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The neurotrophic hepatocyte growth factor attenuates CD8+ cytotoxic T-lymphocyte activity

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Abstract

Background: Accumulating evidence suggests a deleterious role for CD8+ T cells in multiple sclerosis (MS) pathogenesis. We have recently reported that hepatocyte growth factor (HGF), a potent neuroprotective factor, limits CD4+ T cell-mediated autoimmune neuroinflammation by promoting tolerogenic dendritic cells (DCs) and subsequently regulatory T cells. Whether HGF modulates cell-mediated immunity driven by MHC class I-restricted CD8+ T cells remains to be determined.

Methods: Here we examined whether HGF regulates antigen-specific CD8+ T cell responses using an established model of murine cytotoxic T lymphocyte (CTL)-mediated killing.

Results: We found that HGF treatment of gp100-pulsed DCs reduced the activation of gp100-specific T cell receptor (Pmel-1) CD8+ T cells and subsequent MHC class I-restricted CTL-mediated cytolysis of gp100-pulsed target cells. The levels of perforin, granzyme B, IFN-γ, and the degranulation marker CD107a as well as Fas ligand were decreased among CD8+ T cells, suggestive of a dual inhibitory effect of HGF on the perforin/granzyme B- and Fas-based lytic pathways in cell-mediated cytotoxicity. Treatment of CD8+ T cells with concanamycin A, a potent inhibitor of the perforin-mediated cytotoxic pathway, abrogated CTL cytotoxicity indicating that blockade of the perforin-dependent killing is a major mechanism by which HGF diminished cytolysis of gp100-pulsed target cells. Moreover, HGF suppressed the generation of effector memory CTLs.

Conclusions: Our findings indicate that HGF treatment limits both the generation and activity of effector CTL from naïve CD8+ T cells. Complementary to its impact on CD4+ T-cell CNS autoimmunity and myelin repair, our findings further suggest that HGF treatment could be exploited to control CD8+ T-cell-mediated, MHC I-restricted autoimmune dysfunctions such as MS.

Keywords: Hepatocyte growth factor (HGF), Cytotoxic T lymphocytes (CTL), Immune modulation, Central nervous system autoimmunity, Dendritic cells, Multiple sclerosis (MS), Immunological tolerance

Background

Multiple sclerosis (MS), a chronic autoimmune disorder characterized pathologically by central nervous system (CNS) inflammation, demyelination, and axonal damage, has been traditionally attributed to self-reactive CD4+ T lymphocytes that escape tolerance [1]. Growing evidence, however, indicates that autoreactive CD8+ T cells, like their CD4+ counterparts, contribute to the induction, progression,

and pathogenesis of autoimmune neuroinflammation [2,3]. Myelin-specific CD8+ T cells were reported to both aggravate CD4+ T cell-mediated experimental autoimmune encephalomyelitis (EAE) [4], an animal model for MS, and to mediate autoimmune CNS disease on their own [5-7]. In particular, adoptively transferred antigen-specific CD8+ T cells were found to injure the CNS in models of CD8-mediated EAE [5,6] or in mice that selectively express a neo-self antigen in oligodendrocytes [8,9]. Using continuous confocal imaging, axonal loss observed in these models was shown to result from 'collateral bystander damage' by autoaggressive, cytotoxic CD8+ T cells, targeting their cognate antigen processed and presented by oligodendrocytes [10]. Histopathological and neurobiological

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studies in MS also suggest that CD8^{+} T cells hold an active role in disease pathogenesis by targeting oligodendrocytes and the myelin sheath [11].

Due to their ability to function as professional antigen-presenting cells (APCs), CD11c^{+} myeloid DCs play an undisputed role in inciting autoimmunity. In EAE, DCs are critical APCs for the induction of both myelin-specific CD4^{+} and CD8^{+} T cells, and are also a prominent component of CNS-infiltrating cells [12,13]. However, other data indicate that DCs play an important role in initiating tolerance, and that tolerogenic DCs can suppress EAE *in vivo* [14]. Current efforts for the treatment of auto-immune pathogeneses requiring tolerance recovery are focused in the identification of molecules that control the development of tolerogenic DCs [15].

Hepatocyte growth factor (HGF) is a pleiotropic cytokine with potent anti-inflammatory properties shown to act as a potent regulator in multiple animal models of immune-mediated disorders [16-19]. HGF has been shown to govern the development of both human and mouse tolerogenic DCs [20,21]. We recently demonstrated that such a mechanism might account in part for the beneficial action of CNS-restricted overexpression of HGF in myelin oligodendrocyte glycoprotein (MOG)-induced EAE by regulating CD4^{+} T cell-mediated autoimmune responses to MOG [20]. Owing to its strong immunoregulatory and neuroprotective/neurorepair properties [22,23], exogenously supplied HGF was recently further shown to promote recovery in MOG-induced EAE by modulating both the immune response mediated by CD4^{+} T cells and by promoting myelin repair and neural cell development [24]. Because CD8^{+} T cells can further directly mediate motor disability and axon injury in the demyelinated CNS [25] and may actively contribute to neural damage in MS or other CNS inflammatory and degenerative disorders [26], it is important to understand whether HGF could modulate the effector function of antigen-specific CD8^{+} T cells.

To explore the effects of HGF on CD8^{+} T cell functions we used an established *in vitro* model of cytotoxic T-cell-dependent immunity. Our results showed that HGF significantly decreased the generation of effector cytotoxic gp100-petide T cell receptor (Pmel-1) CD8^{+} T cells from naïve splenocytes. HGF greatly reduced the production of inflammatory cytokines and cytolytic enzymes by autoagressive CD8^{+} T cells, including interferon (IFN)-γ, tumor necrosis factor (TNF), perforin, and granzyme B. HGF further diminished the expression of membrane-bound death receptor Fas ligand (FasL), a non-redundant lytic mechanism with cytolytic granule release in cytotoxic T lymphocyte (CTL)-mediated killing. CD8-enriched Pmel-1 splenocytes cultured with HGF demonstrated a considerably lower level of cytolytic activity, as measured by specific killing of antigen-

pulsed target cells. Finally, similar results were obtained when HGF-treated CD11c^{+} DCs were cultured with naïve purified Pmel-1 CD8^{+} T cells. These results suggest that HGF reduces CTL responses via professional APCs and may have important implications for CNS inflammatory diseases including MS.

