Article

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Neural progenitors derived from human embryonic stem cells are targeted by allogeneic T and natural killer cells

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Abstract

Neural progenitor cells (NPC) of foetal origin or derived from human embryonic stem cells (HESC) have the potential to differentiate into mature neurons after transplantation into the central nervous system, opening the possibility of cell therapy for neurodegenerative disorders. In most cases, the transplanted NPC are genetically unrelated to the recipient, leading to potential rejection of the transplanted cells. Very few data provide reliable information as to the potential immune response of allogeneic neural progenitors derived from HESC. In this study, we analyzed *in vitro* the allogeneic immune response of T lymphocytes and natural killer (NK) cells to NPC derived from HESC or of foetal origin. We demonstrate that NPC induce T-cell stimulation and a strong NK cytotoxic response. NK-cell activity is unrelated to MHC-I expression but driven by the activating NKG2D receptor. Cyclosporine and dexamethasone previously used in clinical studies with foetal NPC did not only fail to prevent NK alloreactivity but strongly inhibited the terminal maturation from NPC into mature neurons. We conclude that allogenic transplantation of NPC in the central nervous system will most likely require an immunosuppressive regimen targeting allogenic T and NK cells, whereas possible interference with the differentiation of NPC needs to be carefully evaluated.

Keywords: natural killer cells • T lymphocytes • cellular transplantation • rejection mechanisms • immunosuppressive drugs • neurodegenerative diseases

Introduction

Human embryonic stem cells (HESC) are pluripotent stem cells that are isolated from the inner cell mass of the human blastocyst [1]. Protocols of the differentiation of HESC into progenitors of many types are currently available [2]. Neural progenitor cells (NPC) derived *in vitro* from HESC [2] or of foetal origin [3] have the potential to substitute the damaged nervous tissues in some neurological diseases after transplantation into the brain, having locally differentiated into mature neurons or other subtypes of neural cells. NPC transplantation in the central nervous system has been shown to improve motor symptoms in numerous animal models of Parkinson's disease and spinal cord injury (reviewed in [1]).

Since the transplanted NPC would be genetically unrelated to the recipient, a major hurdle to cell therapy could be the host immune response to the transplanted cells. In addition to the immune reaction to be expected after allogeneic HESC transplantation, products of animal origin used in the differentiation protocols are also thought to amplify the risk of xenogenic antigen inclusion (immunogenic non-human sialoproteins) increasing rejection in the recipient [4]. Multiple steps have been implemented to minimize the amount of animal components during the differentiation process, including the replacement of bovine serum in the medium [4].

Nevertheless, the potential importance of HESC/NPC immune rejection process remains a subject of intense debate. Several

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experimental studies voiced little concern for potential cellular immune problems associated with transplantation of HESC-derived products. Undifferentiated HESC express low levels of HLA class I, which is up-regulated by IFN-γ stimulation or after differentiation into embryoid bodies as well as in teratoma [2, 3, 5], but the level of expression was below those of other somatic cells analyzed [3].

MHC class II and co-stimulatory molecules, however, have not been found (or only at low levels) in these studies, suggesting that HESC lack important molecules to induce T-cell activation or T-cell cytotoxic activity [2, 3, 5]. Conflicting data have resulted from studies analyzing allogeneic T-cell proliferation stimulated by HESC in mixed lymphocyte reaction (MLR). HESC have been shown to induce similar levels of T-cell proliferation as cultured human fibroblasts [2]. In another study, HESC whether undifferentiated or differentiated, failed to stimulate proliferation of alloreactive primary human T cells [5]. More specifically in the case of expanded cells of foetal origin, the expression of MHC class I and II – but not that of the co-stimulatory proteins CD40, CD80 and CD86 – increased significantly after IFN-γ stimulation; peripheral lymphocytes, however, were unresponsive in MLR, suggesting their low immunogenicity despite HLA incompatibility and HLA expression [4].

The absence of MHC class I molecules at the cell surface of progenitors is a potential risk for natural killer (NK) cell cytotoxicity. NK-cell functions are regulated by a complex repertoire of cell surface receptors belonging to different families [6, 7, 8]. Among these, the killer cell immunoglobulin-like receptor (KIR) family is of special interest because of its ligand being MHC class I, HLA-C and HLA-B, and the non-classical HLA-G [6, 7, 8]. Other NK receptors like C-type lectin NKG2A and NKG2C receptors bind to HLA-E, whereas the activating NKG2D receptor recognizes the non-HLA molecules MICA/B and ULBPs [6, 7, 8].

Because most of NK receptors which bind MHC ligands have an inhibitory function, the absence or low expression of the classical MHC class I or non-classical HLA-E, G in HESC, these cells are a good target for elimination by NK. However, a previous study demonstrated that regardless of the differentiation status of the cells and the expression levels of MHC-I *in vitro*, HESC were not destroyed by NK cells, even if the inhibitory HLA-G molecule was expressed [3]. It has therefore been suggested that HESC failed to be eliminated by NK because of lack of recognition rather than inhibition [3]. In a humanized murine model of HESC transplantation, the mice transplanted with peripheral leucocytes of human origin showed only a minimal immune response to HESC whether undifferentiated or differentiated [9]. Despite the putative low level of immunogenicity, the few clinical trials performed in humans using tissue of foetal origin featured empirically immunosuppressive drugs such as cyclosporine [10, 11].

Considering that the immunogenicity of human NPC has not been investigated, we performed an in-depth analysis *in vitro* of T- and NK-cell immune response to allogeneic NPC derived from HESC or of foetal origin. IFN-γ activated NPC-induced significant T-cell proliferation and were destroyed extensively by NK cells due to a mechanism that is MHC class I independent. Moreover, cyclosporine and dexamethasone not only failed to inactivate NK-cell cytotoxicity but also to inhibit the terminal differentiation of NPC into neurons.

Materials and methods

HESC culture

H1 HESC cell line was purchased from WiCell Research Institute (Madison, WI) at low passage under the authorization of the Swiss Public Health Service (OFSP N°R-FP-S-2–0004-00–4). Cells were expanded under non-differentiating conditions and analyzed between passages 10 and 50. Cells were maintained as recommended by the supplier in human HESC cell medium consisting of 80% DMEM/F12, 20% knockout-serum replacement, 2 mM L-glutamine, 1% non-essential amino acids, 0.1 mM β-mercaptoethanol, 4 ng/ml basic fibroblast growth factor (bFGF) (Invitrogen, Carlsbad, CA) at 37°C, 5% CO_2 and high humidity. Mouse embryonic fibroblasts, isolated from embryos of pregnant CF-1 mice (Charles River Laboratories, Wilmington, MA), were used as feeder cells. Fibroblasts were cultured up to passage 2 in DMEM (Invitrogen) supplemented with 10% foetal bovine serum and 1% penicillin/streptomycin (Invitrogen). Cells were mitotically inactivated by irradiation at 40 Gy before seeding onto a gelatin-coated 6-well plate at 1.9×10^5 cells/plate. The HESC culture medium was changed daily, and the cells were plated every 8 days following incubation with type IV collagenase (1 mg/ml; Invitrogen) for 20–30 min. at 37°C and mild mechanical disruption.

ReNcell-VM were provided by Chemicon (Millipore, San Francisco, CA) and expanded as recommended by the manufacturer in maintenance medium in the presence of EGF (20 ng/ml) and bFGF (20 ng/ml).

Neural differentiation of HESC into NPC

For differentiation of HESC into NPC, HESC colonies were detached by incubation with type IV collagenase (1 mg/ml in DMEMF12) for 15 min. at room temperature. Colonies were washed once and maintained in suspension in ultra-low attachment 6-well plates (Costar, Corning Life Sciences, Schiphol-Rijk, the Netherlands) for 1 week in neural induction medium (DMEMF-12, penicillin/streptomycin 1%, N-2 supplement (Gibco, Invitrogen). Thereafter, NPC were expanded by plating of HESC-derived aggregates at low density (10–20 aggregates) on a human laminin (1 μg/ml)-coated tissue culture plate in neural induction medium supplemented with 10 ng/ml human recombinant bFGF and 10 ng/ml human recombinant EGF (R&D Systems, Inc., Minneapolis, MN). After 2 weeks, NPC were dissociated at a single-cell level by enzymatic treatment with Accutase (Sigma-Aldrich, Milwaukee, WI) for 5 min. at 37°C.

