Evidence for CD16a-Mediated NK Cell Stimulation in Antibody-Mediated Kidney Transplant Rejection

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Background. Natural killer (NK) cells localize in the microcirculation in antibody-mediated rejection (AMR) and have been postulated to be activated by donor-specific anti-HLA antibodies triggering their CD16a Fc receptors. However, direct evidence for NK cell CD16a triggering in AMR is lacking. We hypothesized that CD16a-inducible NK cell-selective transcripts would be expressed in human AMR biopsies and would offer evidence for CD16a triggering. **Methods.** We stimulated human NK cells through CD16a in vitro, characterized CD16a-inducible transcripts, and studied their expression in human kidney transplant biopsies with AMR and in an extended human cell panel to determine their selectivity. **Results.** In NK cells, CD16a stimulation induced increased expression of 276 transcripts (FC > 2x, false discovery rate < 0.05), including *IFNG*, *TNF*, *CSF2*, chemokines, such as *CCL3*, *CCL4*, and *XCL1*, and modulators of NK cell effector functions (*TNFRSF9*, *CRTAM*, *CD160*). Examination in an extended human cell panel revealed that *CD160* and *XCL1* were likely to be selective for NK cells in AMR. In biopsies, 8 of the top 30 CD16a-inducible transcripts were highly associated with AMR ($P < 5 \times 10^{-6}$): *CCL4*, *CD160*, *CCL3*, *XCL1*, *CRTAM*, *FCRL3*, *STARD4*, *TNFRSF9*. Other NK cell transcripts (eg, *GNL1*) were increased in AMR but not CD16a-inducible, their presence in AMR probably reflecting NK cell localization. **Conclusions.** The association of CD16a-inducible NK cell-selective transcripts *CD160* and *XCL1* with biopsies with AMR provides evidence for NK cell CD16a activation in AMR. This raises the possibility of other CD16a-triggered effects that are not necessarily transcriptional, including NK localization and cytotoxicity.

(*Transplantation* 2017;101: e102–e111)

Antibody-mediated rejection (AMR) is the major cause of renal allograft failure,¹ but its underlying mechanisms are incompletely understood.² AMR is characterized by microvascular inflammation and circulating donor-specific HLA antibodies (DSA).^{3,4} The potential effector functions of DSA against donor endothelium include direct effects, complement activation, and recruitment of effector cells through engagement of Fc receptors and complement breakdown products.^{5,6} Complement-fixing DSA are more damaging to kidney transplants,⁷ although C4d deposition is not

always evident.^{1,8-14} Leukocytes in the microcirculation in biopsies from patients with AMR suggest an effector role for these cells, but whether such cells are mediators of injury or are recruited because of injury is difficult to establish.

One cell type expressing Fc receptors first identified in our previous studies as being associated with AMR is the natural killer (NK) cell.^{15,16} The principal Fc gamma receptor on human NK cells is CD16a (FcγRIIIa), an activating receptor largely resistant to signals from inhibitory NK receptors.¹⁷ CD16a triggering releases cytokines and cytotoxic molecules that induce injury and target cell apoptosis, a process called antibody-dependent cell-mediated cytotoxicity (ADCC). The association of NK cells with human AMR is well established but the role of CD16a activation, although hypothesized, has not been established. The available mouse models are supportive of a role for NK cells. One study suggested that early production of chemokines was mediated by NK cells in an athymic nude mouse skin allograft model of AMR.¹⁸ Other mouse studies report that Fc receptors and NK cells are involved in AMR in cardiac and kidney allograft models.^{19,20} However, it is difficult to draw a parallel between murine and human Fc receptors because their expression, structure, associated signaling molecules, and affinities for different IgG subclasses differ greatly.²¹⁻²³ Thus Fc receptor involvement in murine AMR may be fundamentally different from Fc receptor involvement in human AMR.

Given the limitations of animal models, we studied CD16a triggering in vitro in primary human NK cells and examined the resulting gene expression changes in human kidney transplant biopsies. We hypothesized that CD16a-inducible NK cell gene expression changes would be distinguishable in biopsies

Received 12 August 2016. Revision received 21 October 2016.

Accepted 2 November 2016.

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P F Halloran holds shares in Transcriptome Sciences Inc., a company with an interest in molecular diagnostics. The other authors declare no funding or conflicts of interest.

