The Unexpected Effect of Cyclosporin A on CD56+CD16- and CD56+CD16+ Natural Killer Cell Subpopulations

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Running Head: Effect of CSA on Human NK cells

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Abstract

Cyclosporin A (CSA) is commonly used to prevent graft-vs-host disease. The influence of CSA on T cell function has been extensively investigated however; the effect of CSA on NK cells is less understood. NK cells were cultured with IL-2 and IL-15 ± CSA for 1 week. Compared to controls, CSA treated cultures showed fewer CD56+CD16+KIR+ NK cells and a reciprocal increase in CD56+CD16 KIR- cells. These changes were mainly due to a reduced proliferation of the CD56dim NK cell subpopulation and a relative resistance of CD56bright NK cells to CSA. Following co-culture with K562 targets, CSA exposed NK cells differed from controls and lacked Ca2+ oscillations, NFAT dephosphorylation and NFAT nuclear translocation. NK cells cultured in CSA retained cytotoxicity against K562, Raji and KIR ligand expressing lymphoblastoid cells. NK cells cultured in CSA showed increases in NKp30 and reductions in NKp44 and NKG2D. Following IL-12 and IL-18 stimulation, CSA treated NK cells showed more IFN-γ producing cells. Using in vitro NK cell differentiation, progenitor cells gave rise to more CD56*KIR* NK cells in the presence of CSA than controls. Collectively, these studies show that CSA influences NK cell function and phenotype which may have important implications for graft-vs-leukemia effects.
**Introduction:**

Following allogeneic hematopoietic cell transplantation (allo-HCT), graft vs. host disease (GVHD) is a major cause of morbidity and mortality. In the late 1980s, drugs such as cyclosporine A (CSA) were introduced as post-transplant immune suppression. CSA is a cyclic oligopeptide derived from the fungus *Tolypocladium inflatum* and was heralded as a revolutionary agent with T cell selective immune suppressive properties. However, it is now clear that CSA modifies the function of many cell types, including non-T cell lymphocyte populations. To date, the effects of CSA on NK cell function have not been fully investigated.

Both murine and human studies show that NK cells mediate a number of potentially beneficial functions following allo-HCT including: eliminating residual malignant cells, removal of host antigen presenting cells (thereby reducing GVHD), and mediating immunity to viral pathogens directly through the cytolysis of virally infected tissues or indirectly by elaborating inflammatory cytokines, such as interferons (IFN). NK cell function is governed by a series of both inhibitory and activating surface receptors (reviewed in 

Recently the Perugia group published results suggesting that NK cells play a critical role in graft vs. leukemia (GVL) reactions. Following conditioning chemotherapy, patients received T cell depleted and CD34\(^+\) selected haploidentical grafts.
Post-transplant immune suppression was not used due to stringent T cell depletion. In some recipient-donor pairs, the donor possessed MHC alleles (i.e., KIR ligands) that were not expressed by the recipient. In this so-called, “KIR ligand mismatched” situation, NK cell clones which are not restrained by host MHC class I can exist and have GVL potential. Recipients with acute myelogenous leukemia (AML) who received a haploidentical transplant from a KIR ligand mismatched donor had a marked reduction in relapse compared to an otherwise similar group of AML patients without this mismatch (0% vs. 75%). In contrast, a large retrospective analysis of unrelated donor, T cell replete transplants from the National Marrow Donor Program showed no decrease in AML relapse following KIR mismatched transplants. Similar results have been found by other groups. There were a number of differences between these two transplant settings including donor source (sibling/parent vs. unrelated), HLA matching (haploidentical vs. matched unrelated), the presence of T cells (severe depletion vs. T cell replete) and the use of post transplant immune suppression (absent vs. present). This latter issue led us to investigate the influence of CSA on NK cell function. Here we show that CSA has a number of profound and unexpected effects on NK cell phenotype and function.

**Methods:**

**CD3<sup>-</sup>CD56<sup>+</sup> NK cell Enrichment**

Peripheral blood mononuclear cells (PBMCs) were isolated from de-identified healthy donor buffy coats using Lymphocyte Separation Media (Mediatech, Herndon, VA). PBMCs were washed and NK cells were isolated by negative selection (Rosett-Sep
NK Cell Isolation, StemCell Technologies, Vancouver, CA) according to manufacturer recommendations, with modifications. Enriched NK cells were >85-90% CD56⁺CD3⁻, (not shown). Samples from de-identified donors were obtained from Memorial Blood Center, St. Paul, MN, in accordance with their protocols.

**Cell culture**

Enriched NK cell populations were cultured in DMEM:Hams F12 (2:1) with 10% human AB⁻ sera (SeraCare, Oceanside, CA), 2-β mercaptoethanol (24 µM), L-ascorbic acid (24 mg/L), sodium selenite (50µg/L), ethanolamine (50µM) and penicillin-streptomycin (100 U/ml of each). At the start of cultures IL-2 (100 U/ml, Chiron, Emeryville, CA), and IL-15 (10 ng/ml, Preprotech, Rockyhill, CT) were added. Cyclosporine A (Bedford laboratories, Bedford, OH) was diluted in ethanol (1 mg/ml) and added at final concentrations from 0.1-5 µg/ml. Vehicle control cultures were set up in parallel using ethanol (EtOH).

**Flow cytometry and FACS sorting**

The following antibodies were used: CD3, CD16, CD56, CD158a, CD158b, CD158e1, NKG2D, IFN-γ, perforin, granzyme B and FasL (BD Biosciences, San Diego, CA), CD158j, NKp30, NKp44 and NKp46 (Beckman Coulter, Fullerton, CA). In some experiments (IFN-γ and perforin/granzyme) intracellular staining was performed with Fix/Perm Buffer (BD Biosciences). Apoptotic cells were detected using annexin V (BD Biosciences) and propidium idodine (Sigma, St. Louis, MO). Cells were analyzed on a FACS Calibur (BD Biosciences). Data was analyzed using Flowjo software.
In some experiments, NK cell subpopulations were obtained by FACS sorting. Briefly, enriched NK cells were stained with antibodies against CD3, CD56, CD16 and sorted on a FACS Diva (BD Biosciences) gating on CD3−CD56\textsuperscript{bright}CD16− and CD3−CD56\textsuperscript{dim}CD16\textsuperscript{+} subpopulations.

