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Role of Natural-Killer Group 2 Member D Ligands and Intercellular Adhesion Molecule 1 in Natural Killer Cell-Mediated Lysis of Murine Embryonic Stem Cells and Embryonic Stem Cell-Derived Cardiomyocytes

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ABSTRACT

The transplantation of cardiomyocytes derived from embryonic stem (ES) cells into infarcted heart has been shown to improve heart function in animal models. However, immune rejection of transplanted cells may hamper the clinical application of this approach. Natural killer (NK) cells could play an important role in this process in both autologous and allogeneic settings by eliminating cells expressing low levels of major histocompatibility complex (MHC) class I molecules. Here we characterize embryonic stem cell-derived cardiomyocytes (ESCM) in terms of their sensitivity to NK cells. We show that despite expression of very low levels of MHC class I molecules, murine ESCM were neither recognized nor lysed by activated syngeneic NK cells in vitro. In contrast, undifferentiated ES cells expressing similarly low levels of MHC class I molecules as ESCM were recognized and lysed by NK cells. This differential susceptibility results from the differential expression of ligands for the major activating natural killer cell receptor natural-killer group 2 member D (NKG2D) and intercellular adhesion molecule 1 (ICAM-1) on ES cells versus ESCM. NKG2D ligands and ICAM-1 were expressed on ES cells but were absent from ESCM. Undifferentiated ES cells were lysed by NK cells in a perforin-dependent manner. However, simultaneous blockade of NKG2D and ICAM-1 by antibodies inhibited this killing. These data suggest that in the course of differentiation ESCM acquire resistance to NK cell-mediated lysis by downregulating the expression of ligands required for activation of NK cell cytotoxicity. STEM CELLS *2009;27:307–316*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Cardiomyocytes derived from murine or human embryonic stem (ES) cells have been extensively examined as a source of cells for regenerating damaged cardiac tissue. In experimental animals these cells have been shown to integrate into host myocardium [1, 2], improve the function of infarcted heart [3–6], and act as a rate-responsive biological pacemaker when injected into hearts with complete atrioventricular block [7, 8]. Despite these promising results, the success of this therapeutic approach will strongly depend on long-term integration of transplanted embryonic stem cell-derived cardiomyocytes (ESCM) into the diseased tissue. Among other factors, immunologic barriers of both the innate and the adaptive immune system might prevent sustained engraftment of transplanted ESCM. Therefore, immunologic properties of these cells need to be characterized.

As an element of the innate immune system, natural killer (NK) cells can be highly cytotoxic without prior immune sensitization to autologous cells expressing low levels of major histocompatibility complex (MHC) class I molecules, as well as to allogeneic cells. The cytolytic activity of NK cells is triggered after integration of signals transmitted from their activating and inhibitory receptors [9]. The engagement of inhibitory receptors by self-MHC class I molecules provides protection for target

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cells that express normal levels of self-MHC molecules on their surface. Accordingly, downregulation of MHC class I molecules on cells infected with certain viruses and on cancer cells can lead to their elimination by NK cells providing that target cells express ligands for stimulatory NK cell receptors [10–12]. Potent stimulatory NK cell receptors include the C-type lectin-like receptor natural-killer group 2 member D (NKG2D). NKG2D is expressed on all resting and activated NK cells of murine and human origin. Another family of activating receptors on NK cells are natural cytotoxicity receptors (NCR), such as NCR1 in mice (also called Ly94 or Mar-1) and their human homologs NKp46, NKp44, and NKp30. Although endogenous ligands for NCR have not been identified yet, NKG2D binds to a family of ligands with structural homology to MHC class I proteins. In mice they belong to a family of five retinoic acid early inducible 1 (Rae-1) proteins (α, β, γ, δ, and ε), as well as the minor histocompatibility protein H-60 and murine UL-16-binding-protein-like transcript 1 (MULT1). Human ligands for NKG2D are the MHC-class I polypeptide-related sequence A and B protein family and the cytomegalovirus UL16-binding protein family. These ligands are expressed in some fetal tissues but not in most adult healthy tissues [13]. However, they can be induced by various types of cellular stress, such as DNA damage, hypoxia, acidosis, inflammation, and heat shock or in response to viral infection or malignant transformation [14, 15]. The expression of these ligands on the surface of some cells can lead to their lysis by NK cells even in the presence of inhibitory MHC class I molecules [16], indicating that they are potent determinants of target cell susceptibility to NK cell killing.

Besides their role in immunosurveillance of virally infected or malignant cells, NK cells also mediate rejection of allogeneic bone marrow transplants [17–20]. In addition, recent studies suggest that NK cells may participate in the acute and chronic rejection of solid organ allografts and xenografts [21]. These data suggest that NK cells may also prevent engraftment of ES cell derivatives. This may be true not only for allogeneic cells but also for autologous grafts expressing low levels of MHC class I molecules, as it may be the case with derivatives of autologous pluripotent stem cells recently established from adult somatic cells (termed induced pluripotent stem cells) [22–25] or pluripotent germline stem cells of the testis [26, 27]. However, little is known about the interaction between NK cells and tissue-specific ES cell derivatives. NK cells have been shown to prevent the engraftment of the hematopoietic progeny of murine ES cells in syngeneic recipients, as well as of hematopoietic derivatives of human ES cells xenotransplanted in NOD/SCID mice [28, 29]. ESCM have not been characterized in this context yet, most probably because of difficulties in obtaining cardiomyocytes from ES cells in sufficient purity and quantity for these types of analyses.

We have recently generated a transgenic murine D3-derived ES cell line (αPIG) for the production of highly purified preparations of cardiac myocytes using an antibiotic selection strategy [3]. Here we characterize the immunologic properties of these cells and show that both ESCM and ES cells express comparable, very low levels of MHC class I molecules. However, only undifferentiated ES cells, not ESCM, were recognized and lysed by NK cells. This was due to high-level expression of activating NKG2D ligands and intercellular adhesion molecule 1 (ICAM-1) by ES cells but not by ESCM. These results demonstrate that immunological characteristics of ES cells can rapidly change during differentiation into specific cell types. Our data suggest that NK cells may not impair the engraftment of ESCM in vivo but may participate in elimination of ES cells.

Materials and Methods

Cells and Mice

The transgenic murine ES cell line αPIG44, derived from D3 ES cells, and the wild-type ES cell lines CCE, HM1, and Bruce4 were maintained on irradiated mouse embryonic fibroblasts (MEF) in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum (FBS), 1× nonessential amino acids, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and 1,000 U/ml leukemia inhibitory factor ([LIF]; ESGRO; Chemicon, Temecula, CA, http://www.chemicon.com). Murine CGR8 ES cells were cultivated on 0.1% gelatin-coated tissue culture plates in Glasgow modified Eagle's medium supplemented with 10% FBS, 1× nonessential amino acids, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and 100 U/ml LIF. All ES cell lines used in this study are of H-2^b MHC haplotype. The YAC-1 lymphoma cells were grown in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% FBS. All cell culture media and reagents were supplied by Invitrogen (Karlsruhe, Germany, http://www.invitrogen.com). Where indicated, cells were incubated with 20 ng/ml interferon γ (IFN-γ) or 1 μM retinoic acid (RA) for 24 or 48 hours prior to analysis as indicated in the figure legends. C57BL/6 (H-2^b) and BALB/c (H-2^d) mice were originally obtained from Charles River Laboratories (Sulzfeld, Germany, http://www.criver.com) and were bred and maintained in a pathogen-free colony unit of our animal facility until use. The use of mice was approved by the ethics committee of the government of Cologne and was performed according to German animal protection law.