Differentiation of NPC into mature neurons

NPC-containing aggregates were cultured for 3 weeks in neural induction medium as described under the section 'Neural differentiation of HESC into NPC' and dissociated at a single-cell level by enzymatic treatment with 0.5% trypsin (5 min. at 37°C) combined with EDTA 0.2 mM. Single-cell suspension was re-plated at low density (5000 cells/cm^2) on 1 μg/ml laminin-coated dishes in neural differentiation medium (neurobasal medium [Gibco, Invitrogen] supplemented with B-27 supplement (Gibco,

Invitrogen), 10 ng/ml human recombinant BDNF (R&D Systems, Inc.,) and 1% penicillin/streptomycin. After 2 weeks, NPC differentiated into neuronal networks. In some experiments, an immunosuppressive drug (CsA, Dexa) was added or not to neural differentiation medium immediately after enzymatic dissociation of NPC-containing aggregates.

Maintenance and neuronal differentiation of ReNcell-VM

Maintenance and neuronal differentiation of ReNcell-VM was carried out in accordance with manufacturer's instructions (Millipore):

ReNcell-VM were maintained in the above neural induction medium supplemented with human recombinant bFGF (20 ng/ml) and human recombinant EGF (20 ng/ml) on laminin (20 μg/ml)–coated tissue culture plates. At confluency, cells were passaged by enzymatic treatment with Accutase (Sigma-Aldrich) for 5 min. at 37°C.

For neuronal differentiation of ReNCell-VM, single cells detached with Accutase were replated at low density on laminin-coated culture plates (5000 cells/cm^2) in the absence of EGF and bFGF. Thereafter, ReNcell-VM evolved into neuronal networks after 2 weeks.

Antibodies

The following antibodies were used to characterize NPC and neurons by immunostaining: rabbit anti Musashi-1, rabbit anti-nestin, mouse anti-vimentin, rabbit anti-Sox-1 (Chemicon, Millipore), goat anti Pax-6, rabbit anti-glial fibrillary acidic protein (GFAP) (Dako, Glostrup, Denmark), mouse anti-βIII-tubulin (Sigma-Aldrich), rabbit anti-βIII-tubulin (Covance, Princeton, NJ). The following mouse anti-human antibodies were purchased from BD PharMingen™ (San Jose, CA): PE-Cy7-conjugated anti-CD3, APC-Cy7-conjugated anti-CD16, FITC-conjugated anti-CD107a and PE-conjugated anti-HLA-I. APC-conjugated anti-CD56 was from Miltenyibiotec (Bergisch Gladbach, Germany). FITC-conjugated anti-HLA-II was supplied by Dako and FITC-conjugated anti-HLA-G by Serotec (Oxford, UK). Biotin-conjugated anti-CD40 was from Diaclone (Besançon, France) and biotin-conjugated anti-CD86 was from Ancell (Bayport, MN). The unlabelled CD86 and CD40 antibodies were stained with streptavidin-ECD from Beckmann Coulter (Beckmann Coulter, Fullerton, CA). FITC-conjugated anti-CD80 and PE-conjugated anti-B7-H1 were supplied by eBioscience (San Diego, CA). Anti-MICA/B was purchased from Santa Cruz Biotechnology (San Diego, CA), and goat anti-mouse alexafluor 546 from Molecular Probes (Carlsbad, CA) was used for detection.

Flow cytometry

The NPC culture aggregates were dissociated by Accutase enzymatic treatment for 5 min. at 37°. NPC were washed once with PBS (completed with 2% FCS) and treated successively with FITC-, PE- and biotin-conjugated antibodies on ice for 10 min. and washed with PBS supplemented with 2% bovine serum. Flow cytometry experiments were performed using FACSAria® and FACS DIVA software (BD PharMingen).

Immunofluorescence analysis

Before analysis, cells were cultured and stimulated on glass cover slips followed by immunofluorescent staining. Samples were fixed in PBS – 4% paraformaldehyde for 30 min. at room temperature and permeabilized for 30 min. with PBS containing 0.2% Triton X-100. The cells were then incubated overnight at +4°C with primary antibodies in PBS containing 1% foetal bovine serum. After washing in PBS, cells were incubated for 1 hr 30 min. at room temperature with the same buffer containing secondary antibodies, washed again and incubated with DAPI 300 nM (in PBS) for 15 more minutes. Sections were finally washed twice in PBS, rinsed with water before mounting in FluorSave® medium (Calbiochem, San Diego, CA) [12].

Mixed lymphocyte reaction

NPC were plated in 96-well plates and irradiated at 3500 rad and co-cultured with lymphocytes from peripheral blood mononuclear cells (PBMCs) isolated from normal blood donors by density-gradient centrifugation. Autologous PBMC were also plated and irradiated as negative controls. After 5 days, cultures were pulsed for 6 hrs with [*methyl*-^{3}H] thymidine (0.5 μCi per well), and cell proliferation was measured by radioactivity incorporation in count per minute using a gamma counter [13]. Similar experiments were performed after pre-incubation of PBMC with various concentrations of CsA and Dexa.

NK-cell isolation

(PBMCs were isolated from normal blood donors by density-gradient centrifugation. NK cells were separated from PBMC by magnetic cell sorting according the manufacturer's instructions (NK cell isolation kit, Miltenyibiotec). Non–NK cells from human PBMC, such as T cells, B cells, dendritic cells, monocytes, granulocytes and erythrocytes were stained with a cocktail of biotin-conjugated antibodies to CD3, CD4, CD14, CD15, CD19, CD36, CD123 and CD123a. A second staining was made using an anti-biotin mAb conjugated with microbeads. NK cells were isolated by depletion of the magnetically labelled cells. The isolated NK cells were cultured for 5 days in RPMI medium supplemented with 10% human AB serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1% MEM non-essential amino acids and 0.1 mM sodium pyruvate, 5 mM β-ME (at 5×10^{-5} M). In some experiments, 25 ng/ml of recombinant human IL-15 (R&D Systems), cyclosporine A (Sigma-Aldrich) and/or dexamethasone (Sigma-Aldrich) were added to the medium.

Cytotoxicity assay

The isolated NK cells were cultured with or without recombinant human IL-15 (25 ng/ml). After 1 week, NK cells were subjected to the cytotoxicity test using a standard chromium (Cr^{51}) cytotoxic assay. K562, NPC and ReNcell-VM NPC treated or not with IFN-γ, were incubated for 1 hr with $^{51}CrO_4$ (Hartmann Analytics, Germany), washed three times and co-incubated for 4 hrs with NK cells. For the blocking assay, NK cells or NPC were pre-incubated for 30 min. with 10 μg/ml of monoclonal blocking antibodies raised against MICA/B and ULBP1,2,3, the ligand of NKG2D (expressed by NPC) or directly against NKG2D (anti-NKG2D), expressed by the NK cells. Then, NK cells were directly incubated with the target cells, that is, NPC or the control K562, for the cytotoxic assay described earlier. The K562 cell line is highly susceptible to lysis by NK cells because it does not express MHC class I.

IFN-γ secretion and CD107a expression by NK cells

For the analysis of the production of IFN-γ and the expression of CD107a, NK cells were incubated for 6 days with or without IL-15 and with or without CsA, Dexa or CsA and Dexa. An IFN-γ capture assay kit (Miltenyibiotec) was used to determine the amount of IFN-γ secreted by NK cells. Briefly, after 7 days of culture with recombinant human IL-15 (25 ng/ml), NK cells were incubated for 45 min. at 37°C together with a bipolar anti-IFN-γ antibody that binds to the cells as well as with IFN-γ secreted at the cell surface. IFN-γ was determined by FACS using a secondary PE-labelled antibody.

For the analysis of CD107a expression, NK cells were incubated with K562, NPC or NPC+IFN-γ cells. After 1 hr at 37°C in 5%CO_2, monensin (6 ug/ml) was added for 5 hrs and CD107a expression was measured by flow cytometry. Cells were washed and stained for IFN-γ and the NK-cell markers as described [14].

Quantitative real-time PCR experiments

Total RNA for quantitative real-time (qRT)–PCR experiments was isolated with Trizol reagent after 7 days of culture. cDNA synthesis was performed with the cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA), according to manufacturer's instructions. Expression of each gene was determined in three independent biological replicates. Reactions were run on an ABI Prism 7900 HT detection system (Applied Biosystems, Carlsbad, CA). ALAS and GusB were used as housekeeping genes. As these genes reacted similarly in all samples examined, data were normalized to ALAS level. Sequences of the primers are described in supplementary Table 1.

Statistical analysis

Statistical analysis was performed using the non-parametric ANOVA–Dunett's test.