**CFSE staining**

NK cells were stained using the Vybrant\textsuperscript{TM} CFDA SE Cell Tracer Kit (Invitrogen, Molecular Probes). Following this, NK cells were cultured and CFSE content was determined by FACS at various time points.

**Cytotoxicity assay**

Cr release assays were performed as previously described\textsuperscript{18}. Briefly, target cells were labeled with 3 x 10\textsuperscript{-4} Ci of 51Cr (Dupont/NEN, Boston, MA) for 1-2 hours at 37°C, 5% CO\textsubscript{2}. Labeled cells were washed three times with PBS and plated in triplicate in 96-well round-bottom plates (10\textsuperscript{4} cells/well). Specified ratios of NK cells were added and incubated for 4 hours at 37°C, 5% CO\textsubscript{2}. Assay supernatant was collected and counted using a gamma counter. The percent lysis was calculated using the following equation:

\[
\% \text{ specific lysis} = 100 \times \frac{(\text{test release} - \text{spontaneous release})}{(\text{maximal release} - \text{spontaneous release})}
\]

Where stated, target cells were treated with the pan-MHC class I blocking mAb HP-1F7 (kindly provided by Dr. M. López-Botet (Hospital de la Princesa, Madrid, Spain)). Briefly, 51Cr labeled target cells were washed and resuspended (10\textsuperscript{6} cells/ml). 25 µg mAb was added to 200 µl of target cells for 30 minutes. Target cells (with or without
Ab) were diluted 10 fold with RPMI 1640 containing 10% FBS and 100 µl was plated in 96-well round-bottom plate. NK cells were added at specified ratios (in 100 µl) and incubated for 4 hours at 37°C, 5% CO2. The final concentration of mAb was 6.25 µg/ml.

**Ca²⁺ imaging**

To assess dynamic [Ca²⁺]i changes in individual NK cells, the Ca²⁺-sensitive fluorophore fura-2 AM (Molecular Probes) was used. NK cells were allowed to adhere onto 25-mm² glass slides and were placed on the stage of an inverted microscope (Nikon Diaphot). Cells were loaded with 10 nM fura-2 AM and 2.5 µg/ml pluronic acid (Molecular Probes) for 20 min, followed by 20 min in Ca²⁺-containing solution to allow for deesterification before the experiment. Following this, K562 cells were added and ratiometric imaging was performed with the excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Imaging was with an intensified charge-coupled device camera (Photonic Science, Robertsbridge, UK) using Metafluor image capture and analysis software (Fryer, Bloomington, MN). Ca²⁺ calibration was achieved by measuring a maximum (with 1 mM ionomycin) and a minimum (with 10 mM EGTA). Intracellular free Ca²⁺ was calculated assuming a dissociation constant of 220. Intracellular free Ca²⁺ was calculated assuming a dissociation constant of 220.¹⁹ For each experiment 10–20 cells were visualized and ratiometric data was acquired from individual cells.

**Western Blotting**

Cell lysates (50 µg) were prepared from NK cells after culture for 7 days using freshly made lysis buffer (10mM Tris-HCl (pH=8), 150 mM NaCl, 1 mM EDTA, 1%
NP-40, 0.5% deoxycholate, 0.1% SDS, protease inhibitor cocktail (complete protease inhibitor, Roche, Indianapolis, IN) and PMSF. Samples were separated on a 4-15% gradient polyacrylamide gel and transferred to a PVDF membrane. Membranes were blocked with 5% non-fat milk. One portion of the membrane was probed with an NFAT-1 antibody (BD Biosciences, CA) while the other portion was probed with an antibody against actin (Santa Cruz Biotechnology, Santa Cruz, CA). Both were probed with a species specific secondary HRP-conjugated antibody and developed using chemiluminescence (ECL, Amersham Piscataway, NJ).

**ImageStream Analysis**

At day +7, NK cells were washed and co-cultured with K562 cells (1:1 ratio) for 10 minutes then fixed with 4% paraformaldehyde, and permeabilized using an equal volume of 0.1% Triton X-100. Cells were then stained with 1 µg NFAT-1 specific antibody, followed by secondary staining with goat anti-mouse IgG. Thereafter cells were blocked with mouse Ig for 30 minutes and stained with CD56 and glycophorin A for 30 minutes. Cells were washed and resuspended in PBS and 5 µl of 7-AAD (0.5 mg/ml) was added. Analysis was performed using an imagestream 100 (Amnis, Seattle, WA) and ~5,000 events/sample was acquired. Nuclear focus vs. intensity was used to examine only cells in focus. DNA content was used to identify single cells, eliminating conjugates and debris. Next, cells were gated on CD56 and then NFAT translocation was evaluated using a similarity score for 7-AAD vs. NFAT (a monontic function of Pearson’s correlation coefficient between the pixel values of two image pairs, described
In total, data was analyzed on ~2,500 events per sample (~50% of the collected sample).

**NK cell Differentiation Cultures**

NK cell differentiation from progenitor cells were performed as described. Briefly, CD34^+^CD38^−^Lin^−^ cells were isolated by CD34 positive selection (Miltenyi Biotech) followed by FASC sorting, gating on the CD34^+^CD38^−^Lin^−^ population. mAbs against CD2, CD3, CD4, CD5, CD7, CD8, CD10 and CD19 (all from BD Biosciences) served as lineage antigens. Cells were cultured in either 24 well or 96 well plates on irradiated (3,000 cGy) AFT024 cells (murine stromal cell line) in media (DMEM: Hams F12 (2:1)) containing IL-3 (5 ng/ml, R&D systems, at the culture initiation only), IL-7 (20 ng/ml, R&D systems), IL-15 (10 ng/ml, R&D systems), FLT-3L (10 ng/ml gift from Amgen, Thousand Oaks, CA) and SCF (20 ng/ml, gift from Amgen).