Generation of ES Cell-Derived Cardiomyocytes

ESCM were generated from a transgenic clone of D3 ES cells (αPIG44) by modification of a previously used mass culture protocol [3]. Cardiomyocytes that can be obtained from this transgenic ES cell clone after puromycin selection are predominantly of atrial-like and pacemaker-like type [3]. To initiate the ES cell differentiation, 1×10^6 ES cells were suspended in 14 ml of differentiation medium (IMDM supplemented with 20% FBS, 10 μM 2-mercaptoethanol, and 1× nonessential amino acids) and incubated in nonadherent plates under continuous horizontal agitation to allow formation of embryoid bodies (EB). After 2 days the EB were diluted into fresh nonadherent plates to a density of 1,000 EB per 14 ml of differentiation medium per plate. The differentiation of EB continued on a horizontal shaker without medium change until enhanced green fluorescent protein (EGFP)-positive EB occurred on days 8–9 after differentiation. At this time, fresh medium supplemented with puromycin (10 μg/ml) was added to select for ESCM. Medium containing puromycin was changed every 2–3 days until pure beating cardiac clusters were collected. For analyses cardiac clusters were enzymatically dissociated into single ESCM after 2–3 days of puromycin treatment (i.e., on day 11 of differentiation) to obtain early-stage ESCM or after 7 or 15 days of puromycin treatment (i.e., on day 16 or 24 of differentiation) to obtain late-stage ESCM [3, 30].

Immunocytochemical Analysis

Puromycin-selected αPIG44 ES cell-derived cardiac clusters were dissociated by 0.05% trypsin/0.2 g/l EDTA treatment, and cardiac myocytes plated on 3.5 μg/ml fibronectin-coated μ-Dish$^{35\ mm,\ low}$ (Ibidi GmbH, Munich, Germany, http://www.ibidi.de) to allow adherence. Cells were fixed in warm 4% buffered paraformaldehyde, permeabilized by Triton X-100, blocked in 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), and stained overnight at 4°C with cardiospecific sarcomeric α-actinin antibodies (clone EA-53, 1:500; Sigma-Aldrich, Steinheim, Germany, http://www.sigmaaldrich.com). After being washed, the sections were incubated with Alexa Fluor 555-conjugated secondary antibodies (Invitrogen) for 1 hour at room temperature. Samples were counterstained with nuclear dye Hoechst 33342 (1:5,000; Invitrogen) mounted to Aqua-Poly/Mount (Polysciences Inc., Eppelheim, Germany, http://www.polysciences.com) to prevent fading and examined using an Axiovert 200M fluorescence microscope and Axiovision 4.5 software (Carl Zeiss, Jena, Germany, http://www.zeiss.com).

Flow Cytometric Analyses

Undifferentiated ES cell colonies and cardiac clusters were dissociated by trypsinization, and 2–5 $\times$ 10^5 cells were stained for 30 minutes at 4°C in 100 μl of 0.1% BSA in PBS containing an appropriate dilution of a desired antibody. Flow cytometric analyses were performed on a FACScan (BD Biosciences, Heidelberg, Germany, http://www.bdbiosciences.com), and data were processed using CellQuest software (BD Pharmingen, San Diego, http://www.bdbiosciences.com/index_us.shtml). Dead cells were excluded by gating on viable cells on the basis of staining with propidium iodide or 7-aminoactinomycin D. At least 10,000 events were acquired for each analysis. Primary antibodies used were phycoerythrin (PE)-conjugated anti-H-2K^b (clone AF6-88.5; BD Pharmingen), PE-conjugated anti-H-2D^b (clone 28-14-8; BD Pharmingen), PE-conjugated anti-CD178/FasL (clones MFL3 and MFL4; eBioscience Inc., San Diego, http://www.ebioscience.com), NKp46/NCR1/Fc chimera (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany, http://www.rndsystems.com), and anti-ICAM1/CD54 (clone YN1/1.7.4; eBioscience/NatuTec GmbH, Frankfurt, Germany, http://www.natutec.com). PE-conjugated NKG2D tetramers were generated as described previously [31, 32]. PE-conjugated secondary antibodies and all isotype control antibodies were purchased from BD Pharmingen or Santa Cruz Biotechnology Inc. (Santa Cruz, CA, http://www.scbt.com).

Reverse Transcription-Polymerase Chain Reaction Analyses

Total RNA was isolated from undifferentiated ES cells, EB at different stages of differentiation, and intact cardiac clusters collected at different time points after initiation of puromycin selection using an RNAqueous kit (Ambion, Darmstadt, Germany, http://www.ambion.com) according to the manufacturer's instructions. For analyses with ES cells maintained on feeders, ES cells were depleted of MEF by serial passaging (three times) on gelatin-coated plates prior to RNA isolation to avoid amplifying MEF-specific transcript. In some experiments, the RNA composed of total RNA from 11 mouse cell lines was used for positive controls (QPCR Mouse Reference Total RNA; Stratagene, La Jolla, CA, http://www.stratagene.com). One microgram of DNase I-treated RNA was reverse-transcribed by Superscript II RTase (Invitrogen) using random hexamers for priming. cDNA was amplified using JumpStart REDTaq ReadyMix polymerase chain reaction mix (Sigma-Aldrich). Negative controls were generated in reverse transcription (RT) reactions in which all reaction components were included except reverse transcriptase. Reactions were terminated at the exponential phase of amplification, and products were analyzed by electrophoresis on ethidium bromide-stained 1.5% SeaKem LE agarose gels (Cambrex, Taufkirchen, Germany, http://www.cambrex.com).

NK Cell Cytotoxicity Assays

Target cells (1×10^6) were labeled with $Na_2{}^{51}CrO_4$ (Hartmann-Analytic GmbH, Braunschweig, Germany, http://www.hartmann-analytic.de) for 1 hour at 37°C and plated in 96-well round-bottomed plates at 3×10^3 targets per well. Poly(I:C)-activated NK cells were plated at the appropriate densities to achieve the desired effector-to-target (E:T) ratios. After 4 hours of incubation, cell culture supernatants (100 μl per well) were removed, and radioactivity was determined in a gamma counter (PerkinElmer Life and Analytical Sciences, Rodgau-Jügesheim, Germany, http://www.perkinelmer.com). The percentage of specific ^{51}Cr-release was determined by the following equation: specific lysis (%) = [(experimental release − spontaneous release)/(maximum release − spontaneous release)] $\times$ 100. In some experiments, the cytotoxicity assay was performed in the presence of saturating amounts of neutralizing antibodies specific for NKG2D (30 μg/ml; clone 191004; R&D Systems) and/or ICAM-1/CD54 (30 μg/ml). Prior to coculture, cells were preincubated for 30 minutes at 37°C with the indicated antibodies. The spontaneous release was <20% in all cytotoxic assays. All groups were run in triplicate, and all experiments were performed at least three times.

Activation and Isolation of NK Cells

In vivo activation of NK cells was performed by i.p. injection of poly(I:C) (200 μg per mouse; Sigma-Aldrich) into wild-type or perforin-deficient (per$^{-/-}$) C57BL/6 mice 48 hours before mice were sacrificed and splenocytes isolated. After lysis of erythrocytes, NK cells were purified using a positive-selection magnetic cell sorting kit (Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com) with antibodies directed against CD49b molecules (clone DX5) specifically expressed on NK cells. The purity of NK cells after selection was determined by flow cytometry and was always greater than 90%.

