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Enhancement of immunoregulation that results in limiting autoimmune diseases is an important problem for clinical medicine. Multiple sclerosis (MS) is a multifactorial autoimmune disease characterized by destruction of myelin and axons within the CNS that is thought to have an autoimmune component. Several defects in immune regulatory mechanisms have been proposed to contribute to the pathogenesis of MS, including a potential role for NK cells, because a high correlation have been proposed to contribute to the pathogenesis of MS, including a potential role for NK cells, because a high correlation

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Materials and Methods

Animals
Female SJL/J mice and C57BL/6 mice were obtained from The Jackson Laboratory, and all animal procedures were approved by the Animal Care and Use Committee of the Medical University of Lodz. Mice were 6–8 wk old when sensitized for EAE.

Reagents
The following Abs were used: anti-Hsp70/hsc70 mouse mAb (StressGen) and anti-Hsp70 mouse mAb and anti-mouse IgG-HRP secondary Ab conjugated with HRP (Santa Cruz Biotechnology). Functional grade purified rat anti-mouse NGK2D Ab (eBioscience) was used for blocking experiments. Mouse Ab: R-PE-conjugated rat anti-mouse CD49b/Pan-NK mAb (DX5 Ab), R-PE-conjugated mAb anti-CD3, PerCP-conjugated anti-CD4, and anti-CD8 mAb, FITC-labeled anti-mouse CD11c, rat anti-mouse H60, rat anti-mouse NGK2D, goat anti-mouse H60, biotin-conjugated anti-rat IgG2a and IgG1, streptavidin-PE (SA-PE) conjugate and appropriate isotype controls, as well as annexin VPE-conjugated and 7-aminoactinomycin (7-AAD) were purchased from BD Pharmingen. 5-(and-6)-carboxyfluorescein diacetate-CEFA-CFSE (CFSE) was purchased from Invitrogen Life Technologies. Proteolipid protein (PLP)139–151 (HSLGKWLGHPDKF) and myelin oligodendrocyte glycoprotein (MOG)35–55 (MEVGWYRSPF SRVHVLRYNRK) were synthesized by the Albert Einstein College of Medicine Sequencing Facility (Bronx, NY). PD10-columns, ADP-, and ATP-agarose were purchased from Sigma-Aldrich, purified protein deriv.

Purification of Hsp70 preparations
Hsp70-peptide complexes (Hsp70-pc) and pure Hsp70 were obtained from CNS tissues of normal mice and mice sensitized for EAE 14 days previously. Hsp70 preparations were isolated and purified according to previously published protocols (21, 22). In brief, CNS tissue was homogenized, centrifuged, and loaded on PD10-column. After these steps the eluant was loaded onto an ADP-agarose column (Hsp70-pc preparations) or ATP-agarose column (Hsp70 alone preparations) then unbound protein was removed and bound material was eluted with buffer D containing 3 mM ADP or 3 mM ATP, respectively. The presence of Hsp70 in the fraction after purification was confirmed by standard SDS-PAGE and immunoblotting with monoclonal anti-Hsp70 Abs. Hsp70 preparations were pooled, concentrated, dissolved in PBS and quantified using a Bradford assay (Bio-Rad).

Proteins were separated by SDS-PAGE according to the Laemmli protocol (23), followed by Coomassie brilliant blue staining or Western blot analysis. Proteins were transferred onto PVDF membranes (Millipore), then blocked in TTBS buffer containing 5% nonfat milk, followed by incubation with primary Ab and then with HRP-conjugated secondary Ab. The blots were developed with chemiluminescence (ECL plus; Amersham Biosciences), according to the manufacturer’s protocol. These procedures were used for assessment of purity and the amount of Hsp70 preparations (data not shown).

The ADP-purified Hsp70-pc isolated from CNS of SJL/J or C57BL/6 mice with EAE were incubated with 10 mM ATP and 3 mM MgCl2 at room temperature for 1 h. The samples of Hsp70-pc preparations were resolved on ready precipitate minipolyacrylamide 10–20% gradient gels with Tris-Tricine and stained with Coomassie brilliant blue (Sigma-Aldrich).