**Statistical analysis**

All statistical analyses were performed with Statistical Analysis System statistical software version 9.1 (SAS Institute, Inc., Cary, NC). For non-normally distributed data the nonparametric Wilcoxon signed rank test and Mann-Whitney rank sum test were used in the evaluation of the statistical differences between vehicle controls (EtOH) and CSA treated cells. One-way and two-way ANOVA were used when the data was approximately normally distributed the general linear models procedure (PROC GLM). Adjustments for multiple comparisons were done with the Tukey’s method. Groups with values of $p \leq 0.05$ were considered to be statistically different.
Results

Effect of CSA on NK Cell Expansion

NK cells were isolated from healthy donor PBMCs using negative selection and cultured in IL-2 (100 U/ml) and IL-15 (10 ng/ml) with increasing concentrations of CSA (0.1 – 5 µg/ml) or vehicle (EtOH). After 1 week there was a dose dependent reduction in NK cell expansion (figure 1a). Following allogeneic hematopoietic cell transplantation, peak CSA levels reach 0.5 µg/ml – 1 µg/ml\textsuperscript{22,23}, thus all further experiments were performed in the presence of CSA at 1 µg/ml. At this dose, NK cell fold expansion was consistently lower than vehicle controls (0.8 ± 0.06 vs. 2.4 ± 1.5, n=10, p<0.01). To investigate whether this reduction in fold expansion in CSA treated NK cells was due to an increase in apoptosis, cultures were analyzed for both fold expansion and the percentages of apoptotic cells at days 3, 5 and 7. As shown in table 1, at day 7, CSA treated NK cells showed a significant reduction in fold expansion and an increase in apoptotic cells relative to controls (n=8).

CSA Differentially Affects NK Cell Subpopulations

NK cells can be separated into subsets on the basis of CD56 receptor density (reviewed in\textsuperscript{24}). The majority of peripheral blood NK cells are CD56\textsuperscript{dim}. Freshly isolated, they co-express CD16, KIR receptors and mediate potent cytotoxicity against K562 cells. In contrast, most CD56\textsuperscript{bright} cells do not express CD16 and have minimal KIR receptor expression. CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cell subpopulations are readily discernable by flow cytometry when freshly isolated. However, after \textit{in vitro} culture distinguishing these subsets may be difficult due to less obvious differences in CD56
intensity. Therefore, CD16 expression was used a marker of CD56\textsuperscript{dim} cells after 1 week of culture. Comparing CSA containing cultures to vehicle controls, there was an increase in the percentage of CD56\textsuperscript{+}CD16\textsuperscript{-} NK cells and a reciprocal reduction of CD56\textsuperscript{+}CD16\textsuperscript{+} NK cells (figure 1b shows a representative donor). Similar findings were observed in 14 donors, with an increase in CD56\textsuperscript{+}CD16\textsuperscript{-} NK cells (22.6% ± 12.3% vs. 47% ± 15.9%, p<0.01) and a decrease in CD56\textsuperscript{+}CD16\textsuperscript{+} subpopulations (73.2% ±12.6% vs. 48.2% ± 16.7%, p<0.01) (figure 1c).

In addition to changes in CD56\textsuperscript{+}CD16\textsuperscript{-} and CD56\textsuperscript{+}CD16\textsuperscript{+} NK cells, there were also alterations in the percentage of KIR expressing cells following culture with CSA. A representative donor is shown in figure 2a, demonstrating a significant proportion of vehicle treated NK cells expressed KIR receptors. In contrast, NK cells from the same donor cultured with CSA showed reductions in CD158a, CD158b, and CD158e1. Gating on CD56\textsuperscript{+}CD16\textsuperscript{-} and CD56\textsuperscript{+}CD16\textsuperscript{+} cell subpopulations after 1 week of culture demonstrated that CD16\textsuperscript{+} cells showed higher percentages of KIR expressing cells, regardless of whether NK cells cultured with or without CSA (figure 2b). These results validate our approach of using CD16 as a marker of CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cell subpopulations after culture. Changes in CD16 and KIR were investigated across a series of donors by determining the percentage of NK cells staining positive for CD16 or a particular KIR and then comparing this to vehicle controls. As shown in figure 2c, significant reductions were consistently noted for CD16, CD158a and CD158b (n=13), while CD158e1 (n=11) showed heterogeneous changes following culture with CSA.

To investigate whether the changes observed after culture with CSA were due to differential proliferation of CD56\textsuperscript{bright} and CD56\textsuperscript{dim} NK cells, freshly isolated NK cells
were labeled with the membrane dye CFSE at the start of cultures. CFSE content was determined by gating on CD56+CD16- and CD56+CD16+ subpopulations on days 3, 5, 7 and 10 of culture. At nearly all time points CD56+CD16- cells showed a similar number of divisions determined by loss of CFSE, regardless of the culture conditions. In contrast, CD56+CD16+ cells cultured in CSA underwent fewer cell divisions than vehicle controls (figure 2d).

