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NKG2A AND CD56 ARE CO-EXPRESSED ON ACTIVATED TH2 BUT NOT TH1 LYMPHOCYTES

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Abstract

NKG2A is commonly expressed on cytotoxic cells but has been found on activated T-Helper (TH) cells. In identifying novel differentiating markers between TH1 and TH2 lymphocytes, we focused on NKG2A expression. TH1 and TH2 cells were negatively isolated from healthy volunteers for microarray analysis and RT-PCR. Flow cytometry of quiescent and activated TH1 and TH2 cells was performed. Isolates were >95% pure CD3+CD4+ cells (TH1=90.3% and TH2=84.1%). Microarrays showed differential expression of NKG2A and NKG2C isoforms between TH1 and TH2 cells. RT-PCR showed greater expression of NKG2A in TH2 cells (4-fold) and NKG2C in TH1 cells (3-fold). Flow studies showed tripling of TH2 NKG2A with activation to 10.76±4.01% (p=0.05), a 23-fold increase in CD56 to 35±14.54% (p=0.03), and an increase in NKG2A+CD56+ double-positive cells to 3.04±1.38% (p=0.04). TH1 lymphocytes did not differ with activation. We identified co-induction of NKG2A and CD56 upon activation of TH2 cells. These cells would likely bind more HLA-E and show increased effector inhibition. Given that certain viruses are known to decrease MHC class I and thus HLA-E production by antigen presenting cells, activated TH2 cells would bind less HLA-E in this scenario. This would likely result in less effector inhibition and a relatively robust TH2 response.

Keywords

TH1 Cells; TH2 Cells; Natural Killer Cells; CD56; Lymphocyte Activation

Abbreviations

NK — Natural Killer Cell; HLA — Human Leukocyte Antigen; TH — T-Helper Cell; IL — Interleukin; IFN — Interferon; TCR — T Cell Receptor; PCR — Polymerase Chain Reaction; MHC — Major Histocompatibility Complex; PBMC — Peripheral Blood Mononuclear Cell

Introduction

The NKG2A receptor is a C-type lectin found primarily on cytotoxic cells such as natural killer (NK) cells and CD8+ T lymphocytes [1,2,3,4]. The binding of NKG2A to its natural ligand, human non-classical class I leukocyte antigen (HLA)-E [5,6,7,8,9,10], induces its immunoreceptor tyrosine-based inhibition motif (ITIM) and suppresses cytotoxic cell effector activity [11,12,13,14]. The inhibitory capability of NKG2A on a bearing cell remains relatively stable over time. This is due to a constant supply of fresh NKG2A receptors from endosomal recycling between the plasma membrane and intracellular compartments [15]. However, NKG2A receptor turnover and therefore surface expression can be altered by extracellular mechanisms. For example, NK cell surface expression of NKG2A is increased in the presence of interleukin (IL) -15 [16].

In addition to its familiar cytotoxic cell expression, NKG2A has recently been discovered on human CD4+CD8-T-Helper (TH) lymphocytes by way of CD3-mediated activation with IL-10 and transforming growth factor (TGF)- β [17]. In addition, non-activated murine Th cells polarized in vitro to the Th1 phenotype were found to bind a single antibody for NKG2A and two other C-type lectin family members [18].

Following from these discoveries, multiple reports emphasize the emerging importance of NKG2A in the cytotoxic response to viruses [19,20,21,22], cancer [23,24], and HIV [25,26]. However, the role of NKG2A on non-cytotoxic cells, like TH cells, and the potential for differential expression between TH1 and TH2 subsets remains unclear. Therefore we report the negative isolation of TH1 and TH2 lymphocytes with a novel antibody cocktail developed by the authors. We followed this with mRNA profiling, quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR), and protein expression studies using flow cytometry. We found moderate levels of NKG2A expression on quiescent TH1 and TH2 cells but only low levels of NKG2C on either subtype. Interestingly, after four hours of phorbol-mediated activation, we found significantly increased TH2 cell expression of NKG2A, CD56, and NKG2A-CD56 double-positivity. This represents the first description of MHC class I recognition capability by TH2 cells as well as raises the possibility of a role for NKG2A in the regulation of TH2 cell effector functions.