EAE induction
Six-week-old SJL/J or C57BL/6 female mice were injected with Hsp70-pc or Hsp70 preparations at dose 1.2 mg/kg, subcutaneously on day 0 and day 7, as described previously (24). On day 14, EAE was induced with PLP139–151. Doses of neutralization Abs were previously defined (24) and confirmed in current experiments. Mice were observed daily and scored for clinical expression of disease as described above.

T cell proliferation assay
Spleen cells (SC) were isolated from mice preinjected with Hsp70 preparations or control EAE animals on day 14. SC were stimulated with 50 μg/ml PLP139–151, or 60 μg/ml PPD and then labeled with [3H]Thymidine (Amersham Biosciences). The uptake was measured by liquid scintillation beta counter (Pharmacia).

ELISA
SC production of IFN-γ was assessed by ELISA (BD Pharmingen). SC were isolated from mice on day 14 after induction of EAE, stimulated with 50 μg/ml PLP139–151. The supernatants were collected after 72 h, frozen at −80 °C and later used for ELISA, according to the manufacturer’s instructions.

Magnetic beads cell sorting
NK cells were negatively selected from SC isolated from mice preinjected with Hsp70-pc or Hsp70 by depletion of non-NK cells using an NK Cell Isolation Kit (Miltenyi Biotec). The NK Cell Isolation Kit enables isolation untouched pure NK cells by removing of magnetic labeled T cells, B cells, granulocytes macrophages, DC, and erythroid cells on magnetic sorter. The efficiency of cell depletion was >90% assessed by staining of isolated cells with NK marker DX5 and flow cytometry analysis using FACS Calibur (BD Biosciences).

In vitro cell cultures
On day 15, SC from mice injected with Hsp70-pc or Hsp70 were isolated and then incubated with blocking anti-mouse NGK2D Ab for 30 min. Next, these preblocked cells were added to PLP-reactive SC isolated from EAE mice at a ratio of 1:4, and simultaneously stimulated with PLP139–151 and cultered for 72 h. To assess the role of NK cells, NK cells isolated from mice injected with Hsp70-pc or Hsp70 preparations were added to PLP-reactive SC isolated from EAE mice, at a ratio of 1:4 and 1:2, and cultured for 72 h. After that, proliferation assays were performed as described above.

NK cells were isolated from SC of mice injected with Hsp70-pc and added to DC isolated from SC of EAE mice, at cell ratio 1:5, 1:1, and 5:1, and then cultured for 3 and 6 days. After that DCs were positively sorted from the coculture cell mixture as described above. These DCs were added to SC isolated from EAE mice, at ratio 1:10 and 1:20, stimulated with PLP139–151 and cultered for 72 h.

In vivo cell transfer
SC or NK cells isolated from mice injected with Hsp70-pc or Hsp70 on day 14 postimmunization were suspended in sterile PBS. A total of 10 × 10⁶ SC or 3 × 10⁶ NK cells were injected into the tail vein of mice on the day of sensitization for EAE with PLP139–151. Mice were observed daily and scored for clinical expression of disease as described above.