Results

NPC derived from HESC and stimulated by IFN-γ express high levels of MHC class I and induce allogeneic T lymphocyte proliferation

Neural differentiation of HESC into NPC was performed *in vitro* in conditions as close as possible to clinical compatibility, being chemically defined and without animal products. Practically, HESC colonies were detached and cultured in chemically defined and animal-free neural induction medium to obtain neural spheres. Neural spheres were then plated at low density on a human laminin substrate to generate NPC. After 3 weeks of differentiation, HESC-derived cells were heterogeneous in terms of phenotype and spatial organization. They consist of various structures such as rosette clusters, floating neural spheres, cell aggregates and neuroepithelial flat cells as recently described [12]. It is noteworthy that some mature neurons – although they were relatively rare, as well as non-neural cells, occurred were present among NPC at this stage (data not shown). To ensure the NPC identity of HESC-derived cells, cultures were immunostained with neuroepithelial markers (nestin, Pax-6) as well as molecules known to be expressed in NPC: βIII-tubulin, vimentin, Sox-1, GFAP, Musashi-1. Table 1 summarizes the phenotypes of the different structures and confirms the neural identity of the derived cells, although non-neural cells were also present within the culture. Adherent flat cells were the majority in the population. These cells proliferated, differentiated further into mature neural cells in appropriate conditions (see Materials and Methods) and expressed the set of neuroepithelial and NPC markers (Fig. 1). Of note, Pax-6 and Sox-1 were not expressed in every cells growing as a monolayer (Table 1). On the other hand, residual spheres were not only heterogeneous aggregates including NPC but also non-neural cells (Table 1). Finally, aggregates including rosette clusters expressed neuroepithelial and NPC markers, and their differentiation into mature neural cells was remarkably efficient (Table 1).

Table 1 Phenotype of cell populations during neural differentiation of embryonic stem cells

	Spheres	Monolayer	Neuronal cells	Rosettes
Nestin	+/−	+	−	+
Musashi-1	+/−	+	−	+
Pax-6	+/−	+/−	−	+
Sox-1	+/−	+/−	+/−	+
Vimentin	+/−	+	−	+
GFAP	low/−	low	−	low
βIII-tubulin	+/low/−	low	+	low
Neurogenicity	++	+		+++

According to co-staining experiments, cells expressing the NPC markers nestin, Sox-1, GFAP and vimentin did not express MHC class I and MHC class II (Fig. 2, left panel; Table 2). It is noteworthy that at times some MHC class I expression was observed in some of the cultured cells MHC class I, especially when flow cytometry technology was used (Table 2). MHC class I expression was increased upon treatment of NPC with IFN-γ for 72 hrs (Fig. 2, right panel; Table 2). The expression of both classical and non-classical MHC as well as co-stimulatory molecules was also analyzed after treatment or not with IFN-γ. Results are summarized in Table 2 (left column). Non-classical MHC (HLA-G and HLA-E) and co-stimulatory molecules were not expressed in non-stimulated cells. Of interest, such treatment does not induce any non-classical MHC and co-stimulatory molecules at the cell

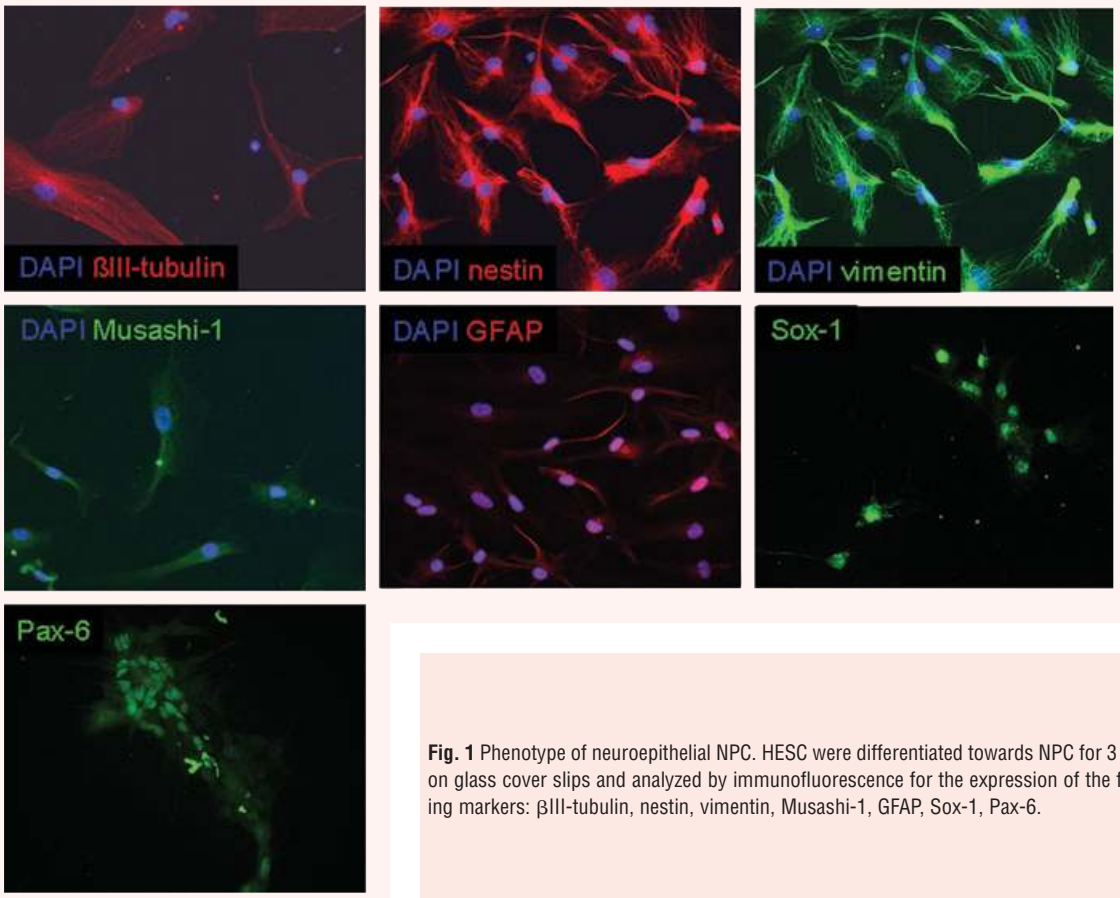

Fig. 1 Phenotype of neuroepithelial NPC. HESC were differentiated towards NPC for 3 weeks on glass cover slips and analyzed by immunofluorescence for the expression of the following markers: βIII-tubulin, nestin, vimentin, Musashi-1, GFAP, Sox-1, Pax-6.

surface, with the exception of HLA-E, which was induced in NPC to a very small extent.

Since NPC differentiate into neurons, the expression of major antigens was also investigated in mature neuronal cells. Neuronal differentiation was performed by re-plating of NPC at low density in neural differentiation medium containing BDNF. Without IFN-γ, neuronal cells expressing βIII-tubulin did not express MHC class I (Fig. 3A). As observed for NPC, MHC class I was significantly induced in neurons after stimulation with IFN-γ (Fig. 3B). As neurons in culture have the tendency to differentiate in clusters, Fig. 3C confirms neuritic expression of MHC class I under IFN-γ treatment in a neuronal network. Finally, a co-staining experiment using MAP-2, another neuronal marker, confirms the ability of mature neuronal cells to increase MHC class I expression upon stimulation with IFN-γ (Fig. 3D). In contrast, MHC class II and non-classical MHC were not expressed in mature neurons (data not shown). Together, these data show that NPC or neuronal cells derived from NPC are capable of increasing MHC I upon stimulation by IFN-γ.

The induction of T-cell proliferation by NPC was tested by MLR. Consistent with the observation that NPC expressed MHC class I in the presence of IFN-γ, the precursor induced T-cell proliferation to a significant extent (Fig. 4A). Non-induced NPC were also able to stimulate T cells but to a smaller extent than the control cells treated by IFN-γ (Fig. 4A).

NK cells kill NPC regardless of IFN-γ stimulation

According to the missing self hypothesis, the absence of MHC class I expression would make NPC a target for lysis by NK cells. To test this hypothesis, NK cells were exposed to NPC in a classical radioactive chromium (Cr^{51}) cytotoxic assay. The MHC class I negative cell line K562 was used as positive control. NK cells stimulated with IL-15 were able to kill NPC treated or not with IFN-γ (Fig. 4B). IFN-γ treatment being associated with the marked expression of MHC class I on NPC, this observation suggests a cytotoxic response of NK that does not depend on MHC class I. Non-stimulated NK cells were also able to kill a small percentage of NPC and K562 cells. Autologous PBL were not killed either by stimulated (IL-15) or unstimulated NK cells (negative control, data not shown).