B3Z Hybridoma Assay

Hybridoma assay was performed as described previously [33]. αPIG44 ES cell-derived cardiac clusters plated on fibronectin-coated plates were exogenously loaded with 1 μM synthetic peptide SIINFEKL for 60 minutes and subsequently washed to remove the excess of SIINFEKL before addition of B3Z cells (2×10^5 cells per well). After incubation for 16–20 hours at 37°C/5% CO_2, the activation of *LacZ* reporter in B3Z cells was determined by the β-galactosidase staining kit (Invitrogen) following the manufacturer's instructions.

Interferon-γ Enzyme-Linked Immunosorbent Assay

The concentration of IFN-γ released by poly(I:C)-activated NK cells into medium after 24 or 48 hours of coincubation alone or with various target cells at an E:T ratio of 50:1 was determined in the cell culture supernatant using Duo Flow IFN-γ ELISA Kit (R&D Systems) according to the manufacturer's instructions. In one group, ESCM were first pretreated with exogenously added IFN-γ (20 ng/ml) for 48 hours to upregulate ICAM-1 expression. Prior to incubation with NK cells these cardiomyocytes were washed extensively to remove the added cytokine and further incubated with NK cells for an additional 24 or 48 hours. At the end of incubation, the cell culture supernatant was collected for IFN-γ measurements. Murine recombinant IFN-γ provided in the kit was used to generate the standard curve.

RESULTS

Generation of Purified ESCM

EB are three-dimensional cell aggregates formed by spontaneously differentiating ES cells. They contain a heterogeneous mixture of cells derived from all three embryonic germ layers and comprise, at most, a few percent of cardiomyocytes [3]. To obtain large amounts of purified cardiomyocytes for their immunological characterization we used the transgenic ES cell clone αPIG44 that expresses the puromycin resistance protein *N*-acetyltransferase and the EGFP under the control of the cardiomyocyte-specific α-myosin heavy chain promoter [3].

Scoring for EGFP-expressing areas in EB revealed that 47% $\pm$ 17% of all EB were positive for EGFP in αPIG44 cultures on days 9 or 10 (cumulative data of six independent experiments with 2,753 EB). Upon addition of puromycin to such EB cultures the noncardiogenic cells were largely eliminated within 3 days, yielding purified cardiac clusters (Fig. 1A). In untreated EB, the frequency of viable EGFP-positive ESCM at day 9 of differentiation varied between 1% and 10% in different cultures (Fig. 1B). However, after 7 days of puromycin selection most of the remaining viable cells were EGFP-positive cardiomyocytes (Fig. 1B), yielding, on average, 3.1 $\pm$ 2.4 cardiomyocytes per ES cell initially taken for differentiation (n = 9 independent differentiations). When these ESCM were plated on fibronectin-coated plates and stained with α-actinin-specific antibodies to verify their purity, 353 of 354 cells scored positive for this cardiac protein (Fig. 1C). The purity of ESCM was also confirmed by RT-polymerase chain reaction (PCR).