Cell death assessment
Two different methods were used to assess cell death. The first one was based on fractional DNA content in the cells and flow cytometry analysis as described previously (25). In brief, spleen cells were collected, spun down, fixed in 70% ethanol, washed, resuspended in DNA staining solution containing propidium iodide, and DNase-free RNase A (Sigma-Aldrich), and incubated for 30 min at room temperature. After that cells were assessed by flow cytometry and DNA content measured in FL2 fluorescence. The second method was used to assess Ag-specific T cell death. For this procedure, SC isolated from EAE mice were first labeled with CFDA-SE (to assess T cells responding to PLP139–151), then stimulated with PLP139–151 and cultured for 3, 4, and 5 days. For CFDA-SE staining, cells were washed and centrifuged twice and then resuspended in 5 μmol/L solution and incubated for 4 min at room temperature. Reaction was stopped by adding cold culture medium and washed twice. Cells were resuspended in culture medium, counted, stimulated with PLP139–151 and used for additional experiments. To assess Ag reactive SC death, SC labeled with CFDA-SE were stained with annexin V conjugated to PE (to detect exposure of phosphatidylserine) and 7-AAD (to detect permeable dead cells) and assessed by flow cytometry. T
cells responding to PLP_{139–151}, detected by CFDA-SE staining, were gated and annexinV-PE and 7-AAD positive cells were counted. To further confirm that CFDA-SE gated cells are CD4 T cells, staining with anti-CD4 conjugated to PE Ab and 7-AAD and flow cytometry analysis were performed.

Flow cytometry

For analysis of surface expression of H60 on SC, SC were isolated from SJL/J and C57BL/6 mice preinjected with Hsp70-pc or Hsp70 or control mice (nonpreinjected with protein preparations) and stained with rat anti-mouse H60 Ab, biotin-conjugated anti-rat IgG2a Ab and streptavidin-PE (SA-PE) conjugate. To define cell populations expressing H60, costaining with anti-CD3 mAb was also performed. For analysis of NKG2D expression, SC were isolated from mice preinjected with Hsp70-pc or Hsp70 or control mice (nonpreinjected with protein preparations) and stained with rat anti-mouse NKG2D Ab, biotin-conjugated anti-rat IgG1 Ab, and with streptavidin-PE (SA-PE) conjugate.

CD4, CD25, and Foxp3 staining was performed as described in manufacture’s protocol (eBioscence). In brief, SC isolated from EAE mice stimulated with PLP_{139–151}, were cultured for three days. Cells were centrifuged and stained with anti-CD4 and anti-CD25. After cell surface staining cells were fixed, permeabilized and stained for forkhead box P3 (Foxp3) expression. Flow cytometry analysis of CD4^{+}, CD25^{+}, and Foxp3^{+} cells was performed. All performed stainings were analyzed with CellQuest software (BD).

Statistical methods

Data are expressed as means with SD. For multiple comparison measures, Student’s t test and two-way ANOVA were applied.

Results

Hsp70-pc prevents clinical EAE in SJL/J but not C57BL/6 mice

In SJL/J mice Hsp70-pc isolated from brains of mice with EAE, contrary to pure Hsp70 or Hsp70-pc derived from brains of healthy mice, prevented the development of EAE clinically and pathologically when administered before PLP_{139–151} immunization, \( p < 0.001 \). In contrast, in C57BL/6 mice preinjected with Hsp70-pc isolated from EAE brain and sensitized for EAE with MOG_{35–55}, we were repeatedly unable to suppress the development of EAE (Fig. 1A). These results indicate that C57BL/6 mice were resistant to the Hsp70-pc-induced inhibition of EAE. In SJL/J mice inhibition of EAE induced with Hsp70-pc preinjection correlated with decreased PLP-induced proliferation of spleen cells, \( p < 0.001 \) (Fig. 1B) in contrary to lack of inhibition of MOG reactive cells observed in C57BL/6 mice (data not shown).

To address Ag specificity of Hsp70-pc-induced tolerance in SJL/J mice, proliferation of SC was assessed in response to another Ag, PPD, present in CFA used for EAE sensitization. In SJL/J mice preinjected with Hsp70-pc and sensitized for EAE, PPD-induced proliferation was also diminished - \( p < 0.001 \) (Fig. 1B) in contrary to lack of inhibition of MOG reactive cells observed in C57BL/6 mice (data not shown).