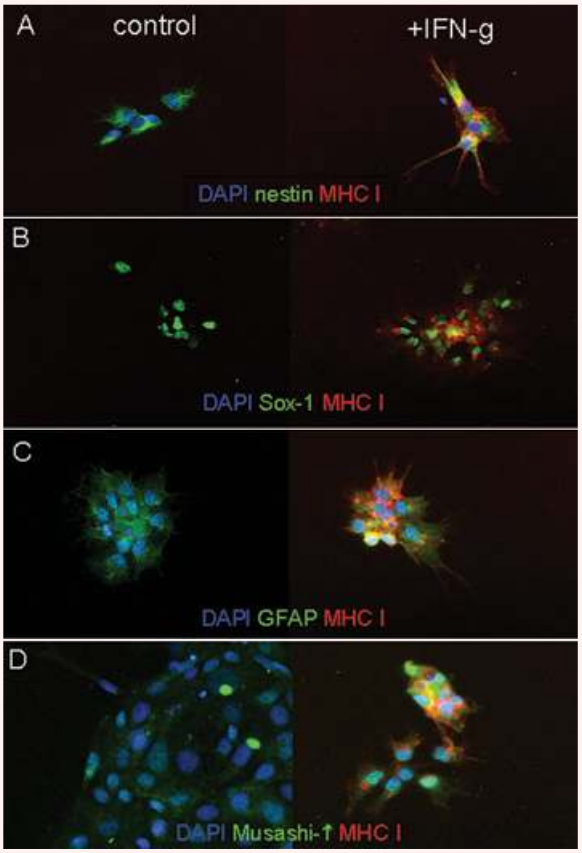

Fig. 2 NPC express MHC class I upon stimulation by IFN-γ. HESC were differentiated towards NPC for 3 weeks on glass cover slips. Then, NPC were incubated or not with recombinant human IFN-γ for 72 hrs and immunostained with MHC class I combined with several NPC markers: nestin (**A**), Sox-1 (**B**), GFAP (**C**) and Musashi-1 (**D**).

Table 2 Expression of lineage's markers and molecules involved in the immune alloreaction in HESC-NPC or ReNcell-VM cell line

	HESC-NPC		ReNcell-VM	
IFN-γ	0	1000 U/ml	0	1000 U/ml
MHC I	−/+[a]	+++	+	+++
MHC II	−	−	−	−
HLA-G	−	−	−	−
HLA-E	−	+[a]	−	−
CD40	−	−	−	−
CD80	−	−	−	−
CD86	−	−	−	−
B7H-1	−	−	−	−
Nestin	+	+	+	+
βIII-tubulin	+	+	+	+
Sox-1	+	+	nd	nd
Pax-6	+	+	nd	nd
Vimentin	+	+	+	+
GFAP	+[a]	+[a]	+	+
Musashi-1	+	+	nd	nd

[a]Weak expression.
nd, not done.

Ligands of the NKG2D-activating receptors ULBP1, ULBP2, ULBP3 and MICA/B were expressed at the surface of NPC (Fig. 5A). This observation suggests the possible implication of NKG2D and its ligands in the killing of NPC by NK. To test this hypothesis, blocking experiments were performed. In the presence of NKG2D or MICA/B, ULPB1, ULPB2 and ULPB3 blocking antibodies, the extent to which NPC were killed by NK cells was reduced (Fig. 5B), confirming the involvement of the NKG2D receptor.

Cyclosporine and dexamethasone do not prevent killing of NPC by NK cells and inhibit NPC differentiation towards neurons

To prevent NK cells from killing NPC by NK cells *in vitro*, we resorted to immunosuppressive drugs, for example, CsA, Dexa or a combination of both CsA + Dexa (CsA+Dexa), traditionally used in solid organ transplantation [15] as well as in a brain cell transplantation study [11]. We confirm first that CsA and Dexa inhibit PBMC proliferation driven by irradiated NPC whether stimulated or not by IFN-γ (Fig. 6A). Then, the functional activity of NK directed against NPC was assessed by CD107a expression at the surface of NK cells [14] and by the secretion of IFN-γ in the presence of immunosuppressive drugs or in their absence. Figure 6B shows a typical example where CD107a is expressed by NK cells in the presence of NPC. The IFN-γ secretion by NK cells exposed to NPC was inhibited by Dexa but not by CsA (Fig. 6C). In contrast, the killing activity was enhanced in the presence of CsA. This effect was corroborated by the killing activity determined by chromium (Cr^{51}) cytotoxic assay (data not shown). Interestingly, the combination of CsA + Dexa slightly reduced the killing activity of NK cells (Fig. 6C, right). It is noteworthy that IFN-γ treatment of NPC did not influence the effect of the different immunosuppressive drugs.

Since the terminal differentiation of NPC into neurons takes place in the recipient's brain (mature neurons cannot be transplanted), the role of immunosuppressive drugs at this final stage needs to be investigated. For that purpose, we undertook the terminal differentiation *in vitro* of 3-week-old HESC-derived NPC into neurons in the presence of various concentrations of drugs or without.

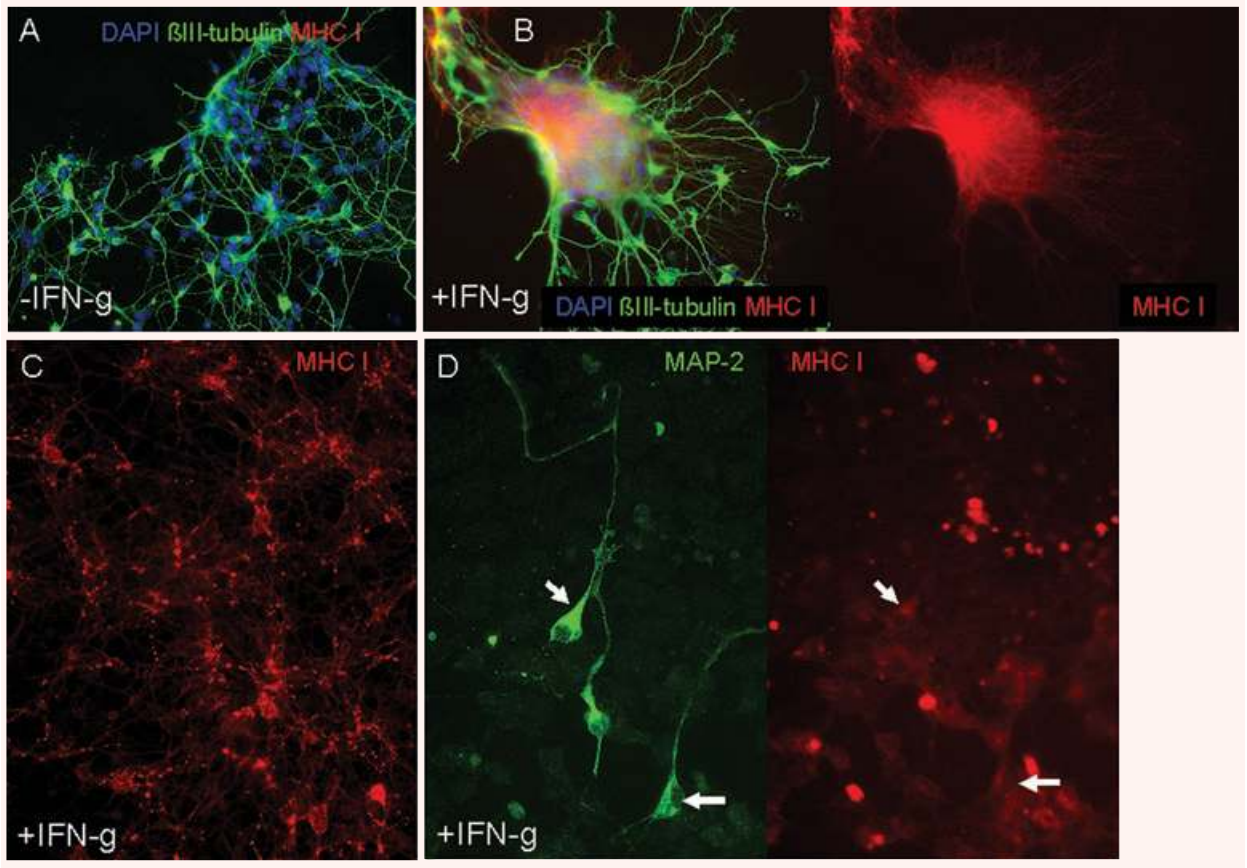

Fig. 3 Neurons differentiated from HESC-derived NPC express MHC class I upon stimulation with IFN-γ. HESC-derived NPC were differentiated towards neurons on glass cover slips for 2 weeks and incubated or not with human recombinant IFN-γ for 3 days, before immunostaining with MHC class I combined with neuronal markers. (**A**) MHC class I expression by βIII-tubulin – positive neurons that were non-stimulated by IFN-γ. (**B**) MHC class I expression by βIII-tubulin – positive neurons that were stimulated by IFN-γ. (**C**) Neuritic expression of MHC class I by neurons organized in large scaffolds. (**D**) MHC class I expression by MAP-2 – positive neurons that were stimulated by IFN-γ (white arrows).