To further prove that CSA differentially affects NK cell subsets, freshly isolated NK cells were FACS sorted into CD56brightCD16- and CD56dimCD16+ fractions and cultured with IL-2/IL-15 ± CSA. As shown in table 2, CSA inhibited the proliferation of both subpopulations; however not equally. The expansion of sorted CD56brightCD16- cells in CSA was 1.3-fold less than vehicle (p<0.05), whereas CD56dimCD16+ cells expanded, on average, 3.5-fold less than controls (p<0.05). Thus CSA induced significantly more inhibition in purified CD56dimCD16+ cells compared to the CD56brightCD16- population (p=0.02). Analyzing the sorted NK subsets for KIR expression showed similar results to those obtained after gating on the CD56+CD16- and CD56+CD16+ fractions of bulk NK cells (as in figure 2b, not shown). Interestingly, while the majority of both sorted NK subsets retained their starting phenotype, some cells either acquired or lost CD16 after a week in culture. This occurred despite a high degree of purity (>97%, a representative donor is shown in figure 2e). Regardless of the starting population, CSA favored the CD56+CD16- phenotype. More specifically, when CD56brightCD16- cells were cultured with CSA, lower percentages CD16 expressing NK cells were observed compared to controls. Likewise, when CD56dimCD16+ cells were cultured in CSA, higher percentages of cells without CD16 were recovered (p=0.06 for
both, figure 2f). Taken together these results show that CSA inhibits proliferation and induces apoptosis of NK cells. This is more pronounced in CD56^{dim}\text{CD16}^{+} cells relative to the CD56^{bright}\text{CD16}^{-} fraction and is the primary explanation for the increase in CD56^{+}\text{CD16}^{-} NK cells after culture in CSA. However, CSA also influences the interconversion of CD56^{+}\text{CD16}^{-} to CD56^{+}\text{CD16}^{+} populations (and vice versa), favoring the CD56^{+}\text{CD16}^{-} phenotype.

**Ca^{2+} Flux and NFAT Translocation in CSA Exposed NK Cells**

NK cells use a series of surface receptors to recognize malignant cells. Following receptor ligation free intracellular Ca^{2+} increases, triggering a series of downstream events including the activation of calcineurin, a calcium regulated phosphatase that dephosphorylates cytosolic NFAT. Dephosphorylated NFAT then enters the nucleus and regulates gene transcription.\textsuperscript{25} By inhibiting calcineurin, CSA blocks nuclear translocation of NFAT.\textsuperscript{26-28} We investigated whether there were differences in Ca^{2+} flux in CSA treated NK cells upon target cell contact, using fura-2 loaded NK cells. Vehicle treated NK cells showed the expected biphasic increase in intracellular Ca^{2+} following the addition of K562 cells.\textsuperscript{29} In contrast, CSA treated NK cells showed a rise in intracellular Ca^{2+} upon contact with K562 cells, but no oscillations were noted (figure 3a).

We next investigated NFAT-1 expression in cultured NK cells and found no significant differences in CSA treated NK cells relative to controls (figure 3b, time 0). Co-culture of NK cells with K562 targets lead to rapid NFAT-1 dephosphorylation in controls, but not in CSA treated NK cells; even after prolonged (30 min) co-incubation with K562 targets (figure 3b and not shown). To further assay nuclear translocation of
NFAT-1 multispectral cell imaging was used. This technique allows for the differentiation of effector and target cells by membrane staining as well as the cellular localization of antigens in individual cells (i.e., cytoplasmic vs. nuclear) (figure 3c). Approximately 45% of control treated NK cells translocated NFAT into the nucleus after a 10 minute incubation with K562 cells (n=1763 events). In contrast, only ~5% of CSA treated cells showed nuclear translocation (n=1674 events) (figure 3d). Collectively, these results show that vehicle control treated NK cells dephosphorylate and translocate NFAT-1 upon interaction with K562 cells, while CSA treated NK cells do not.

**Functionality of CSA Exposed NK Cells**

To investigate the effect of CSA on NK cell cytotoxicity, CSA or vehicle control treated NK cells were used for $^{51}$Cr release assays. Targets included both K562 (MHC class I) and Raji (MHC class I') cell lines. Comparing CSA treated NK cells to vehicle controls, there was a small, but significant increase in average cytotoxicity against K562 cells (62% ± 8.7% vs. 53.3% ± 13.2%, p=0.01, n=11, figure 4a). Likewise, CSA treated cultures showed higher cytotoxicity against Raji cells (57.4% ± 11.8% vs. 42.3% ± 12.1%, p<0.01, n=11, figure 4b). Thus, surprisingly, NK cells cultured in CSA had higher cytotoxicity than controls and this was more pronounced for the MHC class I expressing Raji targets.

Given that CSA exposed cultures had higher percentages of CD56$^+$CD16$^-$KIR$^-$ cells we investigated whether increased killing was secondary to a reduction in KIR. To do this, cytotoxicity was tested against a panel of lymphoblastoid cell lines (LCLs) that express all known KIR ligands (KIR-L), i.e., HLA-Bw4 and HLA C1/C2. The average
cytotoxicity of 4 donors is shown for each target (figure 4c). For 2 of the 4 LCLs tested, there was significant (LCL 16, p<0.01) or near significantly (LCL 7, p=0.08) higher cytotoxicity in CSA treated cultures. No differences between EtOH and CSA treated cells were detected for LCL 19 (p=0.46) and LCL 17 (p=0.63). If cytotoxicity was entirely due to differences in KIR, blocking such interactions should result in equivalent killing. To test this, a pan-MHC class I blocking mAb (HP-1F7) was used.\textsuperscript{30,31} The addition of the blocking mAb resulted in increased cytotoxicity for all 4 LCLs tested, however, CSA treated cells still showed higher cytotoxicity relative to EtOH controls (p<0.01, for all). Given that CSA treated cells had less CD16 expression than controls (figure 1c), such findings are not likely due to antibody dependent cellular cytotoxicity (ADCC). Collectively these results suggest that the reduction of KIR expressing cells (in CSA treated cultures) is not the sole reason for the increased cytotoxicity.