Hsp70-pc induces expression of H60 in SJL/J but not C57BL/6 mice

The mechanism of Hsp70-pc-induced inhibition of EAE in SJL/J mice was dependent on NK cell function and the protection of EAE was transferred with NK cells from Hsp70-pc preinjected mice (21). Therefore, we decided to search for NK cell activating ligands that could potentially stimulate NK cells in Hsp70-pc preinjected mice. To do this, we assessed expression in tissues from SJL/J and C57BL/6 strains of the MHC class I-related proteins, RAE-1 and H60, which have been shown to activate the NKG2D receptor in mice (15). Using Western blot, RAE-1 expression of
only low level was detected on SC from either strain, and neither Hsp70-pc nor Hsp70 induced its expression (data not shown). However, H60 expression was readily detected on SC from SJL/J mice but not on SC from C57BL/6 mice (Fig. 2). More interestingly, in SJL/J mice preinjected with Hsp70-pc, but not with Hsp70, the expression of H60 was up-regulated by eightfold (Fig. 2). However, Hsp70-pc did not induce expression of H60 in C57BL/6 mice (Fig. 2). Our results correspond to published data on the lack of H60 expression in C57BL/6 mice (15, 17). These results suggest that the failure of C57BL/6 mice to develop Hsp70-pc-induced tolerance to EAE might result from a natural and genetically determined deficiency of H60 expression in this strain.

Hsp70-pc EAE inhibition in SJL/J mice depends on H60 expression

To investigate a role of the H60 ligand in induction of Hsp70-pc-induced EAE tolerance, we performed experiments in which H60 was blocked in vivo. Mice preinjected with Hsp70-pc, received anti-mouse H60 Ab i.v. on the day of immunization with PLP139-151. Control mice were injected with the same amount of normal mouse IgG. The results showed reduced expression of H60 on spleen cells (Fig. 3A) and that blockade of H60 in SJL/J mice resulted in loss of the Hsp70-pc-induced protection for EAE, \( p < 0.001 \) (Fig. 3B). In SC isolated from EAE mice expression of H60 was unchanged comparing to naive mice (data not shown). These results suggest that the H60 ligand plays a critical role in Hsp70-pc-induced inhibition of EAE.

To determine the effect of H60 neutralization on reactivity to PLP139-151, we assessed proliferation of SC isolated from mice that had been preinjected with Hsp70-pc, and then received i.v. a blocking Ab to H60 at the time of immunization with PLP139-151. Again, control mice were injected with normal mouse IgG. SC were isolated from mice at 14 day postimmunization and stimulated in vitro with 50 \( \mu \)g/ml PLP139-151 for 96 h. The data showed that proliferative responses to PLP139-151 of SC from mice preinjected with Hsp70-pc was restored by H60 neutralization with anti-H60 Ab, \( p < 0.01 \) (Fig. 3C).
Hsp70-pc-induced inhibition of EAE depends on NKG2D stimulation

H60 is one of the ligands for NKG2D, a stimulatory receptor on NK cells. Therefore, the role of the NKG2D receptor in Hsp70-pc-induced EAE inhibition was assessed by usage of neutralizing Ab to NKG2D, CX5. This mAb blocks binding of NKG2D to its ligands and modulates the NKG2D receptor from the surface of NK cells, but does not deplete NK cells (26). Thus, this experiment allowed us to directly examine the role of the NKG2D receptor in the mechanism of Hsp70-pc-induced EAE inhibition. Mice preinjected with Hsp70-pc, were injected i.v. with the neutralizing anti-NKG2D (CX5) mAb, or a control rat IgG, on the day of immunization with PLP139–151. As expected, mice treated with the CX5 mAb showed reduced expression of the NKG2D receptor on spleen cells (Fig. 4A). Most importantly, mice preinjected with Hsp70-pc and then treated with the blocking NKG2D mAb showed reversion of the Hsp70-pc-induced EAE inhibition, p < 0.001, whereas mice treated with a control rat mAb did not (Fig. 4B). In SC isolated from EAE mice expression of NKG2D was unchanged comparing to naive mice (data not shown).

