redistribution of CD3 in the immunological synapse between nestin+ primary NSCs and CD3+T cells (Figure 5A) which was confirmed by confocal microscopy (Figure 5B). Then we investigated whether the CD80 and CD86 molecules induced on NSCs by IFN- γ could provide functional costimulation to T cells. Irradiated C17.2 or primary NSCs, unstimulated or stimulated with IFN- γ for 24 hours, were used as stimulators in an allogeneic mixed lymphocyte culture with purified responder T cells from SJL/J mice. As shown in Figure 6A, IFN- γ -stimulated primary NSCs (Figure 6, A and B) and C17.2 cells (Figure 6C) were able to stimulate T cell proliferation, and this proliferation was blocked in both cases by CTLA4Ig. Non-IFN- γ stimulated NSCs induced minimal T cell proliferation. When CD28-/- T cells were used as responders, proliferation was significantly decreased compared with wild-type responder T cells of the same background (C57/BL6) confirming the results obtained with CTLA4Ig blockade (Figure 6D). These results indicate that B7 molecules expressed by C17.2 and primary NSCs can provide functional costimulation for T cells, which is inhibited by blocking with CTLA4Ig.

To determine the phenotype of T cells involved in this proliferative response, we repeated the MLR assay with purified CD4+ and CD8+T cells. IFN- γ stimulated NSCs

Figure 8. Stress-induced apoptosis differentially increases CD80 expression by NSCs and CD80 ligation increases apoptosis. **A:** Stress was induced by culturing NSCs with hydrogen peroxide at 100 $\mu\text{mol/L}$ concentration for a 24 to 48 hour period (left histograms) or by media deprived of serum and growth factors (right histograms). Cells were detached using EDTA-Versene, were incubated for 15 minutes at 37°C washed and counted for flow cytometry, 7-amino actinomycin D (7-AAD) staining was used to exclude non-viable cells. **B:** NSCs were incubated with TSA 100 ng/ml for 18 or 24 hours and examined by two-color staining with Annexin V and 7-AAD and anti-CD80 or anti-CD86 (left dot blot). The expression of B7 molecules was measured by gating on early apoptotic cells (Annexin-V positive, 7-AAD negative) *vs.* live cells (Annexin and 7-AAD negative). The green background is the isotype control whereas the red shift is the anti-CD80 or anti-CD86 antibodies. The expression of CD80 is up-regulated in cells undergoing apoptosis (left histograms) as compared with CD86 where a slight up-regulation occurs after a 24-hour time period (right histograms). **C:** Actively proliferating C17.2 NSCs were left untreated, incubated with anti-CD80 (50 μg /ml), TSA (100 ng/ml), TSA plus anti-CD86, TSA plus anti-CD80 (5 to 10 μg /ml), TSA plus CD80 F(ab), TSA plus mIg, or TSA plus rat IgG2a and the percentage of apoptotic cells measured by Annexin and 7-AAD staining. Dot blots of apoptotic cell staining after 18 hours of culture comparing various culture conditions (left). The early apoptotic cells are seen in the lower right quadrant. **D:** Mean $\pm$ SE of percentage of apoptotic cells from triplicate cultures. Apoptotic cells were calculated as the sum of early and late apoptotic cells. These data were reproduced in three independent experiments (each done in triplicate). The effects of TSA alone is demonstrated by an increase in apoptotic cells compared to untreated cells ($P < 0.01$ by Student's paired *t*-test), or cell treated with anti-CD80 alone ($P < 0.001$). The addition of anti-CD80 to TSA-treated cells causes a significant increase in apoptotic cell numbers compared to TSA alone ($P < 0.01$), control mouse Ig ($P < 0.05$), CD80 F(ab) fragment ($P < 0.006$), anti-CD86 ($P < 0.05$), or anti-rat Ig ($P < 0.05$) that do not induce additional apoptosis of TSA-treated cells (right bars). **E:** Mean $\pm$ SE of percentage of primary NSCs with activated caspase-3 after oxidative stress and anti-CD80 treatment. The effect of oxidative stress is demonstrated by an increase in the number of cells with activated caspase-3 ($P < 0.001$). Cells undergoing oxidative stress treated with anti-CD80 have increased expression of caspase-3 ($P < 0.05$).

preferentially induce the proliferation of CD8+ T cells, and this was blocked by treatment with CTLA4Ig and antibodies against MHC-I (Figure 7A). This proliferative response was also confirmed by using non-irradiated NSCs and CFSE-labeled CD8+ T cells (Figure 7B).