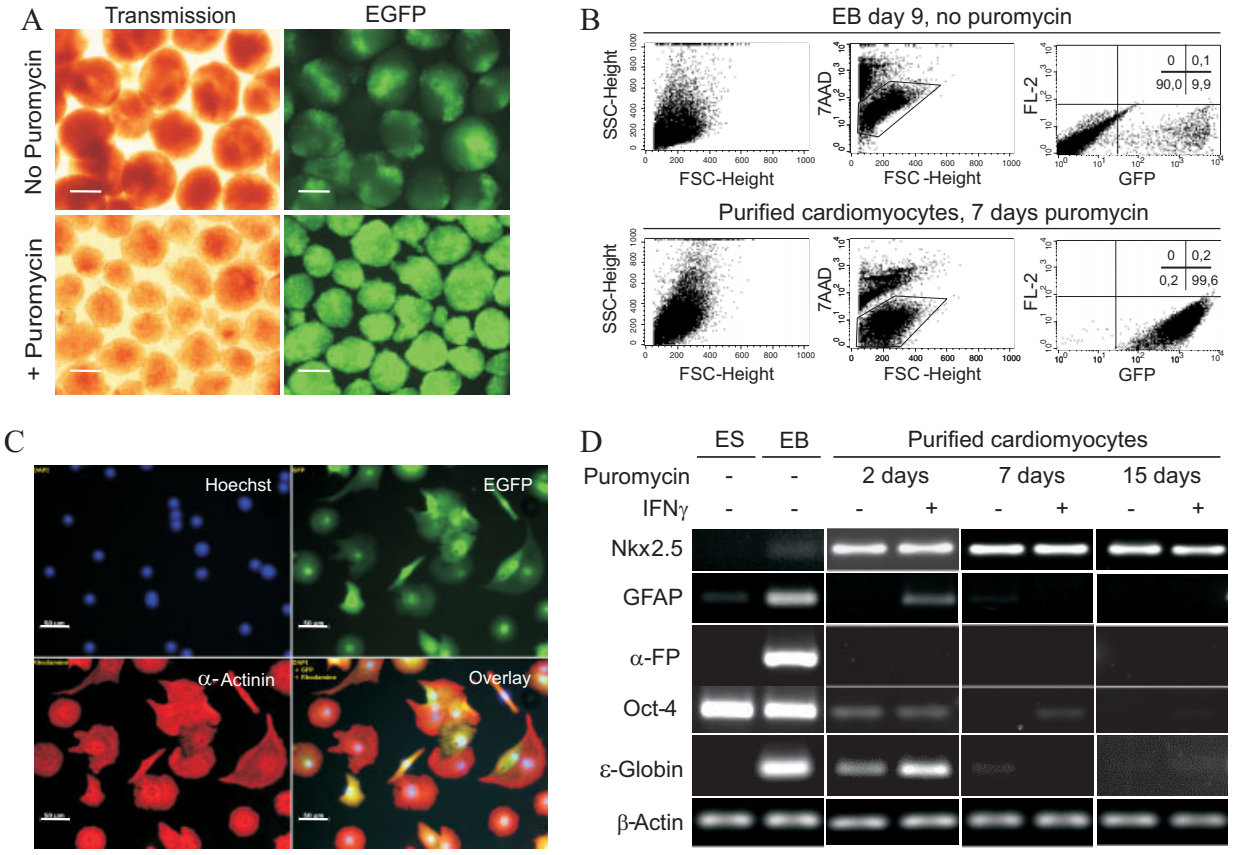

Figure 1. Differentiation and purification of embryonic stem cell-derived cardiomyocytes (ESCM). **(A):** αPIG44 ES cells were induced to differentiate in suspension cultures until embryoid bodies (EB) containing EGFP-positive beating areas appeared on day 8 or 9 of differentiation (upper panels). At this time, puromycin was added for 2 days to eliminate noncardiac cells and generate beating clusters of pure ESCM (lower panels). Scale bars = 200 μm. **(B):** Frequency of EGFP-positive ESCM in nonpurified day 9 EB cells (upper panels) and purified day 16 ESCM treated for 8 days with puromycin (lower panels) was determined by flow cytometry. Approximately 10,000 viable cells were selected for each analysis by gating out 7AAD-positive dead cells (middle panels in both rows). **(C):** Purified day 16 ESCM were plated on fibronectin-coated dishes and stained for α-actinin to determine the percentage of cardiac and noncardiac cells. Of 354 cells, 353 were positive for α-actinin. Scale bars = 50 μm. **(D):** Expression of cardiomyocyte-specific genes and transcripts typically expressed in other cells or tissues was determined by reverse transcription-polymerase chain reaction (RT-PCR) using total RNA isolated from undifferentiated αPIG44 ES cells, whole EB, or ESCM selected by puromycin treatment for 2, 7, or 15 days (days 10, 16, and 24 of differentiation). RT-PCR controls prepared without reverse transcriptase gave no signals (not shown). The amplification of Nkx2.5 and β-actin transcripts was terminated at the exponential phase to better reveal the differences between samples, whereas GFAP, α-FP, Oct-4, and ε-globin transcripts were amplified for 39 cycles to assess with high sensitivity their presence in purified ESCM. Nkx2.5 is a cardiospecific transcription factor, GFAP is an ectodermal marker, α-FP is an endodermal marker, Oct-4 is a marker for undifferentiated ES cells, ε-globin is a marker for erythroid lineage (mesodermal lineage), and β-actin was used as a housekeeping gene. Abbreviations: 7AAD, 7-aminoactinomycin D; EGFP, enhanced green fluorescent protein; ES, embryonic stem; α-FP, α-fetoprotein; FL-2, fluorescence channel 2; FSC, forward scatter; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; IFN, interferon; SSC, sideward scatter.

The expression of transcripts specific for noncardiac cell lineages progressively declined with the duration of puromycin treatment, reaching almost undetectable levels after 7 and 15 days of selection (Fig. 1D).

Expression of MHC Molecules on ESCM

We have previously shown that undifferentiated murine ES cells and cells of EB up to day 20 of differentiation express very low levels of MHC class I molecules on their surface [33]. RT-PCR analyses revealed that both early- and late-stage ESCM constitutively express genes coding for MHC class I heavy chain and β2-microglobulin and strongly increase their expression upon treatment with IFN-γ (Fig. 2A). However, H-2K^b or H-2D^b MHC class I molecules were not detectable on the surface of puromycin-selected αPIG44 ESCM by flow cytometry regardless of the maturation status of ESCM between days 10 and 24 of differentiation (Fig. 2B; supporting information Fig. 1; data not shown). Treatment with IFN-γ strongly induced surface expression of MHC class I molecules on early- and late-stage ESCM, which contrasts to undifferentiated ES cells (Fig. 2B; supporting information Fig. 1; data not shown). The same results were also obtained for ESCM in intact EB, indicating that the expression of MHC class I molecules on ESCM was not altered by puromycin selection (supporting information Fig. 2).

The finding that transcripts of H-2K^b heavy chain and β2-microglobulin genes were expressed in ESCM but MHC class I molecules were not detectable at the cell surface by flow cytometry required a more sensitive analysis of surface expression. For this purpose the B3Z hybridoma T cells that respond with high sensitivity to H-2K^b molecules presenting the ovalbumin-derived epitope SIINFEKL on the surface of target cells were used [34]. The ESCM obtained after 7 days of puromycin selection and exogenously loaded with SIINFEKL were readily recognized by B3Z cells (Fig. 2C). The high specificity of this reaction was demonstrated in controls that were not loaded with SIINFEKL (Fig. 2C). This assay revealed that ESCM constitutively do express MHC class I molecules on their cell surface at very low levels sufficient for detection by antigen-specific T cells.

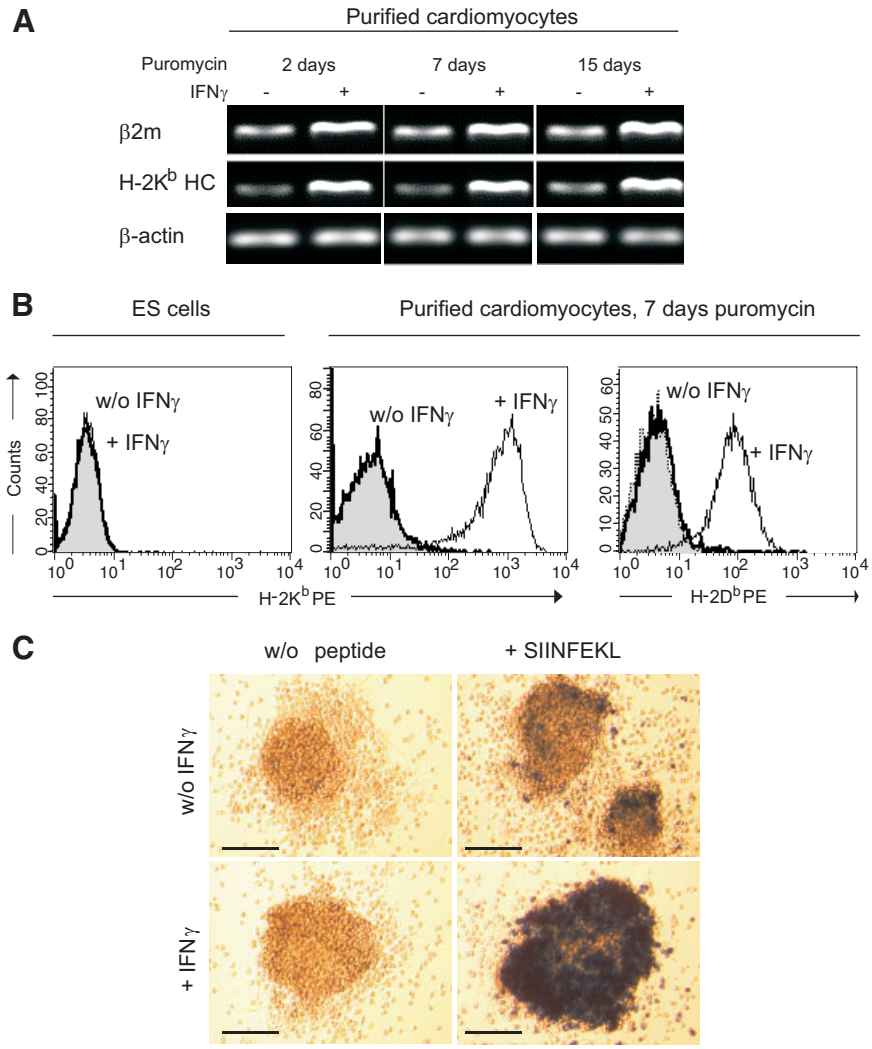

Figure 2. Expression of major histocompatibility complex (MHC) class I molecules on embryonic stem cell-derived cardiomyocytes (ESCM). **(A):** Total RNA was isolated for reverse transcription-polymerase chain reaction (PCR) analysis from puromycin-selected cardiac clusters at day 10, 16, or 24 of differentiation cultured with or w/o IFN-γ. PCR was performed with primers for the heavy chain of MHC class I molecule H-2K^b and β2m. β-Actin served as a loading control. **(B):** Flow cytometric analysis of undifferentiated αPIG44 ES cells and puromycin-selected late-stage ESCM isolated at day 16 of differentiation with (thin lines) or w/o (thick lines) IFN-γ treatment (20 ng/ml for 48 hours). Cells were stained with the indicated antibodies or with the corresponding isotype controls (gray-shaded histograms). All histograms were generated by gating on viable cells. The number of cardiomyocytes analyzed in the middle panel was approximately 10,000, and in the right-hand panel it was approximately 7,000 cells. Dot plot representation of data for H-2K^b molecules is also given in supporting information Figure 1. **(C):** Native or IFN-γ-treated (20 ng/ml for 48 hours) puromycin-selected ES cell-derived cardiac clusters isolated at day 16 of differentiation were left w/o peptide (left panels) or loaded with SIINFEKL (right panels) and incubated overnight with B3Z cells. β-Galactosidase-positive staining (dark blue dots) indicated recognition of H-2K^b/SIINFEKL complexes on ESCM by B3Z cells. Scale bars = 100 μm. Abbreviations: β2m, β2-microglobulin; ES, embryonic stem; HC, heavy chain; IFN, interferon; PE, phycoerythrin; w/o, without.