To determine the effect of NKG2D neutralization on the reactivity to PLP, we assessed the proliferation of SC isolated from mice that had been preinjected with Hsp70-pc and subsequently given anti-NKG2D mAb on the day of immunization with PLP139–151. SC were isolated from mice at 14 day post-PLP immunization and cultured in vitro with 50 μg/ml PLP139–151 for 96 h. The data showed that the reduced responsiveness of SC from mice preinjected with Hsp70-pc to PLP139–151 was restored by NKG2D neutralization with CX5 mAb, p < 0.05 (Fig. 4C).

NKG2D and H60 blockage prevents Hsp70-pc-induced IFN-γ production

We also measured IFN-γ production by SC isolated 14 days after PLP immunization from mice preinjected with Hsp70-pc and treated with neutralizing anti-NKG2D (CX5) or anti-mouse H60 Ab. We detected significantly lower production of IFN-γ in mice preinjected with Hsp70-pc and treated with anti-NKG2D mAb, p < 0.001 or anti-mouse H60 Ab, p < 0.001, than in mice injected with a control rat Ab (Fig. 5). These results confirmed the role of IFN-γ in Hsp70-pc-induced EAE inhibition (21), as well as the instrumental role of H60 and NKG2D in Hsp70-pc-induced IFN-γ secretion.

Neutralization of NKG2D prevents the suppression of PLP-induced proliferation by Hsp70-pc reactive cells

To further assess the mechanism of NK cell-mediated Hsp70-pc-induced tolerance to EAE induction, we investigated the potency of NK cells from mice immunized with Hsp70-pc to influence the reactivity to PLP of SC from EAE animals. For this, we performed experiments in which PLP-reactive SC from EAE mice were cocultured with NK cells from Hsp70-pc or Hsp70-immunized mice, as well as SC from mice that had been pretreated with Hsp70-pc. NK cells from Hsp70-pc, but not Hsp70 preinjected mice, reduced PLP139–151-induced proliferation of SC from EAE mice in a dose-dependent manner, p < 0.01, more effectively than SC from mice pretreated with Hsp70-pc, p < 0.05 (Fig. 6A). The results of these in vitro coculture experiments on NK and SC from

FIGURE 4. Anti-NKG2D mAb reversed Hsp70-pc-induced inhibition of EAE. Mice preinjected twice with Hsp70-pc were injected i.v. with an anti-mouse NKG2D Ab on the day of PLP139–151 immunization. A, Injection with the anti-NKG2D Ab led to a reduction of NKG2D surface expression as assessed by flow cytometry. B, Blocking of NKG2D in vivo abolished Hsp70-pc induced inhibition of EAE. Each point represents the mean of the clinical scores obtained from five to eight animals in each group in three to six experiments. SD was <15%. C, NKG2D blocking in vivo with anti-NKG2D Ab resulted in the restoration of PLP reactivity in vitro as assessed by proliferation assays. Bars, Mean ± SD from three experiments and five to eight animals per group.

FIGURE 5. Hsp70-pc-induced high IFN-γ production was abolished with anti-H60 Ab or anti-NKG2D (CX5) mAb treatment. Mice preinjected twice with Hsp70-pc were given anti-mouse H60 Ab or anti-mouse NKG2D Ab on the day of PLP139–151 immunization. SC were isolated, cultured, restimulated with PLP139–151 and supernatants collected after 72 h. IFN-γ production was measured by ELISA. Bars, Mean ± SD from three experiments and five to eight animals per group.
EAE animals corresponded with in vivo experiments when transfer of NK cells from Hsp70-pc injected mice, but not Hsp70-injected mice, prevented development of EAE in mice sensitized with PLP139–151, p < 0.001 (Fig. 6B).

Next, we assessed the role of NKG2D receptors in the NK cell-induced reduction in PLP reactivity. For this, SC from Hsp70-pc, or Hsp70-immunized mice, were first incubated with neutralizing anti-NKG2D mAb and then added to SC from EAE mice and stimulated with PLP139–151. When NKG2D receptors on SC from Hsp70-pc mice were blocked by the CX5 mAb, restoration of PLP-induced proliferation was observed, p < 0.05 (Fig. 6C). Thus, these results indicate that NKG2D is functionally involved in induction of inhibition of SC reactive to PLP139–151.