Methods

Reagents
Human recombinant HGF (hrHGF) was supplied by T Nakamura (Osaka University, Tokyo, Japan) [27]. Human and murine HGF are cross-reactive. The dose of hrHGF (30 ng/mL) used was chosen based on previous studies analyzing the immunoregulatory effects of hrHGF on CD4^{+} T cell responses [20]. Lower doses were not as effective.

Cells and cultures
Murine EL-4 lymphoma cells were cultured in DMEM complete medium (CM) supplemented with 10% fetal bovine serum, 50 U/mL penicillin, 50 μg/mL streptomycin and 2 mM L-glutamine (all from Invitrogen/Life Technologies). Splenocytes were derived from Pmel-1 T cell receptor (TCR) transgenic mice, whose TCR recognizes an H-2D^b-restricted epitope corresponding to amino acids 25–33 of murine and human gp100 (gp100$_{25-33}$), a self-tumor antigen. Approximately 90% of splenic CD3^{+} T cells in Pmel-1 TCR mice are TCRVβ13^{+} CD8^{+} T cells and demonstrate specificity for gp100 [28].

Purification of mouse splenic CD8^{+} T cells and CD11c^{+} DCs
Mouse splenic CD8^{+} T cells were negatively selected using an anti-mouse CD8^{+} T cell isolation kit (Miltenyi Biotec). To obtain DCs, spleens were minced and incubated with DNase (1 mg/mL) and Liberase HI (Roche) (0.5 mg/mL) for 15 min at room temperature. Cold EDTA was added to a final concentration of 20 mM, and cell suspensions were incubated for 5 min at room temperature before filtering through nylon mesh to remove tissue and cell aggregates. Highly pure mouse splenic DCs were subsequently positively selected using anti-mouse CD11c colloidal superparamagnetic microbeads (Miltenyi Biotec), as reported previously [20]. The purity of CD8^{+} and CD11c^{+} cells, confirmed by flow cytometry, was routinely >95% and >85%, respectively.

CTL stimulation
Single-cell suspensions of pooled spleens from Pmel-1 mice were prepared by gently homogenizing the tissues and passing them through a 70 μm Nylon cell strainer (Falcon, BD Biosciences). Red blood cells were depleted with ACK lysing buffer (BioWhittaker). Cells were resuspended in DMEM CM with 10 μg/mL gp100$_{25-33}$ peptide (KVPRNQDWL) for 1 h at 37°C. After being

washed and counted, hrHGF (30 ng/mL) was added to the cultures every other day starting on day 0. IL-2 (50 U/mL; PeproTech) was added to cultures at days 2 and 4. In certain experiments, purified DCs were treated or not with hrHGF (30 ng/mL) for 24 h at 37°C. After being washed and counted, 2×10^6/mL DCs were resuspended in DMEM CM with 10 μg/mL gp100$_{25\text{-}33}$ for 1 h at 37°C. Purified Pmel-1 TCR tg CD8$^+$ T cells were mixed with gp100$_{25\text{-}33}$-pulsed autologous DCs at a ratio of 10:1 for 5 days. IL-2 (50 U/mL) was added to cultures at days 2 and day 4. Cells cultured for 5 days were tested for functional and phenotypic assays. To evaluate the role of perforin/granzyme-mediated cytotoxicity, the effector cells were pre-treated with 1000 nM concanamycin A (Sigma-Aldrich) for 2 h prior to co-cultures with target cells.

T cell proliferation assay

For antigen-specific stimulation of mouse CD8$^+$ T cells, highly purified DCs treated or not with hrHGF (1–50 ng/mL) for 24 h at 37°C were cultured with gp100$_{25\text{-}33}$ (10 μg/mL) and naïve Pmel-1 TCR transgenic CD8$^+$ T cells for 5 days. Proliferation was measured by incorporation of ^{3}H-methylthymidine (1 μCi/well) during the last 16 h of culture using a filtermate harvester (Packard Instrument Co.) and a 1450 microbeta liquid scintillation counter (PerkinElmer) and was expressed as counts per minute (cpm).

Immunologic markers and flow cytometry

T cells or DCs were incubated in blocking solution (PBS with 10% FCS) for 20 min on ice prior to staining to block non-specific Fc-mediated interactions, and then stained for 30 min at 4°C with FITC, PE, PerCP-Cy5, or APC fluorochromes conjugated with antibodies (Abs) (1:100) against: CD11c, c-Met, CD8, CD28, CD44, CD62L, CD107a, CTLA-4, Fas-ligand (all Abs from eBioscience), and LFA-1 (Biolegend), or appropriate fluorochrome-conjugated, isotype-matched irrelevant Abs to establish background fluorescence. For intracellular staining of IFN-γ, TNF, perforin, and granzyme B (all Abs from eBioscience), CD8$^+$ T cells were stimulated with PMA (1 μg/mL) plus ionomycin (500 ng/mL) in the presence of GolgiStop (10 μg/mL) (BD), and then fixed and permeabilized using BD Cytofix/Cytoperm Plus Kit (BD Biosciences). CD3ζ (eBioscience) staining was evaluated on fixed and permeabilized cells. Live, apoptotic, and dead populations were defined on the basis of 7-AAD Viability Staining Solution from eBioscience according to the manufacturer's instructions. Samples were processed on a FACS Calibur flow cytometer (Becton Dickinson) and analyzed using FlowJo analysis software (Tree Star, Version 9.3.2).