M.D.P. is the primary author of this article and carried out experiments and analyses described within. P.F.H. and L.G.H. are the principal investigators for this study and played significant roles in designing the study, and shaping the content and structure of this article.

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ISSN: 0041-1337/17/10104-e102

DOI: 10.1097/TP.0000000000001586

diagnosed with AMR when compared to other diagnoses. Thus we characterized CD16a-inducible NK cell selective transcripts and examined their associations with human AMR.

MATERIALS AND METHODS

Patient Population and Biopsy Collection

As previously described,²⁴ a set of 703 kidney transplant biopsies collected from 579 patients at 6 kidney transplant centers were histologically classified as per the Banff 2013 report.²⁵ Patient demographics and clinical details for this set have been published.^{26,27} Biopsy collection for this study was approved by the institutional review boards of participating centers. Some biopsies were collected as part of the International Collaborative Microarray study (ClinicalTrials.gov NCT01299168).

Transcript Expression in Biopsies

RNA extraction from biopsies, subsequent labeling, and hybridization to HG-U133 Plus 2.0 GeneChip human gene expression arrays (Affymetrix, Santa Clara, CA) was performed as previously described.²⁷ CEL files were generated with Affymetrix GeneChip Command Console Software version 4.0. Platforms used in analysis include GeneSpring GX 13.0 (Agilent Technologies, Santa Clara, CA), Microsoft Office Excel (Redmond, WA), and "R" software.

Transcript Expression in Cultured Cells

We used a Ficoll-Paque (GE Healthcare Life Sciences, Baie-D'Urfé, Quebec, Canada) density gradient to isolate peripheral blood mononuclear cells (PBMCs) from the blood of healthy volunteers. Cells were purified using EasySep (Stem Cell Technologies, Vancouver, BC, Canada) negative selection kits, and purity was assessed by flow cytometry. Cells were cultured as specified below.

NK Cells

Cells were purified from PBMCs using an EasySep Human NK Cell Enrichment Kit. Data were obtained from 3 separate cultures of NK cells from 3 different donors. Purity of CD45+/CD3-/CD56+ cells as a percent of all viable cells was 83% to 96%. Stimulated NK cell cultures were prepared in plates coated with goat antimouse IgG F(ab')₂ (Jackson ImmunoResearch, West Grove, PA), which was used to cross link anti-CD16a antibodies on the NK cells; unstimulated cells were cultured in uncoated wells. NK cells were coated with anti-CD16a LEAF antibodies (BioLegend, San Diego, CA) before adding them to F(ab')₂-coated plates. Cells were cultured with 200 U/mL recombinant human IL-2 (Affymetrix eBioscience, San Diego, CA). IFNG and TNF enzyme-linked immunosorbent assays were performed on cell-free supernatants to confirm activation. Cells were lysed in TRIzol Reagent (Life Technologies Inc., Burlington, ON, Canada). Total RNA was collected and analyzed on Affymetrix PrimeView GeneChip human gene expression arrays according to manufacturer-recommended procedures. Gene expression data were averaged across the 3 cultures for analysis.

Monocytes

Cells were purified from PBMCs of a single donor using an EasySep™ Human Monocyte Enrichment Kit without CD16a

Depletion. Purity of CD45+/CD3-/CD14+ as a percent of all viable cells was 82%. Cells were stimulated with 500 U/mL recombinant human IFNG (Affymetrix eBioscience). Cells were cultured and harvested at 2-, 4-, and 8-hour timepoints. IL-6 and TNF enzyme-linked immunosorbent assays were performed on cell-free supernatants to confirm activation at each timepoint. RNA extraction and microarray analysis was performed as above. Gene expression data were averaged across the 3 timepoints.

Extended Cell Panel

We used existing Affymetrix HG-U133™ GeneChip gene expression data for a panel of unstimulated primary human B cells, unstimulated immature and lipopolysaccharide-stimulated mature primary human dendritic cells, unstimulated primary human monocytes, allostimulated primary human CD4 and CD8 T cells, human umbilical vein endothelial cells (HUVECs) (ATCC, Manassas, VA) +/- IFNG treatment, human renal proximal tubule epithelial cells (RPTECs) (Lonza, Inc., Allendale, NJ) +/- IFNG, and primary human macrophages +/- IFNG isolated and cultured as previously described.¹⁵

Analysis of CD16a-Inducible NK Transcripts in NK Cells and Biopsies

Gene expression data were preprocessed using robust multi-array averaging. We identified probe sets increased more than 2× across CD16a-stimulated versus 3 unstimulated NK cells isolated from 3 healthy volunteers (Benjamini-Hochberg false discovery rate < 0.05). We eliminated transcripts with expression more than 500 across 4 control nephrectomies, expression less than 200 in stimulated NK cells, and fold change more than 2× in IFNG-stimulated versus unstimulated monocyte cultures. Transcript lists presented are all nonredundant for multiple probe sets.