Given the higher cytotoxicity in CSA treated NK cells (despite MHC class I masking), differences in the expression of effector molecules were tested. As shown in figure 4d, in 3 of 4 donors tested was a slight, but non-significant increase in the geometric mean fluorescence intensity (gMFI) for intracellular granzyme B after culture with CSA compared to vehicle controls (113.5±74.9 vs. 73.5±45.4, p=0.25). Similarly, there was no difference for intracellular perforin (67.0±31.7 vs. 60.8±16.9, NS) or surface FasL (6.7±2.1 vs. 6.2±2.6, NS) between the various culture conditions.

Next, differences in activating receptor expression were investigated. After one week the majority of cells in either condition (CSA or vehicle) expressed NKp30, NKp44, NKp46 and NKG2D. However, differences were noted and a representative donor is shown in figure 5a. To quantify these, the relative change in the gMFI was
calculated across a series of donors (n=16). In CSA treated cultures the gMFI for NKp30 increased relative to controls (p=0.01) (figure 5b). In some donors (7/16), increases were also noted for NKp46, but this was not significant (p=0.36). Interestingly, decreases in the gMFI were noted for NKp44 (p=0.06) and NKG2D (p<0.01) in CSA treated cultures. Similar changes for activating receptors were noted in the FACS purified CD56brightCD16- and CD56dimCD16+ NK cell subsets (as in figure 2e, n=2, not shown).

NK cells link the innate and adaptive immune responses, in part, through the rapid elaboration of cytokines, including IFN-γ. To test the ability of CSA exposed NK cells to secrete IFN-γ in response to monocyte derived cytokines, NK cells were stimulated with IL-12 and IL-18. A higher percentage of CSA treated NK cells secreted IFN-γ compared to controls (58.7% ± 18.6% vs. 39.1% ± 18.0%, n=8, p<0.01) (figure 6a and 6b). Additionally, CSA exposed NK cells had brighter channel fluorescence for IFN-γ (figure 6a, lower right panel) relative to controls, suggesting that on a per-cell basis, each CSA treated NK cell may make more IFN-γ. PMA and ionomycin stimulation served as a positive control and IFN-γ elaboration was seen in the majority of cells regardless of treatment (vehicle or CSA, not shown).

The Impact of CSA on NK Cell Development

Following allo-HCT, CSA may not only affect the transferred mature NK cells but it may also impact NK cells developing from progenitor cells. To investigate the influence of CSA on NK cell development we used an in vitro NK cell developmental system. Briefly, CD34+CD38lin- cells were isolated from umbilical cord blood and cultured on a murine stromal cell line (AFT-024) in the presence of IL-3 (for the first
week), IL-7, IL-15, stem cell factor and FLT-3L. After 5-7 weeks CD56+ cells are generated. Increasing concentrations of CSA had no impact on the percentage of CD56+ cells in the culture (not shown). Interestingly, an increase in the number of cells derived from a fixed number of progenitor cells (10 CD34+CD38-Lin- cells/well) was seen with increasing concentrations of CSA (figure 7a). On the contrary reductions in both the percentage and absolute number of KIR expressing CD56+ cells was observed with increasing CSA concentrations (figure 7b and 7c). These results demonstrate that CSA affects both mature PB NK cells as well as the development of NK cells from progenitor cells. In both cases, NK cells expressing KIR receptors are affected most by CSA.

**Discussion**

During allo-HCT, a complex mixture of lymphocytes and progenitor cells are transferred to the recipient. Mature donor T cells can respond to recipient alloantigens, resulting in GVHD. To prevent GVHD, immune suppressive drugs, such as CSA, are administered. The influence of CSA on T cell function has been extensively investigated (reviewed in ). In contrast, the impact of CSA on NK cell function is not fully established. Here we demonstrate that CSA induces a number of unexpected and significant changes in the phenotype and function of cultured NK cells. Our studies show that CSA results in a dose dependent reduction in NK cell expansion, consistent with other investigators. However, we significantly extend these earlier observations by demonstrating a differential effect of CSA on CD56brightCD16- and CD56dimCD16+ NK subsets. After one week of culture in CSA there were significantly higher percentages of CD56+CD16- cells and a reciprocal decrease in CD56+CD16+ cells. In bulk cultures, there were increased numbers of apoptotic cells in the presence of CSA. Using CSFE
staining, we demonstrate that these effects are predominately on the CD56\textsuperscript{dim}CD16\textsuperscript{+} NK cell population. These results were confirmed using FACS purified CD56\textsuperscript{bright}CD16\textsuperscript{−} and CD56\textsuperscript{dim}CD16\textsuperscript{+} populations. CSA significantly inhibited the CD56\textsuperscript{dim}CD16\textsuperscript{+} cell expansion relative to CD56\textsuperscript{bright}CD16\textsuperscript{−} cell expansion. We also noted that these two populations can inter-convert after a week of culture. Such inter-conversion between CD56\textsuperscript{bright}CD16\textsuperscript{−} and CD56\textsuperscript{dim}CD16\textsuperscript{+} populations has recently been described by other groups \textsuperscript{35,36}. While this occurred in only a minority of purified cells, CSA favored CD56\textsuperscript{−}CD16\textsuperscript{−} cells, regardless of the starting population (either CD56\textsuperscript{bright}CD16\textsuperscript{−} or CD56\textsuperscript{dim}CD16\textsuperscript{+}). Keskin and coworkers have recently reported that the conversion of CD56\textsuperscript{dim}CD16\textsuperscript{−} cells to a CD56\textsuperscript{bright}CD16\textsuperscript{−} phenotype was, in part, mediated by transforming growth factor (TGF) \(\beta\)\textsuperscript{35}. Interestingly, CSA has been shown to induce TGF \(\beta\) secretion/release from T cells \textsuperscript{37-39} however, this has not been investigated in NK cells.