Cytotoxicity assay

Functional activities of antigen-specific CTLs were analyzed with a DELFIA® cell cytotoxicity kit (PerkinElmer) according to manufacturer's instructions. Briefly, target T-lymphocytic leukemia EL-4 cells were pulsed with 10 μg/mL gp100$_{25\text{-}33}$ for 1 h at 37°C in DMEM CM, and then washed. EL-4 (1×10^5 cells) were labelled with 50 μM of fluorescence-enhancing ligand bis(acetoxymethyl)2,2′:6′,2′′-terpyridine-t,6′′-dicarboxylate (BATDA) for 30 min at 37°C. After washing, 5×10^3/well labelled cells were mixed with antigen-specific CTLs at the indicated ratio in 96-well plates. Plates were incubated for 4 h at 37 °C. A total of 20 μL of supernatant were harvested from each well and added to wells containing 200 μL of 50 μM Europium solution (Aldrich Chemical) in 0.3 M acetic acid (pH 4). Plates were shaken for 15 min at room temperature and the fluorescence of the Europium-TDA (Eu) chelates formed was quantitated in a time-resolved fluorometer (DELFIA 1234). All assays were performed in triplicates. Spontaneous release was determined as Eu detected in the supernatant of targets incubated in the absence of effector cells. Maximum release was determined as Eu detected in the supernatants of target cells incubated with lysis buffer instead of effectors. Percent specific lysis was calculated according to the formula: (experimental release - spontaneous release)/(maximum release - spontaneous release) × 100.
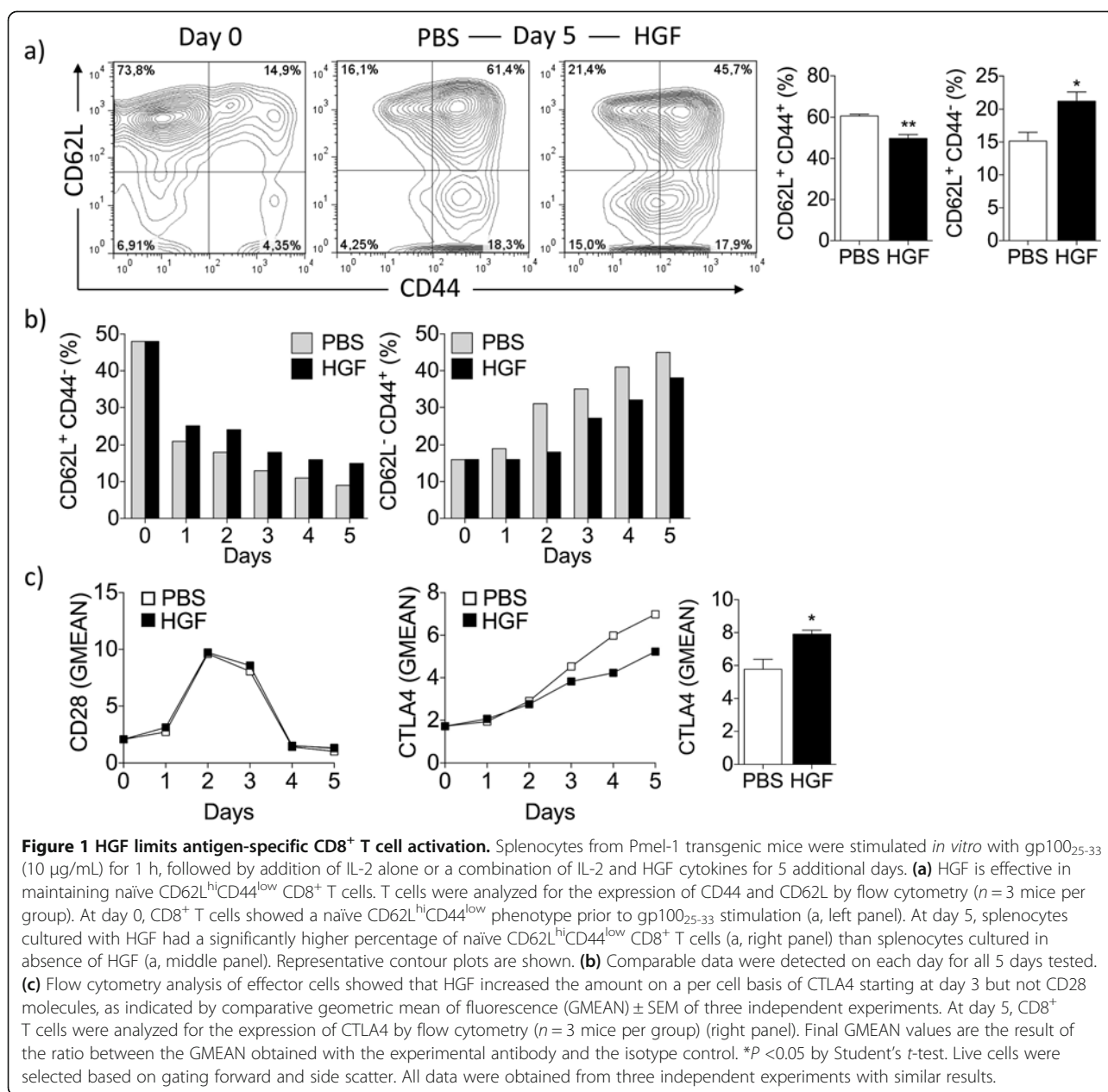
Statistical analysis

Statistical comparisons were done using Student's t-tests. P values <0.05 were considered to be statistically significant. All the statistical analyses were performed by GraphPad Prism for Mac, Version 5.0.

Results

HGF limits effector Ag-specific CTL generation

In order to assess the capacity of HGF to modulate the generation of antigen-specific CD8$^+$ T cells, Pmel-1 TCR transgenic splenocytes were stimulated with gp100$_{25\text{-}33}$ for 1 h, and then cultured with IL-2 alone or in combination with HGF for 5 days. Five days after antigen stimulation with gp100$_{25\text{-}33}$, Pmel-1 splenocyte cultures showed >95% of IL-2 expanded CD8$^+$ T cells (Additional file 1: Figure S1a). As shown by 7AAD staining, a similar percentage of antigen-activated Pmel-1 CD8$^+$ T cells underwent death 5 days after gp100$_{25\text{-}33}$ stimulation when cultured in the absence or presence of HGF (Additional file 1: Figure S1b). These data indicate that HGF has no influence on the viability of the CD8$^+$ T cells during their expansion. At day 0, naïve CD8$^+$ T cells showed a CD62L^{hi}CD44^{low} phenotype prior to gp100$_{25\text{-}33}$ stimulation (Figure 1a, left panel). After 5 days of stimulation, splenocyte cultures receiving HGF maintained a significantly higher percentage of naïve CD62L^{hi}CD44^{low} CD8$^+$ T cells (Figure 1a, right panel) than splenocytes cultured in absence of HGF (Figure 1a, middle