ESCM Are Not Lysed by Activated NK Cells In Vitro

Fulfilling by very low expression of MHC class I molecules an important prerequisite for lysis by NK cells according to the "missing self" hypothesis [35], ESCM should be ideal targets for NK cells. In comparison with YAC-1 cells as standard NK-sensitive murine target cells, four ES cell lines independently derived from the 129Sv mouse strain (αPIG44, CGR8, CCE, HM1) and one ES cell line (Bruce4) of C57BL/6 origin were lysed by poly(I:C)-activated NK cells approximately two- to fourfold less effectively (Fig. 3A). MEF used for the maintenance of αPIG44, CCE, HM1, and Bruce4 ES cells were not significantly lysed by NK cells (Fig. 3A). ESCM were also not lysed by activated NK cells, regardless of whether syngeneic or allogeneic NK cells were used as effector cells, whether early- or late-stage ESCM were tested, and whether ESCM were pretreated with IFN-γ or not (Fig. 3B; data not shown). NK cell-mediated lysis of both YAC-1 cells and undifferentiated ES cells depended on perforin, because NK cells from perforin-deficient mice were not able to lyse these target cells (Fig. 3C). These data show that undifferentiated ES cells and ESCM are differentially killed by the perforin-dependent cytolytic machinery of activated NK cells, although these target cells express similarly low levels of MHC class I molecules. Considering the activating requirements for NK cells, this might be due to expression of killer cell inhibitory ligands or to a lack of activating ligands on ESCM.

Expression of NK Cell Inhibitory Ligands on ESCM and ES Cells

The cytotoxic activity of NK cells is triggered by integration of signals from inhibitory and stimulatory ligands encountered on the surface of target cells [9]. In addition to MHC class I molecules that interact with inhibitory NK cell receptors of the Ly49 family, inhibitory signals can be provided by the nonclassic MHC class I molecule Qa-1 (homolog to HLA-E in humans) that is recognized by CD94/NKG2 heterodimers on NK cells. The transcripts coding for Qa-1 were expressed at low levels in both MEF-depleted ES cells and ESCM selected for 7 days with puromycin (Fig. 4). This low expression on both cell types suggests that Qa-1 is unlikely to inhibit lysis of ESCM by NK cells. Another mechanism that may underlie the differential sensitivity of ES cells and ESCM to NK cells may be the induction of NK cell apoptosis by the engagement of Fas on NK cells by FasL expressed on ESCM, as proposed earlier [36–38]. To examine this possibility, the expression of FasL was compared at the transcript and protein levels on the surface of ESCM and undifferentiated ES cells. FasL transcripts were expressed at low levels in ES cells but could not be detected in three independent preparations of αPIG44 ESCM even after 40 PCR cycles

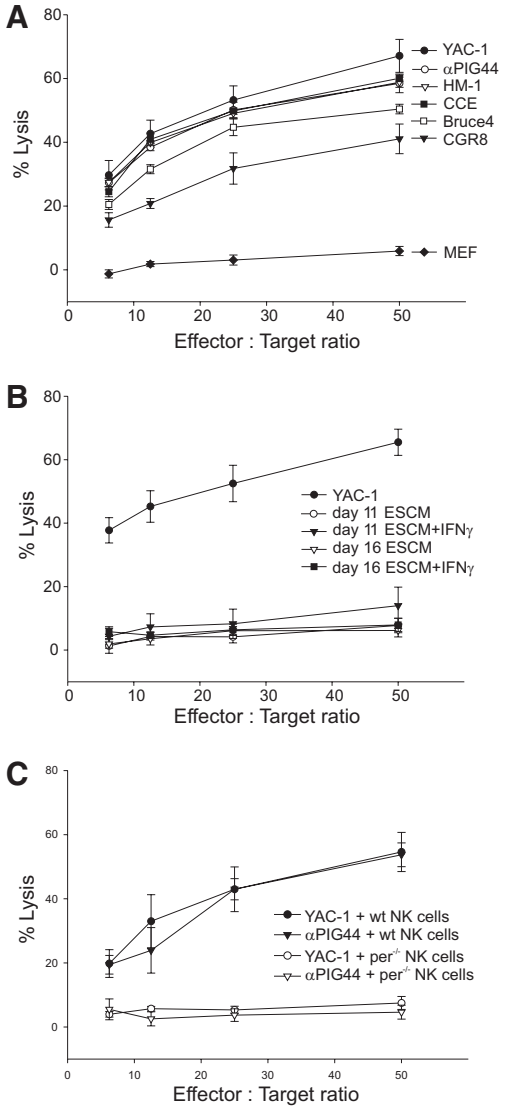

Figure 3. ESCM are not lysed by activated natural killer (NK) cells. A 4-hour ^{51}Cr-release cytotoxicity assay was performed with poly(I:C)-activated syngeneic NK cells (C57BL/6). As target cells, YAC-1 cells **(A–C)**, five independently derived embryonic stem (ES) cell lines (CCE, HM1, αPIG44, CGR8, and Bruce4) **(A)**, MEF **(A)**, and early- and late-stage ESCM **(B)** were used. MEF-dependent ES cell lines contained only a small fraction of contaminating feeders upon trypsinization (5.3% ± 0.7%; $n = 9$). Early-stage ESCM were obtained after 2 days of puromycin selection on day 11 of differentiation, and late-stage ESCM were obtained after 7 days of puromycin selection on day 16 of differentiation. Cardiomyocytes were untreated or treated with IFN-γ for 48 hours at a final concentration of 20 ng/ml. Similar results were obtained with activated allogeneic NK cells from Balb/c mice (not shown). To identify the cytotoxic mechanism NK cells were prepared from poly(I:C)-treated per$^{-/-}$ mice and used in cytotoxicity assay with YAC-1 cells and undifferentiated αPIG44 ES cells **(C)**. Data are given as means ± SEM of at least three independent measurements. Abbreviations: ESCM, embryonic stem cell-derived cardiomyocytes; IFN, interferon; MEF, mouse embryonic fibroblasts; per$^{-/-}$, perforin-deficient.

(Fig. 4). Using flow cytometry, we could not detect FasL protein on any of ES cell lines tested (HM1, CGR8, αPIG44) or on ESCM (data not shown). Therefore, inhibitory signals on ESCM do not appear to explain the differential susceptibility of ESCM and ES cells to NK cell cytotoxicity.