Biopsy and extended cell panel data were obtained using HG-U133 arrays, but CD16a-inducible transcripts were identified using PrimeView arrays, which use different naming conventions to assign probe set IDs to transcripts. To permit comparison between transcripts on the 2 platforms, HG-U133 probe set IDs representing the top 30 CD16a-inducible transcripts' gene symbols were selected on the basis of sequence consensus with PrimeView probe sets, probe set selectivity, and signal intensity.

To incorporate NK cell PrimeView-based expression data for CD16a-stimulated NK cells into the heatmap in Figure 3 HG-U133 probe set expression in each cell type on the panel was normalized to the differences between unstimulated NK cells which were common to both HG-U133 and PrimeView microarrays.

Cytokine & Chemokine Profiling

Primary human NK cells were stimulated with anti-CD16a or left unstimulated as described above. Supernatants were harvested after 2, 4, 8, or 24 hours. Using a Meso Scale Discovery V-PLEX Human Cytokine 30-Plex Kit (Rockville, MD), we measured levels of CCL2, CCL3, CCL4, CCL13, CCL17, CCL22, CSF2, CXCL10, Eotaxin, Eotaxin-3, Flt-1/VEGFR1, IFNG, IL-10, IL-12/IL-23p40, IL-12p70, IL-13, IL-15, IL-16, IL-17A, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, TNF, TNF-β, VEGF-A, and VEGFR-2 in the culture supernatants.

RESULTS

Characterization of the NK Cell Response After CD16a Activation

The algorithm for the analyses in this study is described in Figure 1. We characterized global gene expression changes induced in primary human NK cells after in vitro CD16a stimulation. We looked for highly increased transcripts that were highly expressed. In pilot experiments, we observed that small numbers of contaminating monocytes can alter transcript lists by introducing IFNG-inducible monocyte transcripts which may not be truly associated with NK cell CD16a activation in AMR (data not shown). Thus, we eliminated transcripts increased >2x in IFNG-stimulated versus unstimulated primary human monocytes. We also eliminated CD16a-inducible transcripts that were highly expressed in normal kidneys, as these would not be useful for studying NK cell involvement in kidney transplant biopsies.

Four hundred fifty-seven probe sets representing 276 unique transcripts were increased more than 2× in stimulated versus unstimulated NK cells (false discovery rate < 0.05).

The top 30 most increased transcripts (by fold change vs unstimulated NK cells) with >200 expression in stimulated NK cells are shown in Table 1. CD16a-inducible transcripts included cell surface markers associated with effector cell function *CRTAM*, *TNFRSF9* (4-1BB), and *CD160*; chemokines *CCL3* (MIP-1α), *CCL4* (MIP-1β), and *XCL1* (lymphotactin); and effector cytokines such as *IFNG*, *TNF*, and *CSF2* (GM-CSF). Notably, *TNF* transcripts were increased 14.8× in CD16a-stimulated versus unstimulated NK cells, but *TNF* was also IFNG-inducible in monocytes and thus eliminated from the top transcripts (although of obvious interest as a CD16a-inducible NK cell product).

We validated some of these gene expression changes by measuring protein analytes in NK cell cultures. A multiplex approach was used to measure 30 chemokines, cytokines, and growth factors in culture supernatants (Figure 2). Over a time course of 24 hours, only 5 of the 30 soluble mediators measured were detectable at levels greater than 100 pg/mL in stimulated NK cell cultures: *CSF2* (GM-CSF), *CCL3*, *CCL4*, *TNF*, and *IFNG*. Although we predict that *XCL1* would follow a similar pattern to the other mediators measured, it was

FIGURE 1. Procedural outline of this study.