FIGURE 6. NKG2D-dependent inhibitory effect of NK cells on PLP reactivity and EAE induction. A, NK cells isolated from Hsp70-pc, but not Hsp70, immunized mice showed inhibitory effects on PLP139–151-induced proliferation of SCs derived from EAE mice. The results represent the mean ± SD from four experiments with five to eight animals in each group. B, In vivo transfer of NK cells isolated from Hsp70-pc, but not from Hsp70, immunized mice prevents EAE development. Results represent the mean clinical scores derived from three experiments each with five to eight animals (SD < 15%). C, Preblocking with anti-NKG2D Ab of SC isolated from Hsp70-pc-immunized mice attenuated the inhibitory effect of SC derived from Hsp70-pc-immunized mice on PLP139–151 induced proliferation. Bars, Mean ± SD from three experiments with five to eight animals per group.

FIGURE 7. Anti-H60 or anti-NKG2D prevented the CD3+ cell loss in Hsp70-pc-induced EAE tolerance. A, FACS analysis of apoptotic death of SC after in vitro culture with PLP139–151 (50 μg/ml) for 96 h. Assessment of apoptosis was based on fractional DNA content and the percentage of apoptotic cells calculated from four identical experiments. Bars represent the percentage of apoptotic spleen cells (+ SD) isolated from Hsp70-pc or Hsp70 preinjected EAE mice or control EAE mice. B, FACS analysis of CD3+ cells in SC population. The bars represent the percentage of CD3+ cells in SC isolated from EAE mice, EAE mice preinjected with Hsp70-pc, EAE mice preinjected with Hsp70-pc treated with blocking anti-H60 Ab, or anti-NKG2D mAb, on the day of immunization with PLP139–151. Data are based on five identical experiments.
Inhibition of H60 or NKG2D prevents T cell loss in Hsp70-pc preinjected mice

The inhibitory effect of preinjection with Hsp70-pc on PLP-induced proliferation of SC correlated with an increase in the percentage of cells showing evidence of apoptosis, after PLP stimulation in vitro, \( p < 0.01 \) (Fig. 7A). Similarly, in vivo in mice preinjected with Hsp70-pc, we noted a decrease in the CD3\(^+\) T cell population in the spleen, \( p < 0.01 \) (Fig. 7B). However, in mice that were preinjected with Hsp70-pc and also given anti-H60 Ab, the number of CD3\(^+\) cells in the spleen was not decreased (Fig. 7B). Similarly, inhibition of NKG2D in Hsp70-pc preinjected mice also did not reduce CD3\(^+\) T cell content (Fig. 7B). Collectively, these data suggest that the mechanism of Hsp70-pc-induced EAE inhibition depends on enhanced death of autoreactive cells, and that these phenomena can be reversed by neutralization of H60 or NKG2D.

NK cell mediated loss of PLP reactive cell is not dependent on H60 expression on target cells

To specifically address whether H60 expression on PLP-reactive cells determines their sensitivity to NK cell-mediated cytotoxicity, we measured the loss of CD3\(^+\)H60\(^+\) and CD3\(^+\)H60\(^-\) cells in EAE mice treated with Hsp70-pc, and compared it with control EAE mice. We found that both these populations were lost at relatively the same proportion, 50 and 44%, respectively (Fig. 8, A and B). Thus, we concluded that H60 expression, although critically important for the Hsp70-pc-induced EAE tolerance, does not determine NK cell cytotoxicity against PLP-reactive effector cells. In subsequent experiment, we assessed contribution of CD3\(^+\)CD4\(^+\) and CD3\(^+\)CD8\(^+\) cells to the population loss in mice preinjected with Hsp70-pc. It was found that both fractions of CD3\(^+\) cells were equally affected (Fig. 8C).