Terminal differentiation towards mature neuronal cells was achieved by plating of NPC at low density in laminin-coated tissue culture flasks in the appropriate medium. Under these conditions, clusters of neuritic cells appeared after a few days (supplementary Fig. 1A). Most of the neuritic cells express βIII-tubulin (supplementary Fig. 1B) or GFAP but not vimentin (supplementary Fig. 1C), suggesting these cells were mature neurons and astrocytes, respectively. Neurons were confirmed by the presence of neuritic cells expressing axonal MAP-2 and Synapsin-1 (supplementary Fig. 1D). In rare cases, large clusters of residual NPC were observed because of the presence of cells positive for vimentin (supplementary Fig. 1C) and nuclei expressing Pax-6 and Sox-1 (supplementary Fig. 1E). Of note, these residual NPC expressed also Musashi-1, GFAP and nestin (data not shown). Non-neural cells that were not immunoreactive for NPC and neuronal markers were also present within the culture (data not shown).

Different concentrations of CsA (0.025–5 μg/ml) were tested in the range of plasma levels such as measured in treated patients [16]. None of them proved toxic for the differentiation process with the exception of 5 μg/ml (data not shown). For the various non-toxic concentrations of CsA (0.025–1 μg/ml), number and morphology of mature neurons differed from control cells. In the case of non-treated cells, neurons appeared rapidly and organized in networks of neuritic cells positive for βIII-tubulin (Fig. 7A). As expected, these mature neurons were interspersed with NPC, detected as flat cells that are stained with nestin and without βIII-tubulin expression (Fig. 7A). In contrast, differentiation of NPC into neurons was delayed under CsA exposure. In that case, NPC did not differentiate into large neuronal networks, although isolated or smaller clusters of neurons were observed (Fig. 7A; CsA 0.5 μg/ml) and found to be positive for other neuronal markers MAP-2 and synapsin-1 (data not shown). In addition, neurite

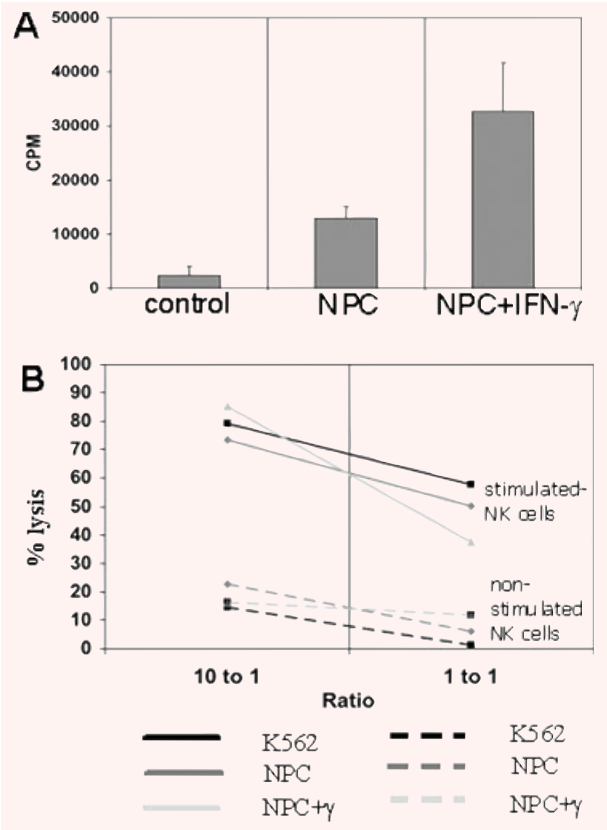

Fig. 4 NPC stimulated allogeneic T and NK cells *in vitro*. (**A**) NPC were mixed with allogeneic peripheral blood lymphocytes in a classical MLR. PBL proliferation is analyzed before and after stimulation of NPC by IFN-γ. Ctrl indicates the spontaneous proliferation of autologous PBL cultured under the same condition. Data are the mean ± S.D. of three independent experiments. (**B**) K562, NPC or NPC stimulated with IFN-γ (NPC/γ) pulsed with ^{51}Chromium were co-cultured for 5 hrs with IL-15-stimulated NK cells (full line) or non-stimulated NK cells (dotted line). Two different NK: NPC ratio were tested (10:1 and 1:1). The percentage of lysis is calculated with the following formula: percentage of specific lysis = [(experimental counts – spontaneous lysis)/(maximal lysis – spontaneous lysis) × 100]. Experiments were done in triplicates and Fig. 2B is representative of three independent experiments.

extensions of neuronal cells were shorter in the presence of CsA, suggesting that these cells were less mature. Similar to CsA, all of the tested concentrations of Dexa reduced the delay before the appearance of neurons, their size and their organization into networks (data not shown).

Another population of non-flat cells presented within the culture was highly positive for nestin and expressed or not βIII-tubulin. These cells harboured neuritic extensions, thus increasing the intensity of nestin immunoreactivity. They might be intermediate cells undergoing neuronal maturation (nestin ^{high}, βIII-tubulin+) or other subpopulations of NPC (nestin ^{high}, βIII-tubulin). In the absence of immunosuppressive drugs, these cells were rare, the majority of cell populations consisting of mature neuron networks and flat cells (Fig. 7B). In the presence of CsA and/or Dexa, however, the majority of cell populations harboured the above phenotypes (Fig. 7B).

In addition to neuronal morphology, the number of neuronal clusters was quantified. It decreased under exposure to CsA, as well as to Dexa (from 0.05 to 5 μg/ml) and to a combination of CsA and Dexa at all concentrations (Fig. 8A). The difference was statistically significant for 1 μg/ml and more of CsA, for 0.05 μg/ml and more of dexa and for all concentrations where CsA and Dexa were associated (Fig. 8A).

Then, the neural maturation of NPC was quantified using the Metamorph Software (Molecular Devices, MDS, Inc. Toronto, Canada). A specific template of the Metamorph Software was created by the BioImaging Core facility (Faculty of Medicine, University of Geneva) to estimate correctly the total neurite length (neurite outgrowth) and the total number of Hcells (total cell body area). Based on four independent experiments, the total neurite outgrowth and total cell body area of neuron derived from HESC-NPC were significantly reduced under treatment with CsA and Dexa (Fig. 8B). The number of undifferentiated NPC colonies was also assessed. These colonies expressed Pax-6 and other NPC markers such as vimentin and Sox-1 and were tested with or without immune suppressants. The presence of CsA and Dexa increased these populations suggesting that the terminal maturation of NPC was also targeted by drugs (data not shown).

Finally, the inhibition of the differentiation process by CsA and Dexa was quantified at the mRNA level. The markers of NPC, vimentin, Pax-6 and nestin mRNAs were expressed at a higher level in the presence of CsA and Dexa compared with PBS, confirming the increased number of non-differentiated NPC. In contrast, the mRNA level of βIII-tubulin was not modified by immunosuppressive drugs since this filament-associated protein was expressed in both NPC and neuronal cells (Fig. 8C).

NPC of foetal origin are also a target of the allogeneic immune system

To confirm the immunogenicity of NPC of foetal origin, the experiments described earlier were also performed using the ReNcell-VM cell line. These NPC correspond to foetal progenitors from the ventral mesencephalon [17, 18]. Results are summarized in Tables 1 (right column) and 2. The induction of MHC class I rather than co-stimulatory molecules upon treatment with IFN-γ was confirmed, as well as the spontaneous MLR and cytotoxicity of activated NK cells. MIC ligands were expressed on ReNcell-VM, but at higher levels than on HESC-derived cells. ULBPs were also found, with a stronger up-regulation of ULBP-2 upon IFN-γ treatment than previously observed with HESC-derived cells. By blocking NKG2D and its ligands, the killing of ReNcell-VM by NK cells was also reduced significantly. Finally, immunosuppressive drugs strongly reduced neuronal differentiation of ReNcell-VM (Table 3). Using the Metamorph Software for quantification, CsA reduced

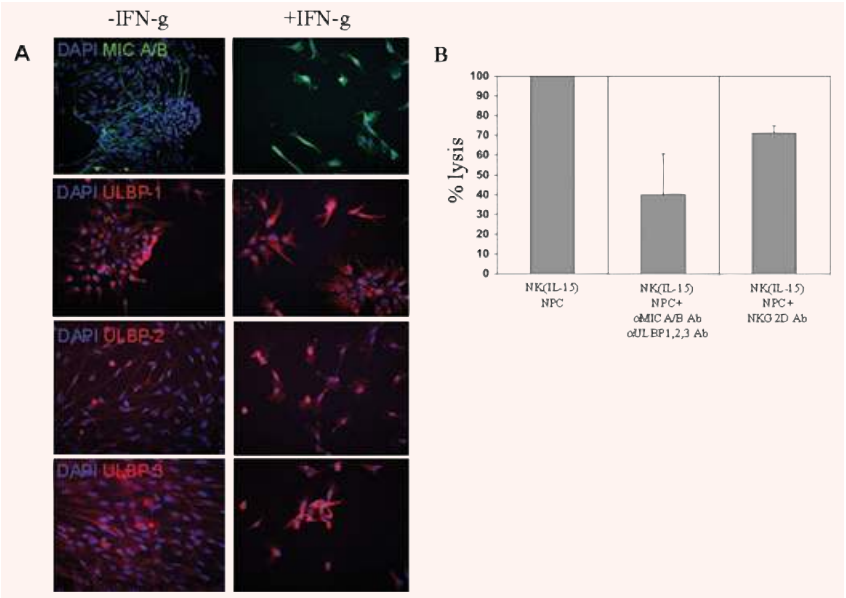

Fig. 5 NPC express ligands for NKG2D involved in the killing of NK cells. (**A**) The NKG2D ligands MICA/B and ULBP1, 2, 3 are detected by immunofluorescence on the cell surface of NPC stimulated or not with IFN-γ 1000 U/ml. (**B**) NK-cell cytotoxicity assay was performed in the presence or absence of blocking antibodies for the NKG2D ligands MICA/B and ULBP1, 2, 3 or by anti-NKG2D blocking antibody. The killing of NPC by NK cells stimulated with IL-15 without blocking antibody arbitrarily correspond to 100% (mean ± S.D. of two independent experiments).