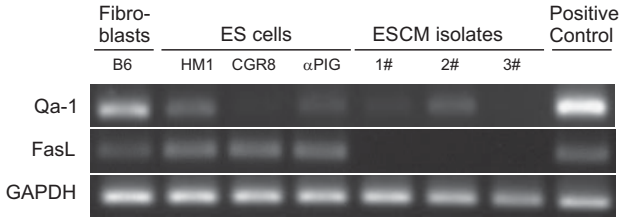

Figure 4. Expression of inhibitory ligands for natural killer cells on ES cell-derived cardiomyocytes (ESCM) and ES cells. Total RNA was prepared for reverse transcription-polymerase chain reaction (PCR) analysis from C57SV fibroblasts, CGR8 ES cells, mouse embryonic fibroblast-depleted HM1 and αPIG44 ES cells, and three independent preparations of late-stage ESCM (lanes 1#–3#) obtained 7 days after puromycin selection on day 16 of differentiation. Commercially available QPCR Mouse Reference Total RNA compiled from 11 different murine cell types served as a positive control. Forty cycles of PCR amplification were used to detect weakly expressed transcripts. Abbreviations: ES, embryonic stem; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Expression of NK Cell Activating and Adhesion Molecules on ESCM and ES Cells

Differential susceptibility of ESCM and ES cells to NK cells might be due to differential expression of activating ligands. Ligands for NCR1 were expressed on YAC-1 cells but were not detectable on the surface of ES cells or ESCM obtained after 7 days of puromycin selection regardless of IFN-γ stimulation, as determined by staining with NCR1/Fc fusion protein (Fig. 5A). Another principal receptor that triggers perforin-dependent NK cell cytotoxicity is the NKG2D receptor. The transcripts of its ligands Rae-1, H-60, and MULT1 were expressed in ESCM and αPIG44 ES cells at similar levels (Fig. 5B). NKG2D tetramers that bind to all these NKG2D ligands were used to determine their expression on ESCM and ES cells. These tetramers strongly labeled YAC-1 cells and to a lesser extent labeled undifferentiated αPIG44 ES cells (Fig. 5A). However, they could not detect NKG2D ligands on ESCM obtained after 7 days of puromycin selection (Fig. 5A). Interestingly, RA strongly upregulated NKG2D expression on ES cells but not on ESCM (Fig. 5A). During the process of differentiation, NKG2D ligands were still expressed to various degrees on EB cells at days 4–16 of differentiation (supporting information Fig. 3). However, the inducibility of their expression by RA was weaker at later stages of differentiation but still detectable (supporting information Fig. 3). IFN-γ did not induce the expression of NKG2D ligands on ES cells or on ESCM even after 3 or 5 days of continuous stimulation (data not shown).

Optimal triggering of perforin-dependent lysis by NK cells via NKG2D receptors requires the interaction of the integrin leukocyte functional antigen 1 (LFA-1) expressed on NK cells and the ICAM-1 on target cells [39]. ES cells express high levels of ICAM-1 on their cell surface (Fig. 5A). However, in the course of differentiation, ICAM-1 levels decreased on EB cells between days 4 and 8 of differentiation but increased again at later time points in most of the cells (supporting information Fig. 3). However, ICAM-1 was not detectable by flow cytometry on purified ESCM (Fig. 5A). Interestingly, IFN-γ treatment for 24 or 48 hours resulted in strong induction of ICAM-1 molecules on approximately 50% of ESCM (Fig. 5A; supporting information Fig. 4) but had only a marginal effect on its expression on nonselected EB cells at all differentiation stages tested (days 4–16) (supporting information Fig. 3). IFN-γ also did not increase ICAM-1 on ES cells, which already constitutively expressed high levels of these molecules. These data suggest that the lack of both NKG2D ligands and ICAM-1 on untreated ESCM might prevent their lysis by NK cells and that isolated

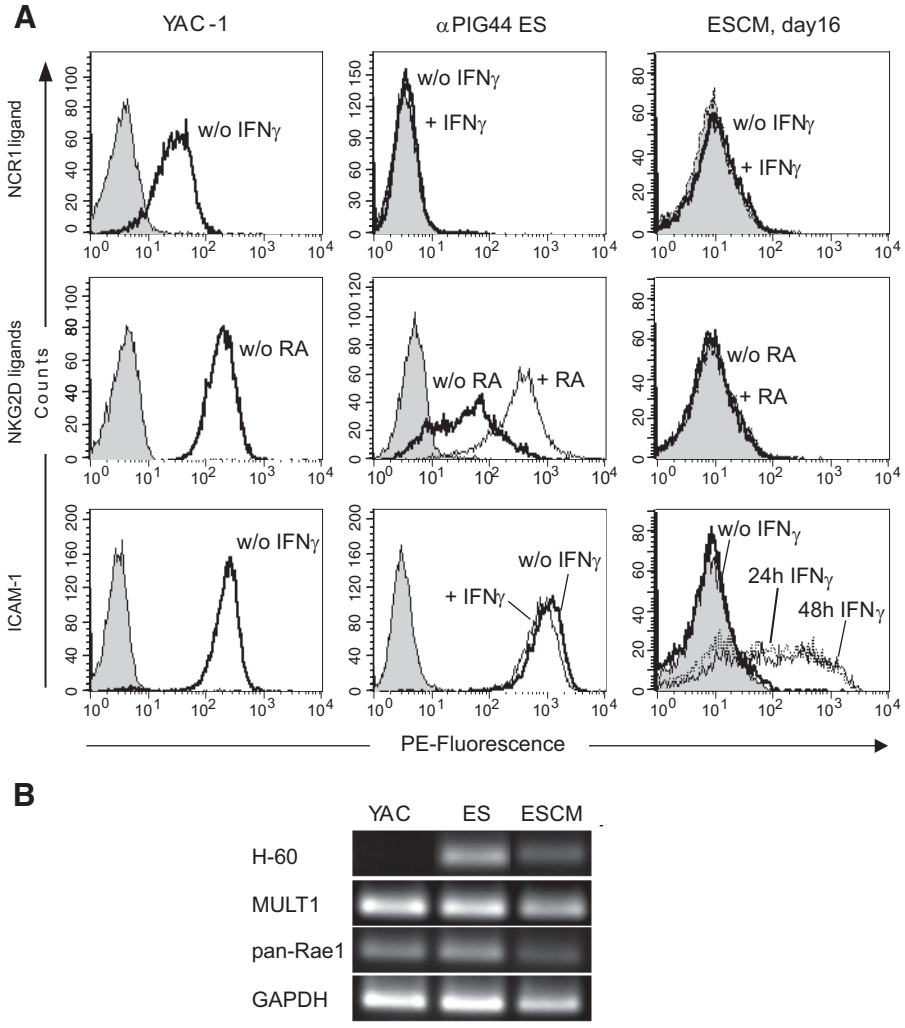

Figure 5. Expression of activating and adhesion ligands for natural killer cells on ESCM and undifferentiated ES cells. **(A):** The expression of NCR1 ligands, NKG2D ligands, and ICAM-1 on the surface of YAC-1 cells, undifferentiated αPIG44 ES cells, and late-stage ESCM obtained after 7 days of puromycin selection at day 16 of differentiation was determined by flow cytometry using NCR1/Fc chimeras, NKG2D tetramers, and anti-ICAM-1 antibody, respectively. Viable cells were gated and selected for analysis. Aliquots of cells were also treated with 1 μM RA or 20 ng/ml IFN-γ for 48 h prior to measurement. ICAM-1 levels on ESCM were determined after 24 or 48 h of IFN-γ treatment. Thin lines show cells treated with the reagent indicated; thick lines show untreated cells; gray-shaded histograms show isotype controls. **(B):** Transcript levels of NKG2D ligands H-60, pan-Rae-1, and MULT1 were determined by reverse transcription-polymerase chain reaction in YAC-1 cells, mouse embryonic fibroblast-depleted αPIG44 ES cells, and late-stage ESCM obtained after 7 days of puromycin selection. Glyceraldehyde-3-phosphate dehydrogenase was amplified as a housekeeping gene. Abbreviations: ES, embryonic stem; ESCM, embryonic stem cell-derived cardiomyocytes; h, hours; ICAM, intercellular adhesion molecule; IFN, interferon; MULT1, murine UL-16-binding-protein-like transcript 1; NCR, natural cytotoxicity receptors; NKG2D, natural killer cell receptor natural-killer group 2 member D; PE, phycoerythrin; RA, retinoic acid; w/o, without.