NK cell promotes DC for elimination of Ag reactive cells

To search for effector mechanism of Hsp70-pc-induced NK cell-mediated tolerance to EAE induction, we investigated NK cell interaction with CD11c\(^+\) DC. CD11c\(^+\) DC preincubated for 72 or 144 h with NK cells from Hsp70-pc but not from control mice showed reduced capability to stimulate PLP\(_{139-151}\) reactive cells, as assessed by proliferation assay, \( p < 0.0005 \) (Fig. 9A). The reduced proliferation of PLP reactive cells correlated with expression of phosphatidylserine by these cells as assessed by annexin V staining and positive staining with 7-ADD indicating enhanced death of PLP-reactive cells, (\( p < 0.001 \) and \( p < 0.005 \), respectively) (Fig. 9B and C). Enhanced death was predominantly observed in the CFDA-SE marked cells corresponding to Ag-driven population of proliferating T cells. The modulation of DC function

FIGURE 8. Distribution of CD3\(^+\)H60\(^+\) and CD3\(^+\)H60\(^-\) cells in Hsp70-pc-induced EAE tolerance. FACS analysis of CD3\(^+\) T cells in the SC population expressing H60 on the cell surface. SC were isolated from EAE mice, EAE mice preinjected with Hsp70-pc, or EAE mice preinjected with Hsp70-pc treated with a blocking anti-H60 Ab or an anti-NKG2D mAb on the day of immunization with PLP\(_{139-151}\). SC were double-stained with anti-H60 mAb and anti-CD3 mAb. A, Representative FACS dot plots of CD3\(^+\) cells in SC population. A clear loss of CD3\(^+\) T cells was detected in SCs isolated from the Hsp70-pc-preinjected mice compared with EAE mice. However, the CD3\(^+\) cell loss was observed equally in cell populations expressing and note expressing H60 on cell surface (6 vs 3% and 23 vs 13%). The data are representative of five identical experiments. Animals treated with anti-H60 or anti-NKG2D Abs had CD3\(^+\) cell counts corresponding to control EAE animals. B, Histogram show mean \pm SD of CD3\(^+\) cell loss in EAE mice preinjected with Hsp70-pc in CD3\(^+\)H60\(^+\) (\( p < 0.001 \)) and CD3\(^+\)H60\(^-\) (\( p < 0.001 \)) cells. C, Representative FACS dot plots of CD3\(^+\)CD4\(^+\) and CD3\(^+\)CD8\(^+\) cell populations in SC isolated from EAE mice or EAE mice preinjected with Hsp70-pc. Both subpopulations showed similar percentage of cell loss.
leading to enhanced death of PLP-reactive cells was observed independently of the ratio of DC:NK cells. Similar inhibition was achieved with the DC:NK ratio 1:5 and 5:1.

**NK cell-mediated EAE tolerance in Hsp70-pc mice do not involved Foxp3 cells**

Data indicating DC involvement of NK cell-mediated EAE tolerance in Hsp70-pc mice prompted us to investigate the role of regulatory T cells in this process. DC were preincubated with NK cells as above and number of Treg cells were assessed in populations of SC by simultaneous staining for CD4, CD25, and Foxp3. However, independently of the preincubation period, 72 to 144 h, as well as the ratio between DC and NK cells, 1:5 and 5:1, there was no clear change in the number of Foxp3 positive cells (Fig. 9D). These data are consistent with no change in Foxp3+ cell found in Hsp70-pc preinjected mice (1.87 ± 0.16) and control EAE mice (2.06 ± 0.20), (p = 0.09).