TABLE 1.
Raw expression values and fold change after CD16a stimulation for the top 30 CD16a-inducible transcripts in primary human NK cells stimulated in vitro

Gene symbol	Gene title	Alias	Unstimulated NK cells (raw expression)	NK cells, anti-CD16a stimulated raw expression	Fold change (stimulated vs unstimulated)
<i>CRTAM</i>	Cytotoxic and regulatory T cell molecule	CD355	45	7361	164.7
<i>IFNG</i>	Interferon, gamma	—	59	6484	110.0
<i>TM6SF9</i>	Tumor necrosis factor receptor superfamily, member 9	4-1BBR	43	3835	90.1
<i>SPRY2</i>	Sprouty homolog 2 (Drosophila)	IGAN3	19	895	46.1
<i>CCL4</i>	Chemokine (C-C motif) ligand 4/4-like 1/4-like 2	MIP-1B	289	12522	43.4
<i>CD72</i>	CD72 molecule	LYB2	20	667	33.5
<i>CSF2</i>	Colony stimulating factor 2 (granulocyte-macrophage)	GM-CSF	34	1093	32.3
<i>CCL3</i>	Chemokine (C-C motif) ligand 3/3-like 1/3-like 3	MIP-1A	322	7433	23.1
<i>ZBED2</i>	Zinc finger, BED-type containing 2	—	15	332	22.7
<i>FEZ1</i>	Fasciculation and elongation protein zeta 1 (zygin I)	—	119	2624	22.0
<i>RGS16</i>	Regulator of G-protein signaling 16	RGSR	50	831	16.8
<i>XCL1</i>	Chemokine (C motif) ligand 1	LTN	93	1344	14.4
<i>NR4A2</i>	Nuclear receptor subfamily 4, group A, member 2	NOT	141	1888	13.4
<i>TRAF1</i>	TNF receptor-associated factor 1	—	55	628	11.5
<i>NR4A3</i>	Nuclear receptor subfamily 4, group A, member 3	CHN	20	220	11.3
<i>IRF4</i>	Interferon regulatory factor 4	LSRF	157	1749	11.2
<i>NAB2</i>	NGFI-A binding protein 2 (EGR1 binding protein 2)	YGL122C	45	479	10.6
<i>CD160</i>	CD160 molecule	BY55	481	4602	9.6
<i>FCRL3</i>	Fc receptor-like 3	CD307c	275	2568	9.3
<i>PTPN6</i>	Protein tyrosine phosphatase, nonreceptor type 6	SHP1	93	836	9.0
<i>SERPINE2</i>	Serpin peptidase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 2	GDN	105	791	7.6
<i>SPRY1</i>	Sprouty homolog 1, antagonist of FGF signaling (Drosophila)	—	38	235	6.1
<i>RAD</i>	Ras-related associated with diabetes	RAD	56	339	6.0
<i>GPR18</i>	G protein-coupled receptor 18	—	606	3641	6.0
<i>TBC1D4</i>	TBC1 domain family, member 4	—	49	293	5.9
<i>STARD4</i>	STAR-related lipid transfer (START) domain containing 4	—	108	643	5.9
<i>RILPL2</i>	Rab interacting lysosomal protein-like 2	RLP2	96	556	5.8
<i>FAM3C</i>	Family with sequence similarity 3, member C	ILEI	209	1196	5.7
<i>BIRC3</i>	Baculoviral IAP repeat containing 3	c-IAP2	157	892	5.7
<i>MYC</i>	V-myc myelocytomatosis viral oncogene homolog (avian)	MRTLc	287	1616	5.6

Results are the average of 3 independent culture replicates each using NK cells from a different donor.

FIGURE 2. Cytokine and chemokine production by CD16a-stimulated NK cells. Positive readouts from a 30-plex analysis of cytokines, chemokines, and growth factors in CD16a-stimulated and unstimulated NK cell culture supernatants are depicted. Positive readouts are defined as readouts well within the limits of or exceeding the detection range of the standard curve for each molecule. All other molecules were negligibly produced, and therefore not shown. Concentration is given in picograms per million NK cells. Cells were cultured for 2, 4, 8, and 24 hours with 200 U/10⁶ cells recombinant human IL2, with a density of 500 000 cells/mL medium at harvest. This experiment was performed once.

absent from the multiplex panel. Thus, CD16a triggering of NK cells produces a limited number of chemokines and effector cytokines that corroborate gene expression changes.