Figure 1 HGF limits antigen-specific CD8+ T cell activation. Splenocytes from Pmel-1 transgenic mice were stimulated *in vitro* with gp100$_{25\text{-}33}$ (10 μg/mL) for 1 h, followed by addition of IL-2 alone or a combination of IL-2 and HGF cytokines for 5 additional days. **(a)** HGF is effective in maintaining naïve CD62L^{hi}CD44^{low} CD8+ T cells. T cells were analyzed for the expression of CD44 and CD62L by flow cytometry ($n = 3$ mice per group). At day 0, CD8+ T cells showed a naïve CD62L^{hi}CD44^{low} phenotype prior to gp100$_{25\text{-}33}$ stimulation (a, left panel). At day 5, splenocytes cultured with HGF had a significantly higher percentage of naïve CD62L^{hi}CD44^{low} CD8+ T cells (a, right panel) than splenocytes cultured in absence of HGF (a, middle panel). Representative contour plots are shown. **(b)** Comparable data were detected on each day for all 5 days tested. **(c)** Flow cytometry analysis of effector cells showed that HGF increased the amount on a per cell basis of CTLA4 starting at day 3 but not CD28 molecules, as indicated by comparative geometric mean of fluorescence (GMEAN) ± SEM of three independent experiments. At day 5, CD8+ T cells were analyzed for the expression of CTLA4 by flow cytometry ($n = 3$ mice per group) (right panel). Final GMEAN values are the result of the ratio between the GMEAN obtained with the experimental antibody and the isotype control. *P <0.05 by Student's *t*-test. Live cells were selected based on gating forward and side scatter. All data were obtained from three independent experiments with similar results.

panel). Augmented naïve CD62L^{high}CD44^{low} phenotype by CD8+ T cells was maintained throughout the entire 5 days of effector generation when incubated with HGF (Figure 1b). These results suggest that HGF may inhibit activation of antigen- and cytokine-stimulated T cells and thus may limit the generation of effector and central memory CD8+ T cells. HGF supplementation did not decrease the expression of T-cell co-stimulatory molecule CD28 on CD8+ T cells (Figure 1c) but was found to upregulate expression of the co-inhibitory receptor cytotoxic T-lymphocyte antigen 4 (CTLA4) by day 3- to 5-cultured CD8+ T cells (Figure 1c), a mechanism likely accounting for the apparent reduced

effector and central memory CD8+ T cell generation over-time.

HGF limits inflammatory cytokine and cytotoxic effector molecule production by activated CD8+ T cells

Since HGF maintained the naïve phenotype of T cells following antigen stimulation, we next examined whether HGF could modulate the effector function of antigen-specific CD8+ T cells. Five days following the initiation of Pmel-1 T-cell cultures, we evaluated the expression of indispensable inflammatory cytokines and content of cytotoxic molecules by T cells cultured in IL-2 alone or in combination with HGF, following antigen-specific

stimulation. As shown in Figure 2a, addition of HGF decreased the expression of IFN-γ, granzyme B, and perforin. Intracellular cytokine staining of antigen-stimulated day 5-cultured CD8^{+} T cells showed that HGF not only decreased the number of T cells producing IFN-γ, granzyme B, and perforin (Figure 2a), but also decreased the amount of IFN-γ, granzyme B, and perforin production on a per cell basis, as indicated by comparative geometric mean fluorescence intensities (GMEAN) (Figure 2b). Similar flow cytometry profiles were observed 4 h following the initiation of T-cell cultures with gp100$_{25-33}$-pulsed EL-4 cells as targets (Additional file 2: Figure S2a, b). This latter effects could not be attributed to changes in cell size, as CD8^{+} T cells cultured in the presence or absence of HGF maintained the same physical cell size as investigated by forward scatter analyses (Additional file 2: Figure S2c).

Treatment with HGF reduces the CD8^{+} cytotoxic-T-lymphocyte response

Five days following the initiation of Pmel-1 T-cell cultures, we evaluated the expression of other CTL-associated effector molecules by T cells cultured in IL-2 alone or in combination with HGF. HGF treatment was associated with reduced content of granzyme B, perforin, and IFN-γ (Figure 3a, b). In addition, HGF also decreased the production of the Th1 cytokine TNF by antigen-specific CD8^{+} T cells. Moreover, HGF reduced

the expression of the death receptor ligand FasL, a non-redundant lytic mechanisms with cytolytic granule release in CTL-mediated killing. Finally, the cell surface molecule lymphocyte function-associated antigen 1 (LFA-1), an important contributor to CTL activation and CTL-mediated direct cell lysis was also significantly reduced. Taken together, these data suggest that HGF significantly reduces both perforin/granzyme B- and Fas-dependent cytotoxicity during an antigen-specific T-cell response. Comparable flow cytometry profiles were detected 4 h following the initiation of T-cell cultures with gp100$_{25-33}$-pulsed EL-4 target cells (Additional file 3: Figure S3a, b). Noticeably, HGF treatment reduced cell surface mobilization of CD107a, a marker commonly used to measure of CTL activity. CD107a is usually found in vesicle membranes, but during CTL-target cell interaction it is mobilized to the cell surface.

HGF restrains cytotoxicity of antigen-specific CD8^{+} cells

Since HGF sustains the naïve activation phenotype of T cells following antigen stimulation and decreases the release of cytolytic granule and pro-inflammatory cytokine mediators and expression of FasL, we next examined whether HGF could modulate the effector function of antigen-specific CD8^{+} T cells. We tested the cytolytic capacity of the cultured T cells using gp100$_{25-33}$-pulsed EL-4 cells as targets. In these experiments, CD8^{+} T cells
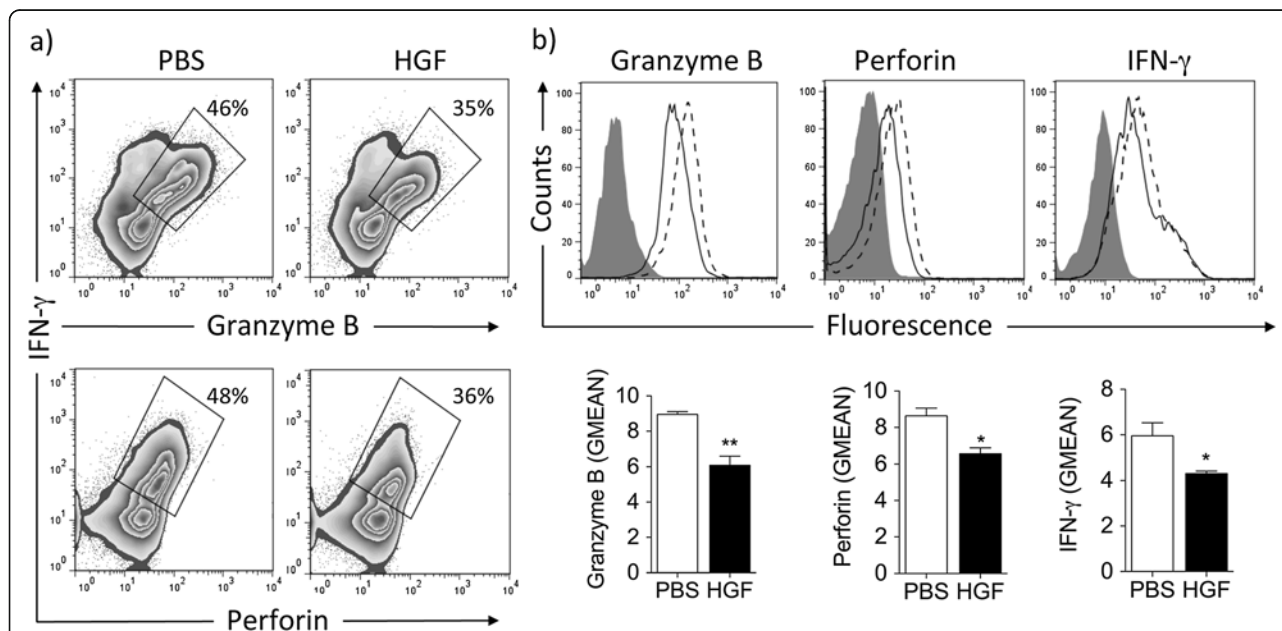