Materials and Methods

Participants

Eleven apparently healthy, non-atopic, non-asthmatic volunteers between the ages of 18 and 50 years had 30 mL of venous blood drawn directly into ethylenediaminetetraacetic acid (EDTA)-containing vials. The participants were representative of both genders and a diverse group of ethnicities and races. The investigation was approved by the Institutional Review Board and General Clinical Research Center (GCRC) Advisory Committee and performed in the Children's National Medical Center GCRC.

Cell Isolation

Cell separation procedures were initiated within 30 minutes of blood collection. Whole blood was emptied into conical tubes and centrifuged at low speed. Platelet-rich plasma was removed and the remaining blood concentrate was diluted to 30 mL total volume with 2% fetal bovine serum (FBS) in 1X phosphate-buffered saline (PBS) then centrifuged over Ficoll Paque PLUS™ (Amersham Biosciences, Piscataway, NJ) density medium to isolate the peripheral blood mononuclear cell (PBMC) layer.

TH1 and TH2 Lymphocyte Isolation

After washing and resuspension of the PBMC layer in PBS+2%FBS, the sample was split into three equal aliquots by volume. Cells were counted by hemocytometer to assure a concentration less than 8×10^7 cells/mL. From each aliquot, either non-differentiated TH cells, TH1 or TH2 lymphocytes were negatively isolated using StemSep™ (StemCell Technologies, Vancouver, BC).

Isolations were conducted according to the manufacturer's protocol except for the addition of novel combinations of monoclonal antibodies we developed and validated for the negative isolation of TH1 and TH2 lymphocytes. For the negative isolation of TH1 cells, approximately 5×10^6 cells were treated for 10 minutes at 4°C with anti-CRTH2-biotin (Clone BM16; Miltenyi Biotec, Auburn, CA) to bind TH2 cells. After washing once, cells were incubated at room temperature for 15 minutes with anti-biotin tetramers (StemCell) and cocktail containing antibodies to CD8, CD14, CD16, CD19, CD56, CCR4, and glycophorin A. Samples were centrifuged, the supernatant removed, and the cell pellets containing antibody bound PBMCs and unbound TH1 cells were suspended in PBS+2%FBS. Following a final incubation for 15 minutes at room temperature with StemCell magnetic colloid, samples were passed through a magnetic column. Eluted TH1 cell isolates were immediately prepared for RNA isolation and/or flow cytometry.

The negative isolation of TH2 cells was similar, except samples were first treated with anti-CD29 ($\beta 1$ integrin)-biotin (4B7R; Research Diagnostics Inc., Flanders, NJ) to bind TH1 cells at 4°C for 10 minutes. Samples were washed and then incubated for 15 minutes at room temperature with an antibody cocktail containing anti-CD8, anti-CD14, anti-CD16, anti-CD19, anti-CD56, anti-CXCR3, and anti-glycophorin A. The remaining isolation procedures were identical to those for TH1 cells.

mRNA Expression Profiling

Three pairs of quiescent TH1 and TH2 samples were used for mRNA expression profiling using PicoPure™ (Arcturus Bioscience, Mountain View, CA) RNA extraction methods. Total RNA yield was determined by spectrophotometer UV absorbance and RNA quality by LabChip® Bioanalyzer electrophoresis. RiboAmp® (Arcturus) amplification was performed for two-rounds and BioArray® (Enzo Life Sciences, Farmingdale, NY) biotin labeling was then performed according to the manufacturer's standard protocols.

Expression profiling data was obtained for each sample individually using the Affymetrix (Santa Clara, CA) U133A GeneChip® (22,283 probe sets querying ~18,000 genes), with standard operating procedures and quality controls as we have published [27,28]. Expression values were calculated using both DNA-Chip Analyzer 1.3 (dChip MBE/mismatch model) [www.dchip.org] and Microarray Suite 5 (MAS5) (Affymetrix) probe set algorithms. Comparative analyses of dChip and MAS5 expression values were performed in GeneSpring® 7 (Agilent Technologies, Palo Alto, CA). Expression values were filtered for probe set present calls in at least two out of the three TH1 and TH2 cell samples. ANOVA (1-way) analyses with no multiple testing corrections and a p-value cutoff of 0.05 were used to further filter the

expression values. Data interpretation was limited to filtered probe sets containing NKG* or KLR* (i.e. C-type lectins) in their primary or alternate names. Probe sets ultimately meeting all filter criteria were examined to determine candidates with consistent differential mRNA expression between TH1 and TH2 cells according to both dChip- and MAS5-derived results. We have previously shown such comparative analyses to be effective in reducing the number of false positives obtained from microarray studies [28].