Identifying CD16a-Inducible NK-cell Selective Transcripts: Expression of NK Cell Transcripts in Other Cell Types

Promiscuous expression in many cell types would make a transcript impossible to interpret as evidence for CD16a triggering in AMR. We examined the selectivity of expression in NK cells among other cell types. Expression of the top CD16a-inducible transcripts was examined in a primary human cell panel composed of B cells, CD4⁺ and CD8⁺ effector T cells, unstimulated and CD16a stimulated NK cells, monocytes,

macrophages +/- IFNG, HUVECs +/- IFNG, RPTECs +/- IFNG, immature and lipopolysaccharide-treated mature dendritic cells (Figure 3).

Multiple CD16a-inducible transcripts were expressed in other cell types in their resting states or after IFNG treatment. *GPR18* and *CD72* were expressed in B cells. *NR4A3* and *IRF4* were expressed by dendritic cells. *SERPINE2* had high expression in RPTECs that overshadowed its expression in other cells. *FEZ1* was expressed in HUVECs and RPTECs. *STARD4* was IFNG-inducible in HUVECs, and highly expressed in all cell types examined. Certain transcripts were relatively selective for NK cells, making them useful as evidence for CD16a activation if they are strongly associated with AMR. *CD160* is of particular interest given its selectivity and high expression in CD16a-stimulated NK cells.

FIGURE 3. Comparative gene expression of the top 30 CD16a-inducible transcripts in NK cells. Top 30 CD16a-inducible transcripts' expression in an extended human cell panel—geometric mean expression for each transcript's representative probe set was calculated for each cell type and then scaled to the ratio of expression in unstimulated NK cells processed on HG-U133 gene chips to unstimulated NK cells processed on PrimeView gene chips. Geometric mean expression values are given in base-2 logarithmic format in each heatmap cell. Color is mapped to the logarithmic geometric mean expression values. Clustering was calculated using a Euclidean distance matrix.

Expression of CD16a-Inducible NK Cell Transcripts in Biopsies With AMR

We examined CD16a-inducible transcripts across 703 kidney transplant biopsies and determined their association with biopsies diagnosed as AMR (N = 110) compared with all other diagnoses except TCMR and mixed rejection (Figure 4). TCMR and mixed rejection were excluded because NK cells and T cells are phenotypically similar and share expression of some of these transcripts. The implications of this overlap are not trivial and thus will comprise a separate manuscript (manuscript in preparation).

Not all of the top CD16a-inducible transcripts were strongly associated with AMR, as expected because some have

other patterns of cell expression. However, 8 of the top 30 CD16a-inducible transcripts showed strong associations with AMR ($P < 5 \times 10^{-6}$): *CCL4*, *CD160*, *CCL3*, *XCL1*, *CRTAM*, *FCRL3*, *TNFRSF9*, and *STARD4* (Table 2).

We reviewed the cell panel data to identify which of these transcripts most selectively reflect NK cell CD16a triggering in AMR. *CD160* was selective for NK cells. *XCL1* was restricted to NK cells and effector CD4+ and CD8+ T cells, but since effector T cells contribute little to the disease process in AMR, *XCL1* expression in AMR selectively supports CD16a triggering.

Other transcripts were more difficult to interpret. *FCRL3* was highly expressed in B cells and T cells. *CCL3*, *CCL4*,

FIGURE 4. Association of CD16a-inducible transcripts in NK cells with antibody-mediated rejection in kidney transplants. Association of the top 30 CD16a-inducible NK cell transcripts with histologically classified AMR versus all other diagnoses except TCMR and mixed rejection—probe sets are plotted by fold change and association with 110 histologically classified AMR biopsies versus 498 biopsies without histologically classified TCMR or mixed rejection.

and *CRTAM* were expressed in T cells and dendritic cells and were IFNG-inducible in monocytes and macrophages. *STARD4* showed widespread expression across most cells in the panel with particular IFNG responsiveness in HUVECs. *TNFRSF9* was moderately expressed in unstimulated macrophages but decreased after macrophage stimulation with IFNG. Despite its robust expression in stimulated NK cells in vitro, *IFNG* was only modestly associated with AMR ($P = 4.6 \times 10^{-3}$) despite its relatively strong expression in biopsies with AMR, likely due to its induction in other tissue injury.²⁸

Some NK Cell Transcripts are Highly Expressed in AMR but not CD16a-inducible

We previously identified a set of 6 NK cell transcripts associated with biopsies from DSA-positive patients that implicated NK cells in kidney AMR: *GNLY*, *MYBL1*, *FGFBP2*, *SH2D1B*, *KLRF1*, and *CX3CR1*.¹⁵ These NK cell transcripts were highly associated with AMR—a finding recently

validated by others²⁹—but it was not known whether they reflect CD16a stimulation. All 6 transcripts show high expression in both stimulated and unstimulated NK cells and are therefore not CD16a-inducible (Table 3).