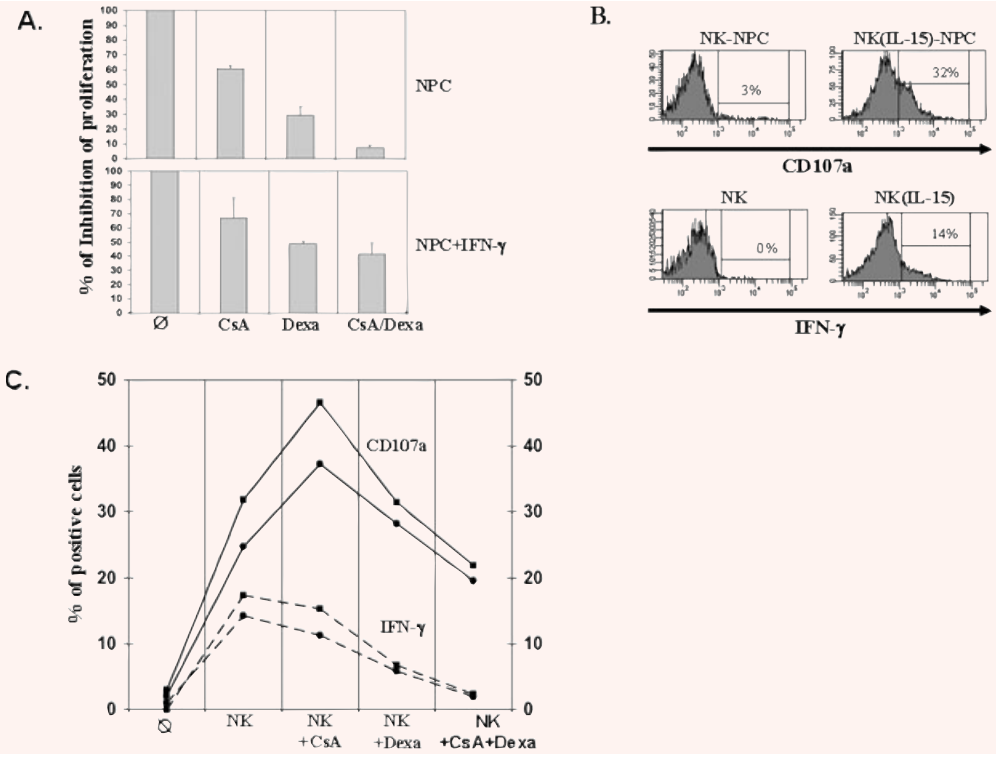

Fig. 6 Effect of cyclosporine (CsA) and dexamethasone (Dexa) on T lymphocytes and NK cells stimulated by allogeneic NPC. (**A**) NPC stimulated or not with IFN-γ were mixed with allogeneic peripheral blood lymphocytes pre-incubated 4 hrs with CsA and/or Dexa in a classical mixed reaction assay (MLR). The percentage in the reduction of the proliferation compared with the proliferation without incubation with immunosuppressive drugs is indicated. Data are the mean ± S.D. of three independent experiments. (**B**) A prototypical example of CD107a expression and IFN-γ secretion by non-activated or IL-15-activated NK cells cultured with NPC. (**C**) Two independent experiments of CD107a expression and IFN-γ secretion by IL-15-activated NK cells cultured with NPC in the presence of CsA, Dexa and the combination of both.

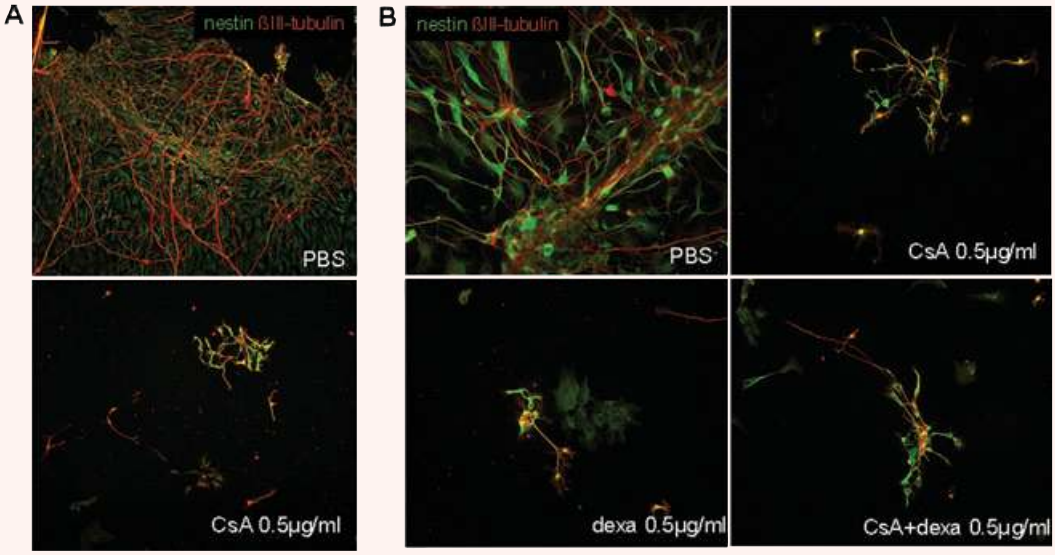

Fig. 7 Neuronal differentiation of NPC is modified by cyclosporin (CsA) and dexamethasone (Dexa). Undifferentiated NPC and mature neurons were analyzed by immunofluorescence for the expression of nestin (green) and βIII-tubulin (red). Undifferentiated NPC expressed nestin and βIII-tubulin, mature neurons harbour thin neuritic extensions that increase the brightness of βIII-tubulin staining but do not express nestin. (**A**) Microscopic view at low magnification of nestin and βIII-tubulin expression after 2 weeks of neuronal differentiation in the presence or not of CsA at 0.5 μg/ml (bottom). (**B**) Nestin and βIII-tubulin expression after 2 weeks of neuronal differentiation in the presence of CsA at 0.5 μg/ml (bottom), Dexa at 1 μg/ml or both.