**Discussion**

The mechanisms that control autoimmune responses are still not fully understood. Based on data from a previous study (21), we postulated that Hsp70-pc-induced tolerance to EAE represented a new natural regulatory mechanism controlling autoimmune diseases within the CNS. This mechanism was dependent on induction of peptides generated during EAE inflammation. However, these peptides had to be chaperoned by stress-induced Hsp70 to exert a tolerogenic effect on EAE. The Hsp70-pc-induced tolerogenic effect was mediated by NK cells, as evidenced by EAE inhibition with transfer of NK cells from mice preinjected with Hsp70-pc. Now, we present data that the NK cell immunoregulatory function induced with Hsp70-pc depends on the presence of the glycoprotein H60, a ligand for the NKG2D receptor and involved altered function of CD11c+ DC. The initial suggestion that H60 might play a role was derived from the observation that Hsp70-pc-induced EAE tolerance was repeatedly unsuccessful in C57/B6 mice, contrary to what we observed in SJL/J mice. One striking difference in NK cell function between these two strains is the lack of H60 expression in C57/B6 mice, contrary to what we observed in SJL/J mice. One striking difference in NK cell function between these two strains is the lack of H60 expression in C57/B6 mice, contrary to what we observed in SJL/J mice. One striking difference in NK cell function between these two strains is the lack of H60 expression in C57/B6 mice, contrary to what we observed in SJL/J mice.
activated by APC in a TCR-specific manner. Blocking of the NKG2D receptor with mAbs prevented the killing of activated, but not resting, T cells by syngeneic NK cells (19). NK cells can also negatively regulate DC (33, 34), and thus influence Ag recognition and effector immune cell reactivity. Most importantly, after CD154 mAb therapy NK cells promote islet allograft tolerance. The mechanism of CD154-induced tolerance was dependent on MHC class I NK cell reactivity (35), however, the “missing self MHC class I” hypothesis was shown not to be involved in the NK cell mediated induction of tolerance. In line with these findings, it has been shown that NK cells in NOD mice, as well as in patients with diabetes, have impaired function that is associated with elevated expression of NKG2D high ligand (26).

In our studies, the NK cell-mediated Hsp70-pc-induced tolerance to EAE involved diminished T cell reactivity to PLP, the Ag used for the induction of EAE (21). The results of the allograft tolerance and our current data on NK cell mediated tolerance to EAE are consistent with the concept that NK cells exert an immunoregulatory effect by down-regulation of T cell responses. To address Ag specificity of the Hsp70-pc-induced immunoregulatory mechanism, we assessed PPD reactivity in mice sensitized for induction of high levels of IFN-γ (43). In this regard, it is of interest to note that a striking finding in diabetes, have impaired function that is associated with elevated expression of NKG2D high ligand (26).

The mechanism of NK cell mediated immunoregulation might involve interaction with DC (36) or direct T cell destruction (37, 38). However, the lack of an increase in H60 expression on T cells from EAE mice immunized with PPL, as well as no enhanced loss of CD3+ H60+ over CD3+ H60− cells in Hsp70-pc-induced EAE tolerance, suggests that direct killing of H60+ PPL-reactive cells might not be of significance in the Hsp70-pc-induced tolerogenic mechanisms. In contrast, when CD11c+ DC were preincubated with NK cells from Hsp70-pc mice their acquired potency to inhibit proliferation of PPL-reactive cells. It is of interest that inhibitory effect of DC on T cell proliferation might involve division-associated loss of Ag specific T cell (39). Similarly, in our experiments NK cell-mediated DC-induced inhibition of proliferation was correlated with enhanced death of PPL-reactive cells. Thus, we postulate that effector mechanism of NK cell-mediated immunoregulation might involve interaction with DC (36) or direct T cell destruction (37, 38). In this regard, it is of interest to note that a striking finding in our experiments with Hsp70-pc-induced EA E tolerance was the induction of high levels of IFN-γ upon stimulation with PPL in mice in which EAE was suppressed. In summary, we have provided evidence that Hsp70-pc induced tolerance for EAE involves activation of the NKG2D receptor on NK cells and that induction of the NKG2D ligand, H60, is necessary to generate regulatory function of NK cells which in turn interact with DC and modulate their activity. These findings might be of significance in tailoring immune regulatory strategies for autoimmune diseases such as multiple sclerosis.

Disclosures

The authors have no financial conflict of interest.

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