DISCUSSION

The present study aimed to determine whether evidence for CD16a-mediated NK cell activation could be found in biopsies diagnosed with AMR by identifying transcripts that were CD16a-inducible, NK cell selective, and strongly associated with AMR. First, we studied CD16a-inducible transcripts in primary human NK cells, and identified a limited number of chemokines, cytokines, and other NK transcripts. Across 703 clinically indicated kidney transplant biopsies, at least 8 top CD16a-inducible transcripts were highly associated with AMR versus other diagnoses, suggesting NK cell CD16a activation in these biopsies. Two in particular,

TABLE 2.

Characteristics of the top 30 CD16a-inducible NK cell transcripts most strongly associated with AMR

Gene symbol	Gene title	Alias	Fold change (stimulated vs unstimulated NK cells)	P value (AMR vs everything except TCMR, mixed)	Cells with >200 expression ^a
<i>CCL4</i>	Chemokine (C-C motif) ligand 4	MIP-1b	43.4	7.97×10^{-25}	NK, DC, T4, T8, MC, MP
<i>CD160</i>	CD160 molecule	BY55	9.6	1.64×10^{-19}	NK
<i>CCL3</i>	Chemokine (C-C motif) ligand 3	MIP-1a	23.1	1.09×10^{-15}	NK, DC, T4, T8, MC, MP
<i>XCL1</i>	Chemokine (C motif) ligand 1	LTN	14.4	1.93×10^{-14}	NK, T4, T8
<i>CRTAM</i>	Cytotoxic and regulatory T cell molecule	CD355	164.7	6.89×10^{-11}	NK, T8, MP
<i>FCRL3</i>	Fc receptor-like 3	CD307c	9.3	1.30×10^{-10}	NK, B, T4, T8
<i>TNFRSF9</i>	Tumor necrosis factor receptor superfamily, member 9	4-1BB	90.1	1.34×10^{-8}	NK, MP
<i>STARD4</i>	StAR-related lipid transfer (START) domain containing 4	—	5.9	3.10×10^{-8}	NK, B, DC, T4, T8, MP, HUVEC, RPTEC

^a Based on the extended cell panel shown in Figure 4.
B, B cells; DC, dendritic cells; MC, monocytes; MP, macrophages; N-, CD16a-stimulated NK cells; T4, allostimulated CD4+ T cells; T8, allostimulated CD8+ T cells.

TABLE 3.

Raw expression values of DSA-selective NK-associated transcripts in CD16a-stimulated and unstimulated NK cells, sorted by decreasing association with AMR

Gene symbol	Gene title	Unstimulated NK cells	CD16a-stimulated NK cells (FC vs unstimulated)	P (AMR vs all diagnoses except TCMR, mixed)
<i>GNLY</i>	Granulysin	15 676	15 049 (0.96)	9.12×10^{-25}
<i>FGFBP2</i>	Fibroblast growth factor binding protein 2	10 404	5410 (0.52)	5.45×10^{-22}
<i>SH2D1B</i>	SH2 domain containing 1B	6512	8402 (1.29)	1.21×10^{-21}
<i>CX3CR1</i>	Chemokine (C-X3-C motif) receptor 1	4154	2825 (0.68)	3.25×10^{-18}
<i>KLRF1</i>	Killer cell lectin-like receptor subfamily F, member 1	6886	7988 (1.16)	2.26×10^{-17}
<i>MYBL1</i>	v-myb myeloblastosis viral oncogene homolog (avian)-like 1	3800	2888 (0.76)	4.33×10^{-14}

CD160 and *XCL1*, are relatively NK cell selective; their association with AMR proves to be the strongest evidence of NK cell CD16a triggering in AMR. However, not all top CD16a-inducible NK cell transcripts showed strong association with AMR, because of promiscuous expression in other cells. We also studied the expression of a set of 6 previously identified NK cell transcripts that provided initial evidence for NK cell involvement in human AMR,¹⁵ and found that they were highly expressed in both stimulated and unstimulated NK cells, but were not strongly CD16a-inducible. Thus, although relatively few transcripts were NK cell selective and strongly associated with AMR, our findings agree with NK cell CD16a stimulation occurring in human AMR.