Figure 2 HGF reduces inflammatory cytokine and cytolytic enzyme production by activated CD8^{+} T cells. Pmel-1 splenocytes were cultured for 5 days in IL-2 alone or in combination with HGF, following antigen-specific stimulation. (**a** and **b**) Intracellular cytokine staining of day 5-cultured CD8^{+} T cells showed that addition of HGF not only decreased (**a**) the number of CD8^{+} T cells producing IFN-γ, granzyme B, and perforin but also decreased (**b**) the amount of IFN-γ, granzyme B, and perforin production on a per cell basis, as indicated by comparative geometric mean of fluorescence (GMEAN) ± SEM of three independent experiments. Final GMEAN values are the result of the ratio between the GMEAN obtained with the experimental antibody and the isotype control. Representative contour plots (**a**) and histograms (**b**) are shown. *P <0.05 by Student's *t*-test. All data were obtained from three independent experiments with similar results.
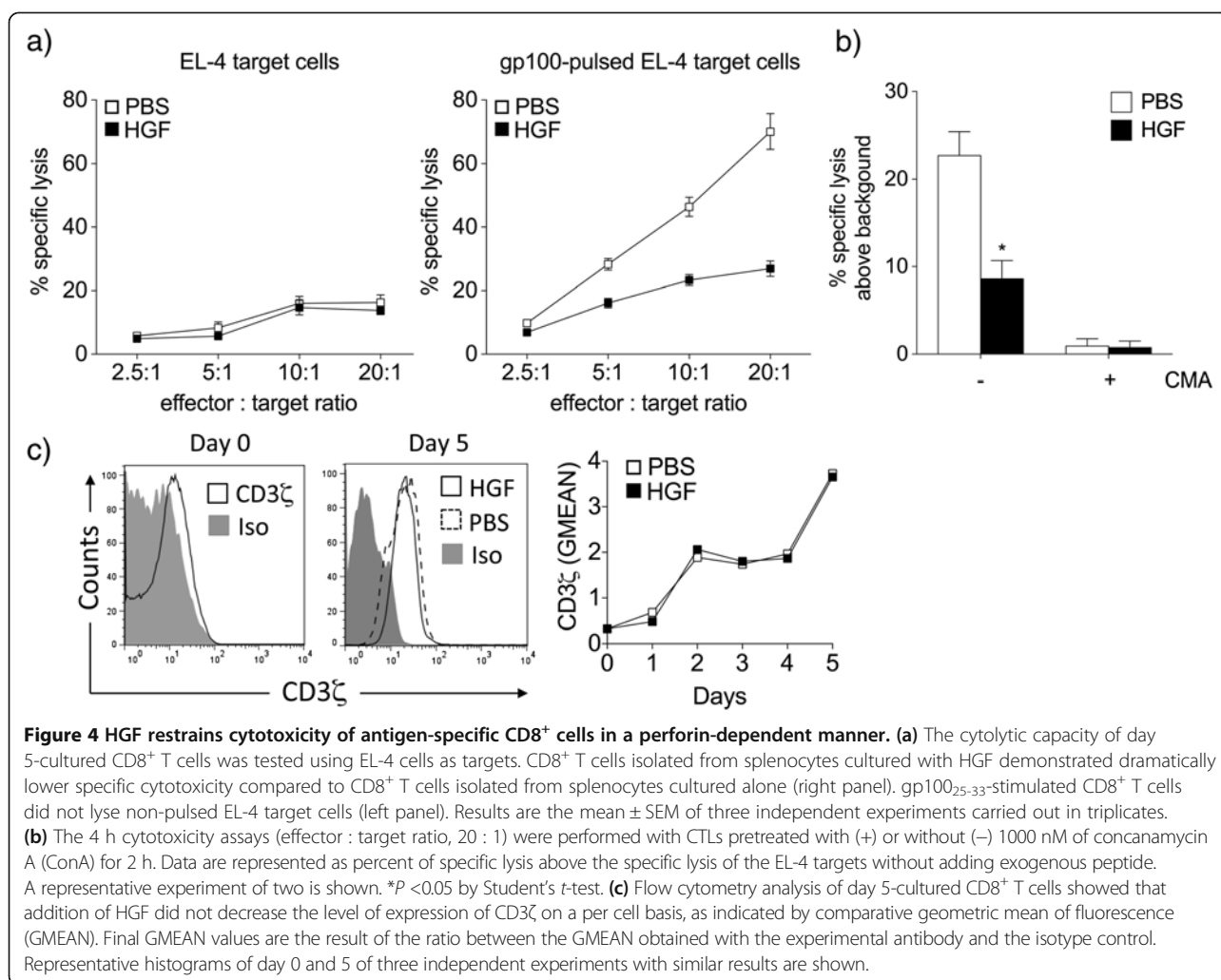
Figure 3 HGF decreases effector molecules by activated CD8$^+$ T cells. Splenocytes from Pmel-1 mice were cultured in IL-2 alone or in combination with HGF for 5 days, following antigen-specific stimulation with gp100$_{25-33}$. **(a and b)** Flow cytometry analysis of effector cells showed that HGF decreased both **(a)** the number of CD8$^+$ T cells expressing TNF, FasL, and LFA-1 and **(b)** the amount on a per cell basis of these molecules that play an important role in CTL cytotoxicity, as indicated by comparative geometric mean of fluorescence (GMEAN) ± SEM of three independent experiments. Final GMEAN values are the result of the ratio between the GMEAN obtained with the experimental antibody and the isotype control. Representative contour plots **(a)** and histograms **(b)** are shown. *$P < 0.05$ by Student's t-test. All data were obtained from three independent experiments with similar results.
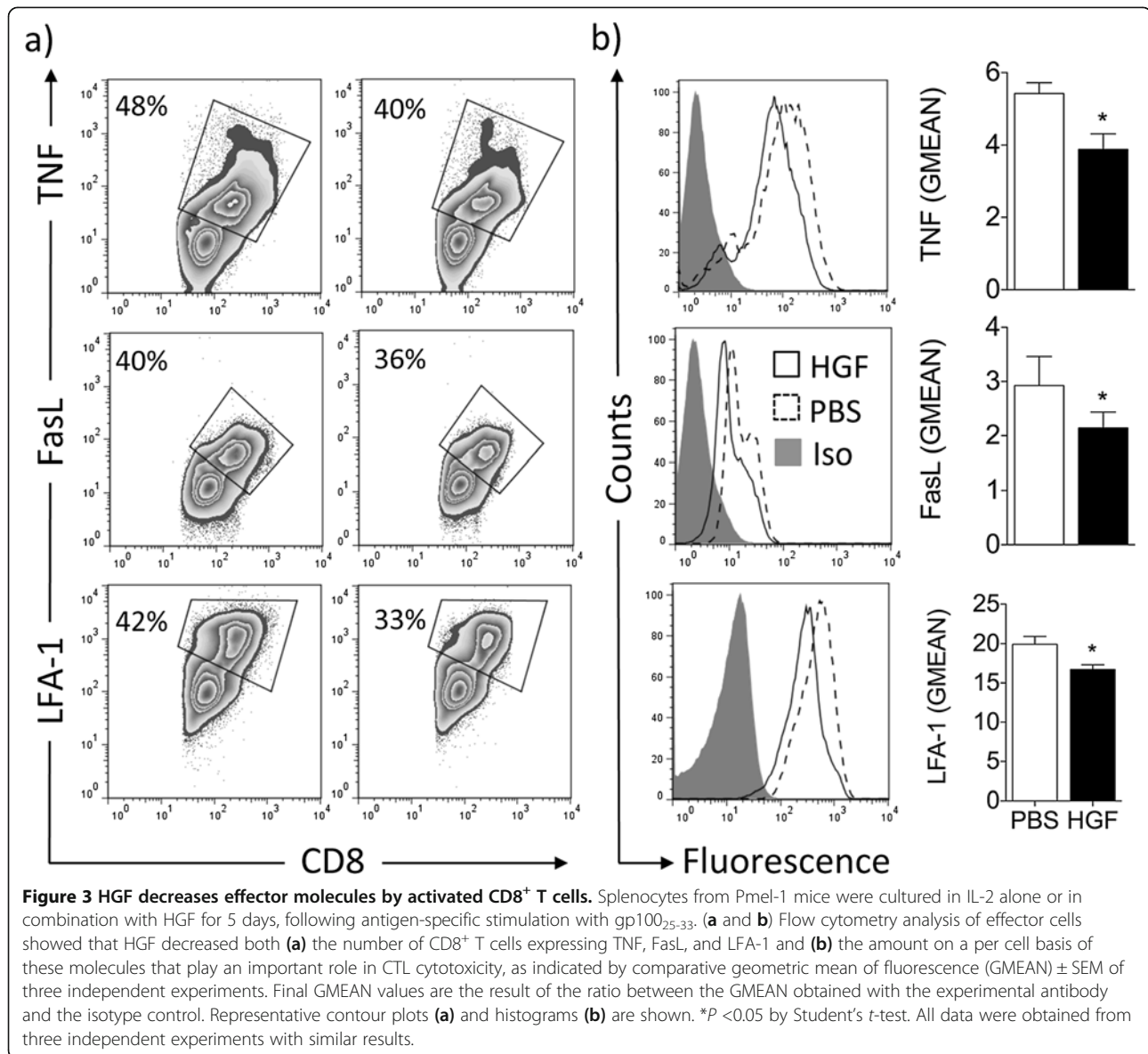
cultured in HGF demonstrated dramatically reduced specific cytotoxicity compared with CD8$^+$ T cells cultured alone (Figure 4a). This decreased cytolytic capacity of CD8$^+$ T cells could be explained by a difference in perforin, granzyme B, or LFA-1 expression, as HGF significantly affected expression of all molecules (Figures 2 and 3). We found that concanamycin A, an inhibitor of the perforin-based cytotoxic pathway, almost completely abolished target cell destruction by CTLs (Figure 4b), stressing the importance of this pathway for immediate lytic function mediated by Pmel-1 CD8$^+$ T cells. HGF pretreatment did not, however, further reduce the residual killing observed using concanamycin A-treated CTLs. These results argue that HGF mainly limits the cytotoxic, anti-tumor function of Pmel-1 CD8$^+$ T cells in