induction of ICAM-1 on ESCM by IFN-γ was not sufficient for enhancing their sensitivity to killing by NK cells.

Blocking of ICAM-1 and NKG2D Molecules Protects ES Cells from Lysis by NK Cells

As mentioned above, optimal triggering of target cell lysis by NK cells via NKG2D receptors requires the simultaneous interaction of NK cells with the ICAM-1 on target cells. To assess the contribution of NKG2D ligands and ICAM-1 molecules to perforin-mediated lysis of αPIG ES cells by NK cells, antibodies against NKG2D were used to block NKG2D on NK cells, and ICAM-1-specific antibodies were used to block ICAM-1 on ES cells. Treatment of ES and NK cells with isotype control antibodies did not affect the lysis of ES cells. However, a single treatment of NK cells with anti-NKG2D or of ES cells with anti-ICAM-1 decreased lysis of ES cells by 4- or 2.5-fold, respectively, compared with the isotype control. Lysis was almost abolished by simultaneous blocking of NKG2D and ICAM-1 (Fig. 6). These data indicate that interaction via NKG2D activating ligands and ICAM-1 is required for lysis of ES cells by NK cells.

Secretion of IFN-γ by NK Cells in Response to ESCM and ES Cells

Stimulation of NK cells via NKG2D receptors engaging their respective ligands on target cells leads to secretion of IFN-γ by

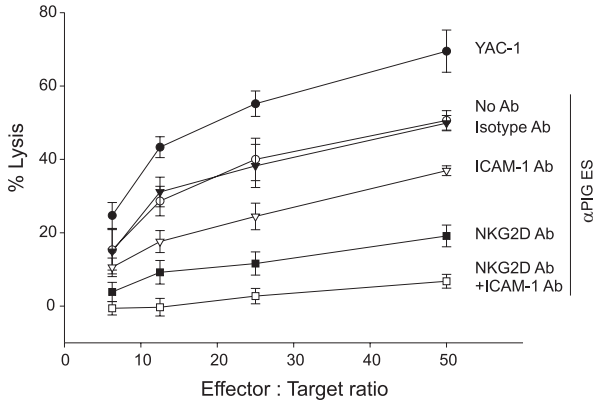

Figure 6. Lysis of undifferentiated ES cells by natural killer (NK) cells depends on interaction via NKG2D and ICAM-1. Poly(I:C)-activated syngeneic NK cells were used as effector cells with YAC-1 and αPIG44 ES cells as targets in a 4-hour ^{51}Cr-release assay. Blocking Abs directed against NKG2D and ICAM-1 molecules were used either alone or in combination at a concentration of 30 μg/ml. Both nonspecific isotype Abs corresponding to anti-NKG2D and anti-ICAM-1 Abs were incubated with control cells at the same concentration as specific Abs. Cells were incubated with or without Abs for 30 minutes prior to and during the assay. Data are given as means ± SEM of at least three independent measurements. Abbreviations: Ab, antibody; ES, embryonic stem; ICAM, intercellular adhesion molecule; NKG2D, natural killer cell receptor natural-killer group 2 member D.

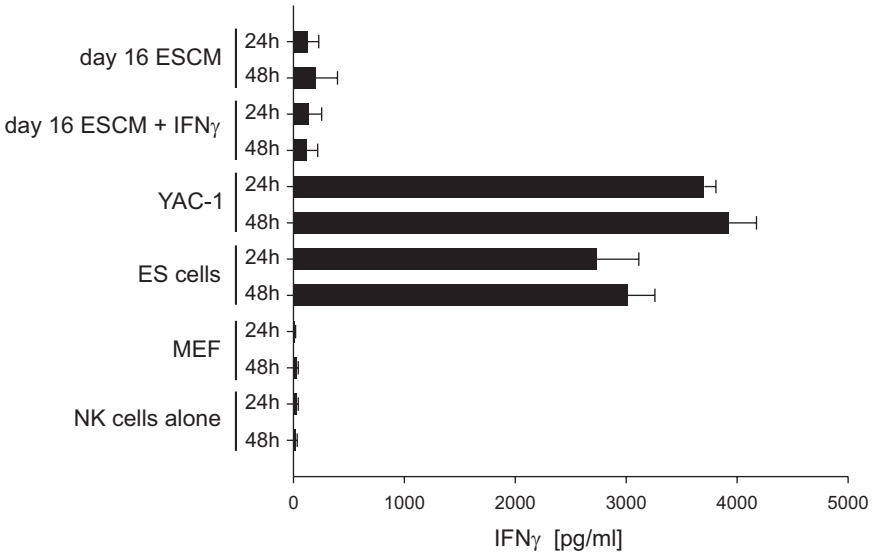

Figure 7. NK cells recognize ES cells but not ESCM. Poly(I:C)-activated syngeneic NK cells were cultured alone or in combination with MEF, YAC-1 cells, αPIG44 ES cells, and day 16 ESCM obtained after 7 days of puromycin selection at an effector-to-target ratio of 50:1 for 24 or 48 h. One group of ESCM samples was pretreated with IFN-γ for 2 days at 20 ng/ml. Prior to incubation with NK cells, IFN-γ-treated ESCM were washed to remove exogenous IFN-γ, and IFN-γ secreted by NK cells into the cell culture supernatant was determined by a specific IFN-γ enzyme-linked immunosorbent assay. Results of three independent measurements are shown for each group as the mean ± SEM. Abbreviations: ES, embryonic stem; ESCM, embryonic stem cell-derived cardiomyocytes; h, hours; IFN, interferon; MEF, mouse embryonic fibroblasts; NK, natural killer.

NK cells. This phenomenon can be used to assess whether ESCM can be recognized by NK cells. As shown in Figure 7, poly(I:C)-activated NK cells incubated alone or with MEF for either 24 or 48 hours did not secrete IFN-γ. However, NK cells incubated with YAC-1 cells or undifferentiated αPIG44 ES cells secreted large amounts of IFN-γ during 24 or 48 hours of coincubation (Fig. 7). In contrast, NK cells alone secreted IFN-γ only at background levels when stimulated with ESCM, regardless of the duration of coincubation. Moreover, even IFN-γ-pretreated ESCM expressing ICAM-1 did not stimulate NK cells to secrete IFN-γ (Fig. 7). These results suggest that ESCM are not recognized by NK cells, that ICAM-1 alone is not sufficient for triggering IFN-γ release by NK cells, and that ESCM are not lysed by NK cells due to the lack of NK cell-activating NKG2D ligands.