Although many transcripts showed increased expression after NK cell CD16a stimulation, few were uniquely expressed in NK cells. Of the 8 strongly AMR-associated CD16a-inducible transcripts identified in this study only *CD160* appeared selective for NK cells. Selection of transcripts expressed in other cell types may reflect induction augmented by the presence of CD16a-stimulated NK cells. For instance, *CCL3* and *CCL4* are CD16a-inducible in NK cells, expressed in monocytes and macrophages, and highly associated with AMR. Thus, *CCL3* and *CCL4* must reflect either the additive effect of production by both NK cells and monocytes, or induction in CD16a-stimulated NK cells augmented by the presence of monocytes.³⁰ Further studies are necessary to determine the reason for the pronounced association of *CCL4* and *CCL3* with AMR.²⁴ *XCL1* expression is restricted to T and NK cells but may be considered NK-selective in the context of AMR. This assumption applies in AMR because T cell-mediated processes are underrepresented. Previous studies showed that T cell transcripts associated with TCMR (eg, *CTLA4*, *ICOS*, *CD28*) are negligibly associated with AMR.^{16,24} Given that *XCL1* is strongly associated with AMR, but key T cell transcripts such as *CTLA4*, *ICOS*, and *CD28* are not, it follows that T cell contribution to the association of *XCL1* with AMR is minimal. Our findings agree with those of Suviolahti et al³¹ who demonstrate that a subset of the CD16a-inducible transcripts we describe also increase in an in vitro ADCC assay. Although their model could not ascertain the NK cell specificity of the gene expression changes, it corroborates our conclusion that the transcripts we identify are responsive to CD16a stimulation in NK cells and are increased in biopsies diagnosed as AMR.

The complex expression of some notable effector cytokines makes it difficult to attribute roles unique to AMR, but such roles cannot be excluded. *IFNG* and *CSF2* are effector cytokines that are potentially NK selective in AMR, but

were not strongly associated with AMR. *CSF2* is expressed in AMR and its protein product is released by stimulated NK cells in vitro, but *CSF2* was not differentially expressed relative to other diagnoses. This is perhaps because *CSF2* is inducible in many cells by proinflammatory cytokines such as TNF that are commonly increased in multiple inflammatory states not modeled in our in vitro studies.³² *IFNG* expression is increased in AMR but it is also increased after tissue injury, weakening the strength of its association with AMR. Furthermore, *IFNG* is regulated by CD16a-inducible receptors *CD160*³³ and *CD72*,³⁴ among others,^{17,35} raising the possibility of regulatory mechanisms engaged in vivo that were not engaged in our model. Nonetheless, endothelial *IFNG*-inducible transcripts such as *CXCL11* and *PLA1A* are strongly associated with AMR, arguing for a major role for NK cell-derived *IFNG* in AMR.^{16,36,37} Effector cytokines, such as *IFNG*, *TNF*, and *CSF2*, play intertwined roles in AMR but investigating the details of their synergies in AMR will require the creation of novel in vitro experiments that better incorporate the immunologic complexities of AMR. A related limitation of this study is that it does not determine whether the CD16a-inducible transcripts specifically result from CD16a-stimulated NK cells; this would be difficult to determine with certainty as other NK cell receptors show a high degree of overlap to CD16a in signaling pathways used.³⁸ However, it is noteworthy that the NK cell gene signature was originally identified in biopsies from DSA-positive patients compared to DSA negative patients,¹⁵ which argues for CD16a stimulation as the most likely source of the CD16a-inducible transcripts in biopsies with AMR in this study.

Our studies suggest that transcripts reflecting the presence of NK cells are predominantly associated with AMR (eg, *GNLY*, *FGFBP2*) and are not necessarily CD16a-inducible. Because the downstream effects of CD16a stimulation promote NK cell localization, these transcripts may be interpreted as indirect indications of CD16a activation. *IFNG*-mediated upregulation of HLA molecules on endothelial cells, for example, provides additional targets for DSA and would enhance physical interactions between NK cells and DSA. AMR-associated chemokines resulting from NK cell *IFNG* effects on endothelium (*CXCL11*) and those directly downstream of CD16a (*XCL1*) also promote NK cell recruitment. The lack of CD16a-inducible transcripts in our initial description of NK cells in AMR may reflect the algorithm used to identify those transcripts. Our previous study focused on the association with the presence of DSA rather than association with a specific diagnosis; thus, the algorithm included

multiple diagnoses in DSA-positive patients. This algorithm would have further diluted the association with AMR.