a perforin-dependent manner in our experimental settings. Impaired CTL killing was further not associated with changes in CD3ζ expression, a crucial molecule for T-cell activation upon antigen recognition (Figure 4c).

HGF limits CTL responses by modulating APC functions

We previously showed that dendritic cells (DCs), required for initiating CTL responses [29], are vulnerable to HGF treatment [20], which can contribute to diminished CTL responses. To address whether this potential mechanism may account for reduced priming of antigen-specific CD8$^+$ T cell responses from Pmel-1 splenocyte cultures, we assessed the effect of HGF on the ability of antigen donor DCs cells to prime naïve antigen-specific CD8$^+$ T cells. Highly purified CD11c$^+$ DCs were cultured for 24 h

Figure 4 HGF restrains cytotoxicity of antigen-specific CD8+ cells in a perforin-dependent manner. (a) The cytolytic capacity of day 5-cultured CD8+ T cells was tested using EL-4 cells as targets. CD8+ T cells isolated from splenocytes cultured with HGF demonstrated dramatically lower specific cytotoxicity compared to CD8+ T cells isolated from splenocytes cultured alone (right panel). gp100$_{25-33}$-stimulated CD8+ T cells did not lyse non-pulsed EL-4 target cells (left panel). Results are the mean ± SEM of three independent experiments carried out in triplicates. **(b)** The 4 h cytotoxicity assays (effector : target ratio, 20 : 1) were performed with CTLs pretreated with (+) or without (−) 1000 nM of concanamycin A (ConA) for 2 h. Data are represented as percent of specific lysis above the specific lysis of the EL-4 targets without adding exogenous peptide. A representative experiment of two is shown. *P <0.05 by Student's *t*-test. **(c)** Flow cytometry analysis of day 5-cultured CD8+ T cells showed that addition of HGF did not decrease the level of expression of CD3ζ on a per cell basis, as indicated by comparative geometric mean of fluorescence (GMEAN). Final GMEAN values are the result of the ratio between the GMEAN obtained with the experimental antibody and the isotype control. Representative histograms of day 0 and 5 of three independent experiments with similar results are shown.

with either HGF or vehicle and then co-cultured with purified Pmel-1 CD8+ T cells in the presence of IL-2 and gp100$_{25-33}$ for 5 days. In contrast to DCs, CD8+ T cells did not express the HGF receptor c-Met (Additional file 4: Figure S4). At day 5, CD8+ T cell measurement of [3H] thymidine incorporation by these cells showed that HGF-treated DCs induced low levels of CD8+ T-cell proliferation when compared to those obtained with untreated DCs (Figure 5a). We tested the cytolytic capacity of the CD8+ T cells co-cultured in the presence of HGF- or vehicle-treated DCs using gp100$_{25-33}$-pulsed EL-4 target cells. As shown in Figure 5b, CD8+ T cells co-cultured with HGF-treated DCs demonstrated dramatically lower specific cytotoxicity compared to T cells co-cultured with control DCs. As expected, CTLs generated in the presence of HGF-treated DCS were associated with reduced expression of cytolytic markers, such as perforin, granzyme B, and FasL. Altogether, these results indicate that HGF acts directly on DCs by reducing their ability to both generate and prime antigen-specific CD8+ T cell responses.

Discussion

HGF is a multifunctional cytokine that blunts inflammation in a variety of inflammatory T-cell-mediated disease models, suggesting that HGF suppresses a common inflammatory process. In MOG-induced EAE, a common model of MS primarily mediated by encephalitogenic CD4+ T cell responses and characterized by demyelination and axonal loss [30], we have previously demonstrated that overexpression of neuronal HGF attenuated disease progression in part via anti-inflammatory signals [20]. Using this MS model, we established that HGF exerts an anti-inflammatory effect through the generation of tolerogenic DCs and the subsequent suppression of autoreactive peripheral Th1 and Th17 cells, leading to reduced CD4+ T cell-mediated CNS injury. Whether HGF modulates cell-mediated immunity driven by MHC class I-restricted CD8+ T cells remained unknown.

In addition to pathogenic CD4+ T cells, multiple observations support the idea that CD8+ T cells are involved in pathogenesis of CNS autoimmunity, as active
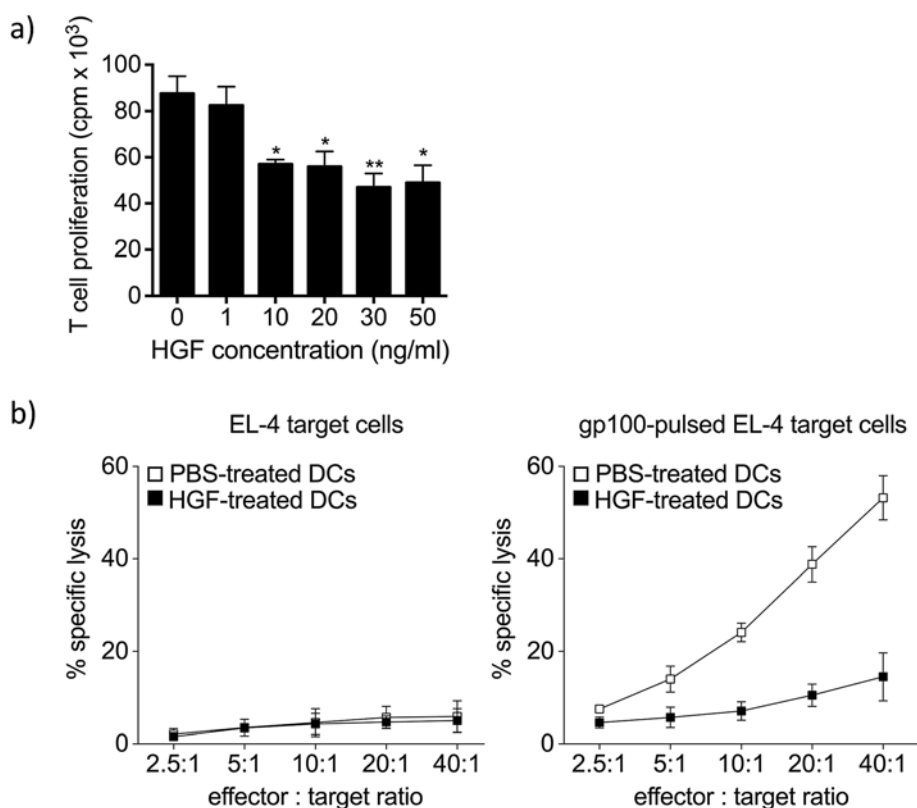
Figure 5 HGF limits CTL responses by modulating APC functions. Highly purified CD11c^{+} DCs were cultured for 24 h with either HGF or vehicle and then co-cultured with purified Pmel-1 CD8^{+} T cells in the presence of IL-2 and gp100$_{25-33}$ for 5 days. **(a)** HGF-treated DCs induced low levels of CD8^{+} T-cell proliferation, as measured at day 5 by [^{3}H] thymidine incorporation. Values are expressed as counts per minute (cpm). Data are representative of three independent experiments with similar results. *P <0.05; **P <0.01 by Student's t-test. **(b)** CD8^{+} T cells co-cultured with HGF-treated DCs demonstrated dramatically reduced specific cytotoxicity compared with T cells co-cultured with control PBS-treated DCs (right panel). gp100$_{25-33}$-stimulated CD8^{+} T cells did not lyse non-pulsed EL-4 target cells (left panel). Results are the mean ± SEM of three independent experiments carried out in triplicates.

contributors to the development of neuroinflammation. In MS, CD8^{+} T cells outnumber by far CD4^{+} T cells in both acute and chronic inflammatory lesions. In addition, while CD4^{+} T cells show a primarily perivascular distribution, CD8^{+} T cells can be detected in the parenchyma [31,32]. Although normally poorly expressed, MHC class I molecules are highly expressed within the MS lesion on astrocytes, oligodendrocytes, and neurons, suggesting that CD8^{+} T cells could be directly engaging these cell types [33-36]. Using an established model of murine CTL-mediated killing we have here examined whether HGF could regulate autoaggressive, cytotoxic CD8^{+} T cell responses.