Conflicting results have been reported regarding the influence of CSA on NK cell cytotoxicity. Introna showed that short-term exposure to CSA (20 hours) significantly inhibited K562 cytolysis\textsuperscript{40}, while Shao-Hsien found no impact of CSA on K562 killing using the identical experimental design.\textsuperscript{41} After culture of PBLs with CSA for three days there was no adverse effect on NK cell cytotoxicity.\textsuperscript{34} In a rodent model, CSA inhibited T cell responses to alloantigen but did not affect NK cell responses, supporting the concept that T cells may be affected at a lower CSA dose compared to NK cells.\textsuperscript{42} More recently Poggi has shown that CSA inhibits NK cell apoptosis upon target encounter.\textsuperscript{43} Across a series of donors, we observed slight increases in the cytotoxicity of CSA treated cultures. Differences in cytotoxicity between CD56\textsuperscript{dim} and CD56\textsuperscript{bright} cells are well documented.
Freshly isolated CD56^{bright}CD16^{-} are weakly cytotoxic against malignant targets, while the opposite is true for CD56^{dim}CD16^{+} cells.^{24,44} However, CD56^{bright} NK cells acquire cytotoxicity comparable to CD56^{dim} cells after \textit{in vitro} culture^{44} or \textit{in vivo} stimulation with IL-2.^{45} While it was tempting to speculate that the increase in cytotoxicity was due entirely to a reduction in KIR expressing cells, higher killing was also observed for MHC class I targets (K562). CSA treated cells did not differ from controls in the intracellular content of granzyme B, perforin or surface expression of FasL. However, we noted significant increases in surface expression of NKp30 and in some donors NKp46 was also increased. This, along with a reduction in KIR expressing cells, could account for increased cytotoxicity observed in our experiments. Moreover, in individual donors, we could demonstrate that an increase in the density of a particular activating receptor translated into higher cytolysis using redirected killing assay (not shown).

Following receptor ligation, one of the earliest phases in NK cell activation is the increase in intracellular Ca^{2+} concentration which links membrane proximal events to downstream signaling pathways.^{46-48} Included in this process is activation of calcineurin, a Ca-dependent serine/threonine phosphatase which dephosphorylates NFAT, facilitating its nuclear translocation.^{26,27} Once in the nucleus, NFAT binds regulatory upstream elements and initiates gene transcription (reviewed in^{28}). CSA exerts its immunosuppressive properties by binding to cyclophilin A and antagonizing the phosphatase activity of calcineurin, preventing the nuclear translocation of NFAT.^{27,49,50} Using single cell imaging, CSA treated NK cells differed from controls, with regard to Ca^{2+} flux. Consistent with others,^{29} control treated NK cells showed an oscillatory pattern of intracellular Ca^{2+} following K562 engagement. In contrast, CSA exposed NK cells
showed a progressive increase in Ca$^{2+}$ and no such oscillations, suggesting that either the activation of calcineurin and/or the nuclear translocation of NFAT may be responsible for the oscillatory pattern of Ca$^{2+}$ translocation following target encounter. Further experiments demonstrated that engagement of K562 cells leads to the rapid dephosphorylation and nuclear translocation of NFAT in control treated NK cells, while neither occurred in cells cultured in CSA. Despite this, CSA treated cells showed target cell killing, suggesting that the effector stage of cytotoxicity (i.e., granule release) is not dependent upon nuclear translocation of NFAT. Other investigators have shown that CSA can inhibit mitochondrial permeability (i.e., cytochrome c release), protecting cells from apoptosis induced by oxidative stress. Protection from apoptosis may also account the increased cytotoxicity seen in CSA treated NK cells, as has previously been reported. However, the lack of nuclear translocation of NFAT in CSA exposed NK cells suggests that following target engagement, the downstream genetic programs driven by NFAT are likely not triggered. What effects this has on later stages of NK cell activation are not well understood.

One of the main immune suppressive actions of CSA is thought to be through inhibition of cytokine gene transcription. Surprisingly, higher percentages of IFN-γ producing NK cells were detected in CSA treated cultures. Considering that CSA exposed NK cell cultures had higher percentages of CD56$^+$CD16$^-$ NK cells and that CD56$^{\text{bright}}$ NK cells secrete more IFN-γ in response to monokine stimulation, such findings may not be entirely unexpected. However, in some experiments there were more IFN-γ producing cells than there were CD56$^+$CD16$^-$ cells. Interestingly, CSA treated T cells show similar increases in IFN-γ after T cell receptor/CD28 stimulation or
CD3 triggering with exogenous IL-12.\textsuperscript{53} These findings may be due to elevated c-fos expression and increased AP-1 binding to the IFN-γ promoter.\textsuperscript{53} Increased IFN-γ synthesis and secretion following CSA exposure may have important consequences both for preserving GVL activities and exacerbating GVHD.\textsuperscript{54-57}

Here we show that CSA induces a dose dependent and selective inhibition in the IL-2 and IL-15 induced proliferation of CD56\textsuperscript{dim} NK cells and that CD56\textsuperscript{bright} NK cells are relatively resistant to this effect. This calcineurin inhibitor does not reduce cytotoxicity and leads to higher quantities of IFN-γ secreting cells. As a result, the repertoire of NK cells in CSA treated patients following allo-HCT may be expected to reflect these features. In fact, some investigators report such observations. For instance, Chklovskiaia\textsuperscript{58} and Jacobs\textsuperscript{59} both show that following transplantation the ratio of CD56\textsuperscript{bright} and CD56\textsuperscript{dim} cells is reversed for 4-6 months. We recently have observed that at D+100, recipients of allo-HCT have fewer KIR expressing cells that secrete more IFN-γ compared with the donor.\textsuperscript{60} Likewise, Shilling demonstrates that KIR expressing NK cells increase slowly (over first nine months of transplant).\textsuperscript{61} Such reductions in KIR expressing cells (due to CSA) may, in part, explain the inconsistent findings regarding any effect of KIR-L mismatch on leukemia relapse following T cell replete transplants.\textsuperscript{11-16} Previously, reductions in KIR and increase in CD56\textsuperscript{bright} NK cells seen after transplant were ascribed to a response to cytokines present during the period of lymphopenia during transplantation or perhaps the NK cell developmental processes itself (i.e., CD56\textsuperscript{bright} giving rise to/or preceding CD56\textsuperscript{dim} cell reconstitution). Our studies suggest that calcineurin inhibitors may also account for these findings and that these drugs impact NK cells in unexpected ways.
References:

Figure Legends:

Figure 1: Culture with CSA reduces overall NK cell fold expansion and results in higher percentages CD56+CD16− NK cells. A) Fold expansion of NK cells cultured in IL-2 (100 U/ml) and IL-15 (10 ng/ml) either in the presence of vehicle control (EtOH) or increasing concentrations of CSA (0.1, 0.5, 1, and 5 µg/ml) after 7 days. Results are the average ± SD from 4 donors. Asterisk (*) indicates values that showed significantly less proliferation (p<0.05) compared to vehicle controls using ANOVA with Tukey adjustment. B) FACS plots from a representative donor showing staining for CD56 and CD16 before (left) and after 1 week of culture with IL-2 (100 U/ml) and IL-15 (10 ng/ml) and either vehicle control (EtOH)(middle) or CSA (1 µg/ml) (right). C) The percentages of CD56+CD16− and CD56+CD16+ NK cell subpopulations after 7 days of culture in IL-2 (100 U/ml) and IL-15 (10 ng/ml) ± CSA or either vehicle (EtOH). Results of 14 individual donors are shown (gray dashed line) as well as averages (heavy solid line). Statistical analysis was performed using Wilcoxon signed rank test.

Figure 2: Following culture with CSA there are fewer KIR expressing cells due to a reduction in CD56+CD16− cell proliferation. A) Results from a representative donor after 7 days of culture with IL-2 (100 U/ml) and IL-15 (10 ng/ml) ±CSA, showing a reduction in CD16 or KIR (CD158a, CD158b, CD158e1) in the CSA treated cultures (bottom), relative to controls (EtOH) (top). Results are representative of >3 individual donors. B) Cultured CD56+CD16− cells differ in KIR expression compared to CD56−CD16+ cells regardless of whether they are cultured in CSA or vehicle (EtOH).
Purified NK cells were cultured with IL-2 (100 U/ml) and IL-15 (10 ng/ml) ± CsA for 7 days and then analyzed by FACS. Results are from a single donor and are representative of >3 individuals. C) Reduction in CD16 and KIR on NK cells exposure to CSA. NK cells were cultured in IL-2 (100 U/ml) and IL-15 (10 ng/ml) ± CSA for 7 days and analyzed by FACS. Shown is the percent relative change in CD16 or KIR between CSA and vehicle control treated NK cultures (percent relative change = 100 x (%receptor expressing cells in CSA - %receptor expressing cells in control) / % receptor expressing cells in control). Results are the average of 13 healthy donors for CD16, CD158a and CD158b and 11 donors for CD158e1. Donors that expressed <5% CD158e1 were considered to not express this gene and were excluded (n=2). Wilcoxon signed rank test was used to calculate statistics. D) CSA differentially affects the proliferation of CD56^CD16^- and CD56^CD16+ NK cell subpopulations. NK cells were freshly isolated and stained with the membrane dye CFSE. Cells were analyzed at days 3, 5, 7, and 10 of culture after staining with CD56-APC and CD16-PE. CFSE content was determined after gating on the CD56^CD16^- and the CD56^CD16+ subpopulations and the differences between CSA and vehicle control treated cultures are shown as overlaid histograms. Results are representative of 3 individual donors. E and F) CD56^{bright}CD16^- and CD56^{dim}CD16^+ NK cell subpopulations were FACS purified from healthy donor buffy coats. Post sort purity was >97%. After culture in IL-2 (100 U/ml) and IL-15 (10 ng/ml) ± CSA for 7 days cell phenotype was determined using FACS. As shown, some CD56^{bright}CD16^- cells acquired CD16 and some CD56^{dim}CD16^+ NK cells lost CD16. E) A representative donor is shown F) The average percentage of CD56^+CD16^+ cells found after CD56^{bright}CD16^- cells were cultured with (striped) or without (solid) CSA (left).
average percentage of CD56<sup>+</sup>CD16<sup>-</sup> cells generated after culture of the CD56<sup>dim</sup>CD16<sup>+</sup> purified fraction either with (striped) or without (solid) CSA (right). Results are the average +/- SD for 5 separate donors.

**Figure 3: CSA-exposed NK cells do not show Ca<sup>2+</sup> oscillations, NFAT dephosphorylation and/or nuclear translocation following engagement with K562 cells.** A) NK cells were cultured with IL-2 (100 U/ml) and IL-15 (10 ng/ml) ± CSA for 7 days and used to assess dynamic intracellular calcium changes in individual cells (described in Methods). Following the addition of K562 target cells, vehicle treated (EtOH) NK cultures showed the expected calcium oscillations. In contrast, CSA treated NK cells showed no oscillations. Results are representative of 5 individual donors. B) The addition of K562 cells leads to rapid NFAT dephosphorylation in control (EtOH) treated cells, but not in CSA-exposed NK cells. Freshly isolated NK cells were cultured for 7 days with IL-2 (100 U/ml) and IL-15 (10 ng/ml) ± CSA for 7 days. Following this, NK cells were co-cultured with K562 target cells (E:T = 1:1) for 5 and 10 minutes. Controls included K562 cells alone (left) and NK cells without the addition of K562 (time 0). Immediately at the time point stated (5 and 10 minutes) cells were lysed and the subjected to Western blotting. Actin served as the loading control. Results are representative of 3 individual donors. C) and D) Multi-spectral imaging demonstrating that NFAT nuclear translocation is inhibited following K562 engagement in CSA-treated NK cells, but not vehicle treated control cultures. C) Individual images of vehicle (EtOH) control (top) and CSA-treated cells (bottom) showing nuclear NFAT translocation and non-translocation after co-culture with K562 cells (10 min.),
respectively. Shown are bright field, FL-1, FL-4, FL-2, and composite images from left to right. D) NFAT vs. 7-AAD similarity score for vehicle control (upper row) and CSA (lower row) treated NK cultures both at baseline (left columns) and following co-culture with K562 cells (right columns) after 10 min. Gates identify cells that have not (left) and have (right) undergone NFAT translocation. Results are representative of 2 experiments on ~2,500 events for each condition.