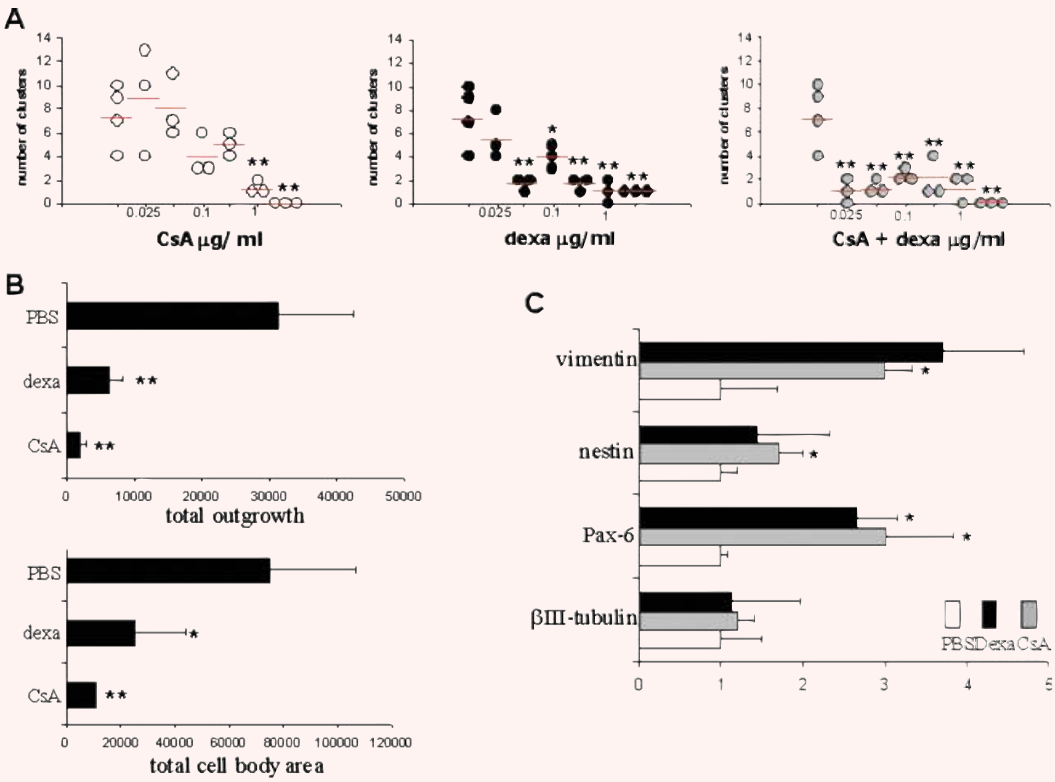

Fig. 8 Neuron quantification and expression of specific genes in the presence of cyclosporine (CsA) and dexamethasone (Dexa). (**A**) Quantification of the absolute number of neuronal clusters (>50 neuronal cells/cluster) in the presence or not of CsA (from 0.1 to 5 μg/ml), Dexa (from 0.05 to 5 μg/ml) or CsA + Dexa in several independent experiments (* for $P < 0.05$; ** for $P < 0.01$). (**B**) The total neurite outgrowth and total cell bodies areas were calculated using a specific template in the Metamorph Software. Neurite outgrowth is indicative of the total length of neuritis (arbitrary unit). The total cells body area estimate the total number of cells, including clusters (arbitrary unit) (* for $P < 0.05$; ** for $P < 0.01$). (**C**) Fold increase of gene expression of vimentin, PAX6, nestin and βIII-tubulin after 7 days of NPC's culture in the presence of CsA and Dexa compared with PBS. The figure is representative of two experiments performed in triplicates (* for $P < 0.05$).

Table 3 Comparison of the immunogenicity of HESC-derived NPC and foetal ReNcell-VM cell line

	HESC-NPC		ReNcell-VM	
IFN-γ	0	1000 U/ml	0	1000 U/ml
[a]Costimulatory molecules	−	−	−	−
[b]Non-classical HLA	−	−	−	−
MHC I	−	+++	−	+++
MHC II	−	−	−	−
[c]MLR	+	+++	+	+++
NK-cell killing	+++	+++	+++	+++
MIC A/B	+	+	++	++
ULBP-1	+	+	+	++
ULBP-2	+	+	+	+
ULBP-3	+	++	+	++
NK killing after blocking of NKG2D and its ligands	+	+	+	+
NK killing with immunosuppressants	+++	+++	+++	+++
Inhibition of neuronal differentiation with immunosuppressants	++	nd	+++	nd

[a]CD40, CD80,CD86, B7H1.
[b]HLA-G, HLA-E.
[c]Mixed lymphocyte reaction.
nd, not done.

significantly neuronal differentiation of ReNcell-VM, as assessed by total neurite outgrowth and total cell bodies area (supplementary Fig. 2). Together, these results confirm the significant immunogenicity *in vitro* of NPC of foetal origin, their targeting by both T lymphocytes and NK cells and the inhibitory effect of the immunosuppressive drugs.

Discussion

These data report that NPC derived from allogeneic HESC or NPC of foetal origin stimulated a strong immune response *in vitro*, suggesting that they might play a part graft rejection after transplantation. We demonstrate the potential importance of NK cells in the rejection process and the role of commonly used immunosuppressive drugs that do not only prevent the killing of NPC by NK cells but also inhibit their maturation into mature neurons.

Cell therapy is a promising approach to curing neurodegenerative disorders like Parkinson's disease [19]. Such an approach would involve preparing NPC *in vitro* and implanting them in the brain before their final differentiation into mature neurons. This concept has been proven feasible in different animal models (reviewed in [1]). Significant progress has been made recently in re-programming autologous cells into pluripotent

stem cells capable of differentiation into neural precursors *in vivo* (reviewed in [20]). However, many problems remain to be addressed before envisaging to use such a strategy in clinical trials [20], and the use of allogeneic HESC still remains an available option.

The risk of rejection is mainly due to the immune system recognizing and killing the transplanted tissue, while in stem cell and solid organ transplantation (SOT) the mechanism of rejection is mainly mediated by T cells [21, 22]. The role of NK cells in the rejection process is emerging. In haematopoietic stem cell transplantation the role of NK cells in the graft-*versus*-host reaction has been clearly demonstrated in rodents and by human studies [23, 24]. In xeno-transplantation, NK cells have been shown to play an important role and should be targeted by immunosuppressive regimens [15]. In human SOT, NK cells also play a role but it is less marked than that of T cells in immune rejection [25]. Our data highlight the importance of NK cells in destroying neural precursors by a mechanism that does not depend on MHC class I, but which is, at least to some extent, mediated by the C-type lectin-activating receptor NKG2D. NKG2D has been implicated in the killing of tumours, and its different ligands, MICA A/B and ULBP1,2,3, are expressed constitutively at the cell surface or induced by stress [26, 27]. Our data illustrate that by blocking NKG2D or its ligands expressed at the surface of NPC, the elimination of such precursors can be prevented. Similar experiments consisting of blocking the natural cytotoxic receptors (NCR) NKp44, NKp46 and NKp30 [6], failed to inhibit the killing of HESC-NPC and ReNcell-VM (data not shown). The possibilities of genetic modification of NPC in reducing the expression of NKG2D ligands with a view to preventing the killing activity of NK should be explored.

These observations corroborate a recent animal study that demonstrates the important role of NK cells and NKG2D by killing teratoma induced by ESC in a mouse model of transplantation [28]. The brain is supposed to be an anatomical site with a certain degree of immune privilege [29], raising the question of the relevance of our *in vitro* results to immune rejection *in vivo* after transplantation. Although the alloreactivity in the brain is poorly described, the immune reaction of a recipient to transplanted cells was already reported some 20 years ago [30, 31].

The brain is composed of different cell types of which several have the potential to present antigens and to induce an alloimmune response. The presence of T and NK cells has been demonstrated in numerous rodent and human studies [32, 33] and the autoimmunity of the brain has been well described [33]. Finally, the stereotactic surgical procedure in NPC transplantation induces a breach of the haemato-encephalic barrier and, as a consequence, is thought to contribute to local inflammation.

For these reasons, the first clinical trial protocols of foetal tissue transplantation featured immunosuppressive drugs administered to the transplant recipients [34, 35]. Classical immune suppressants like steroids or calcineurin inhibitors (*i.e.* cyclosporine) are a logical choice, but our data strongly suggest that they do not prevent cell lysis by NK. Several reports have already shown that *in vitro* human NK cells were not significantly affected by immunosuppressive drugs [36–38], but this has never been reported in the context of HESC or NPC. More important, our data demonstrate that CsA and Dexa inhibit neuronal differentiation at different levels. First, drugs hamper the maturation of neuronal cells since (*i*) their appearance is delayed, (*ii*) their morphology and phenotype match more precursor or intermediate cells rather than mature neurons, (*iii*) they result in few neuronal networks. Besides, drugs reduce the proliferation and differentiation process of NPC, the number of undifferentiated NPC and neurons being decreased, just like the number of clusters of mature neurons.

As expected, Dexa decreased the secretion of IFN-γ by NK cells [39]. Corticoids are involved in many different biological pathways that are subject to DNA-dependent regulation like cytokine transcription or non-genomic activation [39]. It is therefore unlikely that just one protein or molecule should achieve the inhibition of differentiation that we observed in the presence of Dexa alone. In contrast, the main target of CsA is cyclophillin A. The cyclosporin A–cyclophillin A complex inhibits a calcium calmodulin-dependent phosphatase – calcineurin, whose inhibition suppresses T-lymphocyte activation [40]. Previous reports emphasize the importance of cyclophillin A in the differentiation of human and mouse neuronal cells [41, 42]. Chiu *et al.* have demonstrated that the neuronal differentiation correlated with the nuclear translocation of cyclophillin A, and the abrogation of endogenous cyclophillin A expression dramatically reduced the expression of neural-specific markers like βIII-tubulin. Therefore, interference with calcineurin seems to be strongly deleterious to neural differentiation and cyclosporine or steroids might not be the best choice for NPC transplantation. The different stages of neurogenesis are very complex [43], and additional studies need to be performed to determine which stages of the differentiation process are targeted by CsA and Dexa. Interestingly, our results corroborate a previous study consisting of a xenogenic model of mice bone marrow stromal cells (BMSC) transplanted into rat brain with or without CsA. Mice BMSC can survive in intact and stroke brain, and may even exhibit long-distance migration and increased outgrowth processes in the group without CsA [44]. Further studies are necessary to determine if other calcineurin inhibitors like tacrolimus have a similar effect.