In this study, we found that HGF treatment of DCs reduced the generation and functions of cytotoxic effector CD8^{+} T lymphocytes and subsequent MHC class I-restricted CTL-mediated cytolysis of target cells. The development of naïve cytotoxic CD8^{+} T cells into CTLs requires specific recognition of antigen:MHC class I complexes on professional APCs in conjunction with co-stimulatory signals. Secondary recognition of antigen: MHC class I complexes on a target cell by a CTL leads to the death of the target cell. Our findings indicate that HGF treatment interfered with the development of autoaggressive CTLs and not their capacity to recognize their target cells. In particular, we found that HGF treatment increased the levels of the inhibitory counter-receptor CTLA4 molecules expressed on CD8^{+} T cells but did not affect the expression of the CD3ζ molecules. CTLA4-mediated negative co-stimulation together with other regulatory mechanisms mediated by tolerogenic APCs likely accounts for maintenance of high frequencies of naïve CTL precursors incapable of cytotoxicity in splenocyte cultures supplemented with HGF.

CTLs mediate the killing of target cells via two major pathways, a granule-dependent (perforin/granzyme B) and independent (FasL induced cell death) mechanism. Here we found that HGF treatment decreased the levels of the effector CTL molecules IFN-γ, TNF, perforin, and granzyme B as well as the expression of CD107a, a

marker of CD8^{+} T-cell degranulation following stimulation. Using a potent inhibitor of the perforin-based cytotoxic pathway, concanamycin A, we found that HGF potently inhibits CTL-mediated killing through interference with the granule exocytosis pathway. Our data further revealed that HGF treatment reduced CTL-bound FasL expression on CD8^{+} T cells, suggestive of an action of HGF on the dual perforin/granzyme B- and Fas-based CTL-mediated cytotoxicity. As both the perforin/granzyme B-dependent granule exocytosis pathway [37-41] and the Fas signaling [42,43] have been implicated as potential mechanisms in oligodendrocyte and/or axonal injury and demyelination in MS, our findings taken together suggest that HGF might be effective in a potential therapeutic approach to reduce CTL effector function in CTL-mediated human autoimmune disorder of the CNS.

Conclusions

Altogether, our findings indicate that HGF treatment limits both the generation and effector functions of CTLs. Complementary to its impact on CD4^{+} T-cell CNS autoimmunity, our findings further suggest that HGF treatment could be exploited to control CD8^{+} T-cell-mediated, MHC I-restricted autoimmune dysfunctions such as MS. By coupling immunosuppressive properties on both CD4^{+} and CD8^{+} T cell effector responses and neurorepair actions, HGF appears thus to be a promising candidate for the treatment of inflammatory demyelinating neurodegenerative diseases such as MS. One must, however, point out that such observations are preliminary, and do not establish the safety of HGF administration over the long term, which may include potential adverse events. In particular, additional research is warranted to evaluate the impact of HGF therapy in anti-tumor immunity as the potent immune inhibition exerted by HGF may help tumor cells to escape from immune surveillance.

Additional files

Additional file 1: Figure S1. HGF does not affect *in vitro*-expanded CD8^{+} T cell viability. (**a**) Five days after stimulation with gp100$_{25-33}$, Pmel-1 splenocyte cultures showed >95% of IL-2 expanded CD8^{+} T cells. (**b**) A similar percentage of antigen-activated Pmel-1 CD8^{+} T cells underwent death 5 days after gp100$_{25-33}$ stimulation when cultured in the absence or presence of HGF, as shown by 7AAD staining. Representative contour plots are shown. All data were obtained from three independent experiments with similar results.

Additional file 2: Figure S2. HGF restrains CTL effector molecule expression. *In vitro*-expanded CD8^{+} T cell were cultured for 4 h with gp100$_{25-33}$-pulsed EL-4 target cells. (**a** and **b**) Intracellular cytokine staining of CD8^{+} T cells showed that addition of HGF to Pmel-1 splenocyte cultures not only decreased (**a**) the number of CD8^{+} T cells producing IFN-γ, granzyme B, and perforin but also decreased (**b**) the amount of IFN-γ, granzyme B, and perforin production on a per cell basis, as indicated by comparative geometric mean of fluorescence. (**c**) Forward scatter analyses of CD8^{+} T cells indicate that HGF supplementation did

not affect cell size. Data are presented overlapping the control analysis. Representative contour plots (**a**) and histograms (**b, c**) are shown. All data were obtained from three independent experiments with similar results.

Additional file 3: Figure S3. HGF dampens effector molecules by activated CD8^{+} T cells. *In vitro*-expanded CD8^{+} T cell were cultured for 4 h with gp100$_{25-33}$-pulsed EL-4 target cells. (**a** and **b**) Flow cytometry analysis of effector cells showed that addition of HGF to Pmel-1 splenocyte cultures decreased both (**a**) the number of CD8^{+} T cells expressing CD107, TNF, FasL, and LFA-1 and (**b**) the amount on a per cell basis of these molecules that play an important role in CTL cytotoxicity, as indicated by comparative geometric mean of fluorescence. Representative contour plots (**a**) and histograms (**b**) are shown. All data were obtained from three independent experiments with similar results.

Additional file 4: Figure S4. DCs but not CD8^{+} T cells show cell-surface expression of the HGF receptor c-Met. Expression of c-Met at the cell surface of CD11c^{+} DCs and CD8^{+} T cells was examined by flow cytometry. Illustrative histograms depict the expression of c-Met (open histograms) and control staining with isotype-matched antibody (shaded histograms).

Authors' contributions
MB, NM, and PHL designed research, analyzed data, and wrote the paper; PW analyzed data, gave conceptual advice, and discussed the results at all stages; MB and GS performed the experiments; PW provided the Pmel-1 mice; and PHL supervised the study. All authors read, commented, and approved the final manuscript.

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