**Figure 4: CSA treated NK cells have higher cytotoxicity, in part, due to the reduction in KIR expressing cells.** Cytotoxicity against: A) K562 cells or B) Raji after 7 days of culture. The results for individual donors (n=11, light gray dashed lines) and average cytotoxicity (black bar) (E:T=5:1) are shown. Statistics calculated using the Wilcoxon singed rank test. C) Cytotoxicity of NK cells (cultured with vehicle or CSA) against KIR-L expressing LCLs (HLA-B Bw4, and HLA-C C1/C2). The average cytotoxicity of 4 donors ± SE is shown (E:T=5:1). Statistics calculated using the Wilcoxon singed rank test. D) FACS histograms for granzyme B (left), perforin (middle) and FasL (right) for cells cultured CSA (heavy line, open histogram) or vehicle (EtOH, dotted line, closed histogram). Isotype controls are shown in dashed line and open histogram. Granzyme B and perforin was detected using intracellular staining, while surface staining was used for FasL. Results are representative of 4 donors.

**Figure 5: Changes in NCRs (NKp30, NKp44, and NKp46) and NKG2D after culture with CSA.** A) NK cells were cultured in IL-2 (100 U/ml) and IL-15 (10 ng/ml) (± CSA for 7 days) and analyzed by FACS. NKp30 staining for a representative donor is
shown. Geometric mean fluorescent intensity (gMFI) is listed in right lower portion of figure. B) Relative change in gMFI for NKp30, NKp44, NKp46 and NKG2D after culture with CSA. Relative change in gMFI = 100 x (gMFI of receptor in CSA - gMFI of receptor in control)/ gMFI of receptor in control. Results are the average of 16 healthy donors. Statistics calculated using the Wilcoxon singed rank test.

**Figure 6: Intracellular IFN-γ Detection in CSA-exposed NK cells.** NK cells were cultured with IL-2 (100 U/ml) and IL-15 (10 ng/ml) ± CSA for 7 days and then IFN-γ production in NK cells was induced by culturing cells in IL-12 (10 ng/ml) and IL-18 (100 ng/ml) for 18 hours. Brefeldin A 10 µg/ml final concentration was added for the last 4 hours. Samples were stained with CD56 first, permeabilized and stained with IFN-γ. A) A representative donor. B) The percentage of IFN-γ expressing cells in 8 consecutive donors. Black bars represent mean. Statistics calculated using the Wilcoxon singed rank test.

**Figure 7: Hematopoietic progenitor cell-derived NK cells express less KIR in the presence of CSA.** UCB-derived CD34+CD38Lin− cells cultured on a stromal cell line (AFT024) in the presence of cytokines (IL-3, IL-7, IL-15, SCF and FLT-3L) and increasing amounts of CSA (0.1-5 µg/ml) as described in Methods. On day +50, cultures were examined for A) Total cell number, B) percentage and C) absolute number of KIR-expressing NK cells. Results are the average of 4 donors ± SEM. Asterisk (*) indicates values that were significantly less than controls (p<0.05) by Mann-Whitney rank sum test.
Table Legends

Table 1: CSA induces a reduction in NK cell fold expansion due to an increase in apoptosis. NK cells were isolated and cultured in IL-2 (100 U/ml) and IL-15 (10 ng/ml) ± CSA. Cells were enumerated at days 3, 5, and 7 of culture and the average ± SD is shown (n=8). Apoptosis was evaluated by FACS staining for annexin V and PI at the above time points. Cells were considered apoptotic if annexin V+/PI⁻ or annexin V+/PI⁺ and the average % of apoptotic ± SD is shown (n=8). NS, not significant

Table 2: Fold Expansion of FACS sorted CD56<sup>bright</sup>CD16⁻ and CD56<sup>dim</sup>CD16⁺ NK cells with vehicle or CSA. NK cells were isolated using negative selection from buffy coat preparations and cells were then stained with CD56 and CD16 and CD3 and FACS sorted into CD3⁻CD56<sup>bright</sup>CD16⁻ and CD3⁻CD56<sup>dim</sup>CD16⁺ populations (see figure 2E for representative experiment). Cells were then cultured with CSA or vehicle (EtOH). After 7 days, fold expansion was determined by enumeration. Sorted cells were also examined by FACS and the majority (>85%) retained the sorted phenotype (i.e., CD56<sup>bright</sup>CD16⁻ and CD56<sup>dim</sup>CD16⁺ see figure 2E for representative donor).
Figure 1

A) Fold Expansion

B) Percentage Positive

CD56+CD16-

CD56+CD16+

Day 0

Day 7 EtOH

Day 7 CSA

CD56

CD16

p < 0.01

p < 0.01

EtOH CSA

EtOH CSA
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
**Figure 7**

A) Abs. Number of KIR+ cells

B) Percentage of KIR+ cells

C) Abs. Number of KIR+ cells
## Table 1

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Table 2

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The unexpected effect of cyclosporin A on CD56+CD16- and CD56+CD16+ natural killer cell subpopulations

Hongbo Wang, Bartosz Grzywacz, David Sukovich, Valarie McCullar, Qing Cao, Alisa B. Lee, Bruce R. Blazar, David N Cornfield, Jeffrey S. Miller and Michael R. Verneris