The detailed mechanism of how NK cells and DSA damage microvascular endothelium in AMR remains incompletely understood. Although NK cells have roles in antiviral activity, anti-tumor responsiveness, and immunoregulation in pregnancy,³⁹ these roles are nondeleterious and probably not CD16a-dependent, making AMR a human disease uniquely driven by NK cell CD16a activation. The effects of CD16a activation are deleterious for the graft, and pathology may result from unresolvable injury in response to inflammatory stimuli, ADCC, or both. Endothelial cytotoxicity could occur by NK cell degranulation or by NK cell CD16a-inducible cytokines.^{40,41} Indeed, TNF-mediated cytotoxicity is enhanced by IFNG⁴²⁻⁴⁴ and other molecules may enhance chronic allograft injury by NK cells.²⁰ This does not preclude a role for complement in AMR, either through lysis or through leukocyte recruitment via complement receptors. The role for complement and NK cells in AMR are joint given the preference of human CD16a for complement fixing IgG subclasses; IgG1 and IgG3.⁴⁵ NK cells have complement receptors CR3, CR4, C3aR, and C5aR,⁴⁶ suggesting that complement receptor engagement enhancement of CD16a-mediated NK cell activation also requires further study. Complement-fixing DSA predict a greater risk of kidney allograft loss⁷ and direct effect of DSA binding to endothelial cells may also play a role.⁴⁷ These effects could be explored in an in vitro model of cell stimulation that includes endothelial cells, NK cells, and complement-fixing DSA. The overlap between gene expression changes induced by CD16a in NK cells and those induced by T cell receptor stimulation in T cells should also be explored, as these cognate antigen recognition systems signal activation through similar pathways.

In light of these data, we propose a model of AMR wherein NK cell localization and activation at the microcirculation endothelium is mediated by CD16a engagement to DSA. CD16a stimulates increased expression of molecules that regulate proliferation, cytotoxicity, and soluble mediator production (eg, CD160, CD72, TNFRSF9, and CRTAM). CD160 is a GPI-linked membrane protein that enhances NK cell cytotoxicity and IFNG production.^{33,48-50} However, CD160 is cleaved from the membrane by metalloproteases in the presence of IL-15, which is produced by activated monocytes,⁵¹ so it may have the opposite effect on NK cells in AMR by blocking DSA interactions with class I HLA.⁵² CD16a also triggers NK cells to secrete inflammatory cytokines and chemokines that encourage margination and proliferation of other leukocytes: CCL3 and CCL4 are potent chemoattractants for monocytes and macrophages,⁵³ CSF2 is a growth factor that encourages macrophage activation and proliferation,⁵⁴ and XCL1 attracts additional NK cells.⁵⁵ IFNG and TNF activate monocytes, macrophages, and endothelial cells,⁵⁶⁻⁵⁸ and may mediate their own cytotoxic effects in AMR.⁴⁴ In this model, monocytes recruited by stimulated NK cells produce chemokines CCL3, CCL4, and CCL5 together with NK cells. Monocytes also engage DSA directly through Fc receptors, further enhancing their cytotoxic effects. CD16a-stimulated NK cells may cause some microvascular damage through ADCC, although it should be noted that endothelial cell remodeling and injury response—potentially related to angiogenesis—is probably the principal determinant of graft dysfunction after NK cell interaction

with endothelium in AMR rather than endothelial cell apoptosis.^{36,59} Taken together, our current and previous studies suggest that CD16a-inducible transcripts would be a useful measure of NK cell activity in a biopsy already diagnosed as AMR, which may have prognostic implications.

ACKNOWLEDGMENTS

This research has been supported by funding and/or resources from the Roche Organ Transplant Research Foundation, and in the past by Genome Canada, the University of Alberta Hospital Foundation, Roche Molecular Systems, Hoffmann-La Roche Canada Ltd., Canada Foundation for Innovation, the Alberta Ministry of Advanced Education and Technology, and Astellas. Special thanks to Anna Hutton, Jeffery Venner, and Vido Ramassar for their assistance with data collection and analysis.

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