Stress Differentially Increases CD80 Expression in NSCs

Oxidative stress is a key element of cell damage during multiple sclerosis²⁶ and other neurodegenerative diseases. We investigated the effect of stress on the expression of CD80 and CD86 molecules by NSC. Previous studies have shown costimulatory molecules to be up-regulated on a variety of cells under stress conditions.^{27,28} Stress was induced by exposing the C17.2 NSCs to hydrogen peroxide (H₂O₂), or serum deprivation. Using flow cytometry analysis, we examined the expression of CD80 and CD86 molecules during stress conditions. 7-AAD was used to exclude necrotic cells from the analysis. We found that up-regulation of CD80 occurred within 24 hours of oxidative stress exposure. CD86 expression was not significantly affected by these conditions (Figure 8A). CD80 was also up-regulated within 24 hours of serum deprivation (Figure 8A).

CD80 Expression Increases in NSC Undergoing Apoptosis

Previous studies have shown up-regulation of CD80 on tumor cells during pro-apoptotic stimuli,²⁹ however a direct link between apoptosis and CD80 expression has not been reported. We investigated the dynamics of CD80 and CD86 molecule expression on apoptotic C17.2 NSCs. We used Trichostatin A (TSA) a highly specific histone deacetylase inhibitor that induces apoptosis by modifying the transcriptional program of neural cells and increasing the DNA-binding activities of AP-1, CREB, and NF- κ B transcription factors.^{17,30} CD80 and CD86 expression were measured in populations of live (7-AAD⁻, Annexin-V⁻) and early apoptotic cells (7-AAD⁻, Annexin-V⁺). We found that CD80 expression was up-regulated on apoptotic cells by 18 hours of culture (Figure 8B), while CD86 expression was only slightly up-regulated by 24 hours (Figure 8B).

Cross-Linking of CD80 Enhances NSC Apoptosis

Having established that oxidative stress-induced differential up-regulation of CD80, we next assessed the consequences of cross-linking costimulatory molecule on the surface of NSCs. Engagement of CD80 induces tyrosine phosphorylation and growth arrest in B cells.³¹ Thus, we asked whether CD80 was signaling the NSCs in a similar manner. Control NSCs or NSCs incubated with a high concentration of anti-CD80 (50 μ g/ μ l) alone did not undergo apoptosis. TSA alone induced significant amount of apoptosis compared to control ($P < 0.01$) or anti-CD80

($P < 0.001$), however, when added in the presence of TSA, anti-CD80 (5 to 10 μ g/ μ l) had a synergistic effect on apoptosis induction as demonstrated by Annexin-V staining, compared with TSA alone ($P < 0.01$) (Figure 8, C and D). The F(ab) fragment of anti-CD80 did not enhance TSA-induced apoptosis indicating that cross-linking of anti-CD80 was necessary for this effect. Anti-CD86 did not enhance TSA-induced apoptosis (Figure 8, C and D). It was recently reported that T cells that acquire membrane expression of CD80 underwent apoptosis³² and that cross-linking of CD80 can decrease cell growth and induce apoptosis in B cells by a signaling cascade that involves caspase-3 activation.³³ We examined the activation of caspase-3 in the cells undergoing apoptosis after oxidative stress and anti-CD80. We found a significant increase in activated caspase-3 after treatment with anti-CD80 ($P < 0.05$), suggesting that caspase-3 pathway is involved in the NSC apoptosis induced by CD80 signaling (Figure 8E). The above-described experiments were performed with C17.2 cells and with primary NSCs isolated from the SVZ with similar results.