In conclusion, the ideal immunosuppressive regimen after transplantation would inhibit killing activity by NK cells while preserving the neuronal differentiation of NPC *in vivo*.

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References

1. **Morizane A, Li JY, Brundin P.** From bench to bed: the potential of stem cells for the treatment of Parkinson's disease. *Cell Tissue Res.* 2008; 331: 323–36.
2. **Grinnemo KH, Kumagai-Braesch M, Mansson-Broberg A, *et al.*** Human embryonic stem cells are immunogenic in allogeneic and xenogeneic settings. *Reprod Biomed Online.* 2006; 13: 712–24.
3. **Drukker M, Katz G, Urbach A, *et al.*** Characterization of the expression of MHC proteins in human embryonic stem cells. *Proc Natl Acad Sci USA.* 2002; 99: 9864–9.
4. **Odeberg J, Piao JH, Samuelsson EB, *et al.*** Low immunogenicity of in vitro-expanded human neural cells despite high MHC expression. *J Neuroimmunol.* 2005; 161: 1–11.
5. **Li L, Baroja ML, Majumdar A, *et al.*** Human embryonic stem cells possess immune-privileged properties. *Stem Cells.* 2004; 22: 448–56.
6. **Lanier LL.** NK cell recognition. *Annu Rev Immunol.* 2005; 23: 225–274.
7. **Parham P.** MHC class I molecules and KIRs in human history, health and survival. *Nat Rev Immunol.* 2005; 5: 201–214.
8. **Bashirova AA, Martin MP, McVicar DW, *et al.*** The killer immunoglobulin-like receptor gene cluster: tuning the genome for defense. *Annu Rev Genomics Hum Genet.* 2006; 7: 277–300.
9. **Drukker M.** Immunogenicity of embryonic stem cells and their progeny. *Methods Enzymol.* 2006; 420: 391–409.
10. **Spencer DD, Robbins RJ, Naftolin F, *et al.*** Unilateral transplantation of human fetal mesencephalic tissue into the caudate nucleus of patients with Parkinson's disease. *N Engl J Med.* 1992; 327: 1541–48.
11. **Winkler C, Kirik D, Bjorklund A.** Cell transplantation in Parkinson's disease: how can we make it work? *Trends Neurosci.* 2005; 28: 86–92.
12. **Preynat-Seauve O, Suter DM, Tirefort D, *et al.*** Development of human nervous tissue upon differentiation of embryonic stem cells in three dimensional culture. *Stem Cells.* 2009; 27: 509–20.
13. **Kraus AB, Shaffer J, Toh HC, *et al.*** Early host CD8 T-cell recovery and sensitized anti-donor interleukin-2-producing and cytotoxic T-cell responses associated with marrow graft rejection following nonmyeloablative allogeneic bone marrow transplantation. *Exp Hematol.* 2003; 31: 609–21.
14. **Uhrberg M.** The CD107 mobilization assay: viable isolation and immunotherapeutic potential of tumor-cytolytic NK cells. *Leukemia* 2005; 19: 707–9.
15. **Kaufman DB, Shapiro R, Lucey MR, *et al.*** Immunosuppression: practice and trends. *Am J Transplant.* 2004; 4: 38–53.
16. **Barbari AG, Masri MA, Stephan AG, *et al.*** Cyclosporine lymphocyte maximum level: a new alternative for cyclosporine monitoring in kidney transplantation. *Exp Clin Transplant.* 2005; 3: 293–300.
17. **Donato R, Miljan EA, Hines SJ, *et al.*** Differential development of neuronal physiological responsiveness in two human neural stem cell lines. *BMC Neurosci.* 2007; 8: 36.
18. **Hoffrogge R, Mikkat S, Scharf C, *et al.*** 2-DE proteome analysis of a proliferating and differentiating human neuronal stem cell line (ReNcell VM). *Proteomics.* 2006; 6: 1833–47.
19. **Newman MB, Bakay RA.** Therapeutic potentials of human embryonic stem cells in Parkinson's disease. *Neurotherapeutics.* 2008; 5: 237–51.
20. **Jaenisch R, Young R.** Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. *Cell.* 2008; 132: 567–582.
21. **Rogers NJ, Lechler RI.** Allorecognition. *Am J Transplant.* 2001; 1: 97–102.
22. **Villard J.** Immunity after organ transplantation. *Swiss Med Wkly.* 2006; 136: 71–7.
23. **Ruggeri L, Capanni M, Urbani E, *et al.*** Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science.* 2002; 295: 2097–100.
24. **Ruggeri L, Mancusi A, Capanni M, *et al.*** Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: challenging its predictive value. *Blood.* 2007; 110: 433–40.
25. **Vilches C, Parham P.** Do NK-cell receptors and alloreactivity affect solid organ transplantation? *Transpl Immunol.* 2006; 17: 27–30.
26. **Moretta A, Bottino C, Vitale M, *et al.*** Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annu Rev Immunol.* 2001: 19; 197–223.
27. **Moretta L, Bottino C, Pende D, *et al.*** Human natural killer cells: molecular mechanisms controlling NK cell activation and tumor cell lysis. *Immunol Lett.* 2005; 100: 7–13.
28. **Dressel R, Schindehutte J, Kuhlmann T, *et al.*** The tumorigenicity of mouse embryonic stem cells and in vitro differentiated neuronal cells is controlled by the recipients' immune response. *PLoS ONE.* 2008; 3: e2622.
29. **Simpson E.** A historical perspective on immunological privilege. *Immunol Rev.* 2006; 213: 12–22.
30. **Mason DW, Charlton HM, Jones AJ, *et al.*** The fate of allogeneic and xenogeneic neuronal tissue transplanted into the third ventricle of rodents. *Neuroscience.* 1986; 19: 685–94.
31. **Widner H, Brundin P.** Immunological aspects of grafting in the mammalian central nervous system. A review and speculative synthesis. *Brain Res.* 1988; 472: 287–324.
32. **Moynihan JA, Santiago FM.** Brain behavior and immunity: twenty years of T cells. *Brain Behav Immun.* 2007; 21: 872–80.
33. **Segal BM.** The role of natural killer cells in curbing neuroinflammation. *J Neuroimmunol.* 2007; 191: 2–7.
34. **Olanow CW, Goetz CG, Kordower JH, *et al.*** A double-blind controlled trial of bilateral fetal nigral transplantation in Parkinson's disease. *Ann Neurol.* 2003; 54: 403–14.
35. **Freed CR, Greene PE, Breeze RE, *et al.*** Transplantation of embryonic dopamine neurons for severe Parkinson's disease. *N Engl J Med.* 2001; 344: 710–9.
36. **Chiossone L, Vitale C, Cottalasso F, *et al.*** Molecular analysis of the methylprednisolone-mediated inhibition of NK-cell function: evidence for different susceptibility of IL-2- versus IL-15-activated NK cells. *Blood.* 2007; 109: 3767–75.
37. **Wang H, Grzywacz B, Sukovich D, *et al.*** The unexpected effect of cyclosporin A on CD56+. *Blood. 2007*; 110: 1530–9.
38. **Wai LE, Fujiki M, Takeda S, *et al.*** Rapamycin, but not cyclosporine or FK506, alters natural killer cell function. *Transplantation.* 2008; 85: 145–9.
39. **Rhen T, Cidlowski JA.** Antiinflammatory action of glucocorticoids–new mechanisms for old drugs. *N Engl J Med.* 2005; 353: 1711–23.
40. **Liu J, Farmer JD, Lane WS, *et al.*** Calcineurin is a common target of cyclophillin-cyclosporin A and FKBP-FK506 complexes. *Cell.* 1991; 66: 807–15.

41. **Nahreini P, Hovland AR, Kumar B, *et al.*** Effects of altered cyclophilin A expression on growth and differentiation of human and mouse neuronal cells. *Cell Mol Neurobiol.* 2001; 21: 65–79.
42. **Chiu R, Rey O, Zheng JQ, *et al.*** Effects of altered expression and localization of cyclophilin A on differentiation of p19 embryonic carcinoma cells. *Cell Mol Neurobiol.* 2003; 23: 929–43.
43. **Suter DM, Krause KH.** Neural commitment of embryonic stem cells: molecules, pathways and potential for cell therapy. *J Pathol.* 2008; 215: 355–68.
44. **Irons H, Lind JG, Wakade CG, *et al.*** Intracerebral xenotransplantation of GFP mouse bone marrow stromal cells in intact and stroke rat brain: graft survival and immunologic response *Cell Transplant.* 2004; 13: 283–94.