DISCUSSION

The few previous immunological studies on ES cells have been focused on undifferentiated ES cells or on the heterogeneous cell populations present in whole EB or teratoma. These studies have delivered results that range from those claiming unique immune-privileged, immunosuppressive, and tolerance-inducing properties for ES cells [33, 36–38, 40–42] to those refuting these conclusions [43–48]. Although the issue of immunogenicity of pluripotent ES cells still needs further clarification, the results obtained with ES cells or mixtures of different cell types present in EB and teratomas may not necessarily reflect the properties of pure populations of therapeutically more relevant, nontumorigenic ES cell derivatives, such as cardiomyocytes. In this study we use highly purified ES cell derivatives to determine their antigenic and immunogenic properties, focusing on interaction of ESCM with NK cells in vitro. Our data reveal that ESCM and undifferentiated ES cells greatly differ in some of their immunological properties. MHC class I molecules were expressed on purified ESCM and on ES cells at similar very low levels that were detectable only by a highly sensitive T-cell hybridoma assay [33]. According to the "missing self" hypothesis, cells expressing very low levels of MHC class I molecules should be lysed by NK cells [35, 49]. In agreement with this paradigm, five different lines of undifferentiated ES cells derived from 129Sv and C57BL/6 mouse strains were readily lysed by activated NK cells. In contrast to ES cells, both untreated and IFN-γ-treated ESCM were not sensitive to lysis by activated NK cells irrespective of their developmental stage.

The sensitivity of ES cells and ESCM to NK cells correlated with the expression of ligands for NK cell activating receptor NKG2D on their cell surface. These molecules, which promote lysis of diverse types of cancer [39, 50, 51] and infected cells [52] as well as rejection of bone marrow grafts [19], were highly expressed on ES cells but were not detectable on ESCM. The Rae-1 family of NKG2D ligands was also detected by others in undifferentiated ES cells [38, 53] and in embryonal carcinoma F9 cells [13]. Rae-1 transcripts were found in early mouse embryos in many tissues, including heart [13]. However, at later stages of development Rae-1 genes were preferentially expressed in the brain but not in the heart [13].

In addition to NKG2D ligands, our experiments revealed that the LFA-1 ligand ICAM-1 is expressed on ES and on cells of intact EB, corroborating the data of Tian et al. [54]. We extended these observations to purified ESCM, which completely lacked the ICAM-1 expression. Strong adhesion to target cells through ICAM-1/LFA-1 interaction is critical in triggering NK cell cytotoxicity [39, 55, 56]. The key role of stimulatory NKG2D ligands and ICAM-1 in lysis of ES cells was demonstrated in blocking experiments with NKG2D- and ICAM-1-specific antibodies, which acted synergistically and almost completely prevented ES cell lysis by activated NK cells. These data and the lack of expression or low-level expression of NK cell inhibitory ligands (MHC class I molecules, Qa-1 and FasL) on ESCM strongly suggest that the inability of NK cells to lyse ESCM was due to insufficient stimulation rather than predominance of inhibition of NK cells. This conclusion was also corroborated by the finding that untreated ESCM or ESCM pretreated with IFN-γ did not stimulate secretion of IFN-γ by activated NK cells.

NKG2D ligands can be induced in healthy cells by various types of cellular stress [14, 15] or by mediators such as retinoic acid [13, 57] and the inflammatory cytokine IFN-γ [58]. Undifferentiated ES cells and ESCM differed greatly in their response to these two factors. Retinoic acid upregulated the levels of NKG2D ligands on undifferentiated ES cells but was without effect in ESCM and had only weak effect on noncardiac cells in intact EB. In contrast, IFN-γ strongly induced ICAM-1 and H-2K^b molecules but not NKG2D or NCR1 ligands on ESCM and did not affect these molecules on ES cells. However, despite upregulation of ICAM-1 on ESCM, these cells were still not susceptible to lysis by NK cells. Therefore, isolated induction of

the coactivatory ICAM-1 molecules on ESCM is not sufficient to override the inhibitory signals from MHC class I molecules in the absence of activatory NKG2D ligands. This is in agreement with the finding that the sensitivity of many target cells to killing by NK cells is reduced, rather than enhanced, by IFN-γ treatment [59]. NKG2D ligands were also not upregulated on ESCM even after exposure to environmental conditions that they may encounter after transplantation into infarcted heart, such as hypoxia (1% O_2 for 3 days) (our unpublished observation). In addition, NKG2D ligands remained undetectable on ESCM 1 day after heat shock (43°C, 30 minutes), which is usually used as a preconditioning strategy prior to transplantation to increase the survival of ESCM in vivo [2, 5]. These data imply that ESCM may not be susceptible to rejection by NK cells in vivo even if exposed to the hypoxic and inflammatory microenvironment of the infarcted heart.

Although published studies on interactions between NK cells and ESCM are not available, several groups have reported that murine ES cells are susceptible to lysis by activated NK cells [53]. In the most recent study, it was demonstrated that murine ES cells are highly susceptible to in vitro killing by freshly isolated rat and IL-2 stimulated mouse NK cells [53]. Others have shown that lysis of allogeneic murine ES cells in vitro can be achieved with activated NK cells [38] and that human ES cells are moderately lysed in vitro by human cytokine-activated NK cells [60]. In contrast to these results, Koch et al. reported that murine C57BL/6 ES cells resisted lysis by activated syngeneic and allogeneic NK cells [41]. The reason for this discrepancy is currently unknown, but it is unlikely to be due to different ES cell lines used and their genetic backgrounds because in our hands the susceptibility of five different ES cell lines, including one of C57BL/6 origin, to lysis by NK cells was comparable.

In vivo, NK cells also appear to affect the engraftment of transplanted ES cells. Bonnevie et al. reported that rejection of murine ES cells transplanted into infarcted hearts of immunocompetent baboons was mediated in part by NK cells [48]. NK cells have also been found to play a role in preventing the engraftment of undifferentiated and differentiated human ES cells in NOD/SCID mice that lack functional B and T cells [29]. In this study, depletion of NK cells accelerated teratoma formation from intramuscularly injected human ES cells and improved the engraftment of intravenously administered human ES cell-derived hematopoietic stem cells. Dressel et al. also provided strong evidence that NK cells may play a role, at least partially, in controlling tumorigenicity of mouse ES cells in cyclosporine conditioned rat recipients [53]. Although NK cells appear to participate in regulating teratoma formation in xenogeneic settings [29, 48, 53], syngeneic and allogeneic NK cells were not capable of inhibiting teratoma formation, although they were cytotoxic against ES cells in vitro after stimulation [53]. Therefore, it remains to be determined whether in vitro susceptibility of ES cells to killing by activated NK cells can be translated into in vivo strategies for preventing teratoma formation in syngeneic and allogeneic constellations.

Conclusion

The results of this study demonstrate that immunologic properties of ES cell derivatives suitable for therapeutic transplantation may greatly differ from those of undifferentiated ES cells and will most likely depend on the cell type being investigated. These data also suggest that differential sensitivity of ES cells and ESCM to NK cells may be harnessed for selective elimination of residual ES cells in grafts of differentiated cells to prevent teratoma formation. However, whether NK cells in vivo can selectively eliminate undifferentiated ES cells and spare autologous or allogeneic ESCM or other differentiated cells remains to be determined.

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Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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