Discussion

Currently used immunomodulatory therapies partially alter the disease course of MS during the relapsing-remitting phase, however the secondary progressive phase of disease remains resistant to such treatments. Thus, it is becoming clear that repair should be considered as a goal for treatment. NSCs constitute a natural reserve of cells that can participate in repair of the CNS. There are specific germinative zones in the embryonic and adult mammalian CNS³⁴ that can be considered as sources of NSCs. These zones include the ventricular zone (VZ), the subventricular zone (SVZ), the external germinal layer of the cerebellum, the subgranular zone of the dentate gyrus, and the ependymal layer of the spinal cord.³⁵ EAE, an animal model of MS, is characterized by an infiltration in the CNS of autoreactive T cells and macrophages, accompanied by the production of pro-inflammatory Th1 cytokines⁶ and oxidative injury mediators.⁷ During EAE there is increased proliferation of cells in the SVZ, suggesting that stem or progenitor cells in this area may proliferate in response to demyelinating damage.⁸ In chronic EAE there is proliferation of cells from the SVZ, migration to the corpus callosum, and differentiation to astrocytes and small numbers of oligodendrocytes.²⁰ One hypothesis to explain the lack of repair observed in chronic MS, is that there is injury to the NSC pools in the CNS that are normally responsible for repair. We have observed expression of CD80 and CD86 in the SVZ during the course of EAE. We show that this expression is occurring on nestin+ NSCs and is induced by IFN- γ and TNF- α . These cytokines are prototypical Th1 cytokines and are increased during the course of EAE.^{6,36} It is not surprising that costimulatory molecules are expressed in the CNS by microglia and reactive astrocytes during the course of an inflammatory response;^{6,15} these cells have long been known to play a role in CNS inflammation. There is increasing evidence, however, that costimulatory

molecules are expressed by various cell populations not traditionally viewed as APCs, including human myoblasts and muscle cells,^{37,38} neurons,⁶ and murine fibroblasts,²⁸ suggesting a role in the control of the local immune response.

In this report, we show constitutive and inducible expression of the costimulatory molecules CD80 and CD86 by neural stem cells by RT-PCR and FACS analysis. Class I but not Class II MHC expression was up-regulated after IFN- γ stimulation, which is in concordance with a previous report of undifferentiated neural progenitors cells.³⁹ We show that costimulatory molecules are immunologically functional, ie, neural stem cells are able to costimulate T cells. Specifically, purified CD8+ T cells proliferate to stimulated NSCs and this proliferation is blocked by MHC class I. T cell proliferation was partially inhibited by adding CTLA4Ig, or using CD28-/- T cells, indicating a non-exclusive role for B7-CD28 interactions, with other costimulatory molecule pairs likely providing the residual costimulation. In addition, we find that oxidative stress up-regulates CD80 in neural stem cells. Our results extend to the nervous system reports suggesting that stress and/or injury can induce the up-regulation of CD80 and CD86 molecules in other tissues. Takada et al,⁴⁰ found that during cold ischemia-reperfusion injury there was an increase in costimulatory molecule expression in the kidney, which was ameliorated by CTLA4Ig treatment. Morel et al^{27,29} found up-regulation of CD80 in tumor cells in response to two types of stress: γ -irradiation and H₂O₂-induced oxidative stress. Others have found up-regulation of CD80 expression in fibroblasts during stress induced by serum starvation.²⁸ Thus, stress induced by the inflammatory milieu of EAE may participate in the up-regulation of CD80 and CD86 on the surface of NSCs.

The role of costimulatory molecule expression by NSCs during EAE is unclear. Recent reports link CD40 (another costimulatory molecule) with neuronal apoptosis.⁴¹ CD40 is known to signal through the cytoplasmic tail increasing the expression of CD80. We demonstrate that anti-CD80 synergizes with TSA and increases apoptosis of NSCs. Others have shown that CD80 cross-linking induces cell cycle arrest in Raji B cells and induces downstream signal transduction via tyrosine protein kinases.³¹ How does cross-linking of CD80 on the surface of NSCs occur? One possibility is that infiltrating T cells may interact with NSC during the course of EAE and induce apoptosis of NSCs. Here, we demonstrate that T cells can interact with NSCs *in vitro* and form conjugates, it is not clear whether this interaction happens *in vivo*. Another possibility is that NSCs become exposed to inflammatory cytokines (IFN- γ and TNF- α) during the course of EAE and up-regulate CD80 and CD86. Our finding that cross-linking of CD80 on the surface of NSCs enhances apoptosis of these cells has important clinical implications for the repair potential of these cells. Understanding the molecular mechanisms of the effect of inflammation on NSC function is an important step in developing therapies that enhance the potential for repair in the CNS.

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