Quantitative Real-Time Reverse-Transcriptase PCR

RNA was extracted by Trizol®/chloroform phase separation for RT-PCR. Total RNA quantities were verified by UV absorption at 260 nm and integrities by 18s and 28s ribosomal bands using LabChip® analysis on a 2100 Bioanalyzer (Agilent, Palo Alto, CA). SuperScript™ III First-Strand Synthesis System (Invitrogen, Carlsbad, CA) was used for cDNA synthesis.

T-bet:GATA-3 mRNA expression ratios were used to confirm the enrichment for TH1 and TH2 cell fractions as adapted from methods described by Chakir, et al. [29,30]. Briefly, DNA primers were designed according to known gene sequences as follows: T-bet (Forward) 5'-CAA CGC CCG GCT GCA TAT CG[FAM]T G-3'; T-bet (Reverse) 5'-TGG TAG GCA GTC ACG GCA AT-3'; GATA-3 (Forward) 5'-CAG AGG ACC CTG TCT GCA ATG CCT G [FAM]G-3'; GATA-3 (Reverse) 5'-TCC TCC AGT GAG TCA TGC ACT TT-3'. T-bet and GATA-3 PCR samples were run in multiplex and results were calculated using the C_t method [31]. A formula for the purity of TH1 cells in a given sample was derived [$Pu=R/(R+1)$, where $Pu=TH1$ purity and $R=T\text{-bet:GATA-3 ratio}$] and used to calculate the enrichment for TH1 and TH2 cells in each sample.

Additional primers were designed for human NKG2A and NKG2C as well as a primer set designed to mimic the single Affymetrix Probe Set (PS) 206785_s_at recognizing both transcripts. Original DNA primers for this expression profile validation step were designed according to known gene sequences as follows: NKG2A (Forward) 5'-CTA CTC AGG GGC AGA TTC AGG TCT GAG [FAM]AG-3'; NKG2A (Reverse) 5'-TGG CCT CTC CAC TAA AGG ATG-3'; NKG2C (Forward) 5'-GAA TCA TCA TCA GGA CAA TGC AAA TGA [FAM]TC-3'; NKG2C (Reverse) 5'-GCC AAG GTT TAC TGC CAC CT-3'; Probe Set 206785_s_at (Forward) 5'-GAG CCA TCC TAG CAT TTG TGT CGG GC[FAM]C-3'; Probe Set 206785_s_at (Reverse) 5'-CCC TCA GAC ATT GGC AAC CA-3'. NKG2A, NKG2C, and Probe Set PCR samples were run using the Standard Curve Method [31].

All PCR reactions were completed on quiescent cells with cycles of 95°C, 55°C, and 72°C. JOE-labeled human TATA-binding protein (TBP) (Invitrogen)-containing wells served as positive controls and polymerase-free wells as negative controls. Reactions were run using an ABI PRISM® 7700 PCR instrument (Applied Biosystems, Foster City, CA) and relative gene expression levels were calculated using Sequence Detection System Software (ABI). Amplification curves reaching threshold after 40 cycles were excluded from analysis. Standard errors of means were calculated using the "Delta Method" [32]. Gel electrophoresis was used to confirm appropriate amplicon sizes of PCR products and absence of primer-dimer contamination.

Flow Cytometry

TH1 and TH2 cell isolates from eight subjects were halved. One half of each cell isolate was activated for 4 hours with 50 µg/µL of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO) in the presence of 1 µg/µL of ionomycin (Sigma) and 1 µg/µL of brefeldin A (Sigma).

Non-activated and activated cells underwent a series of incubations with fluorescent-labeled monoclonal antibodies, as well as permeabilization where appropriate, and then fixation with 1% paraformaldehyde. Four-color flow cytometry (FACSCalibur™ System, BD) was performed with varying combinations of anti-CD3-PerCP (BD), anti-CD4-FITC (BD), anti-CD4-APC (BD), anti-CD8-FITC (BD), anti-CD29-biotin (RDI), anti-CD56-APC (BD), anti-CXCR3-APC (BD), anti-CRTH2-PE (Miltenyi), anti-CCR4-PE (BD), anti-NKG2A-APC (R&D Systems, Minneapolis, MN), anti-NKG2C-PE (R&D Systems), anti-interferon (IFN) – γ (BD), anti-interleukin (IL) –4 (BD), and anti-Biotin-APC (BD) using appropriate isotype and negative (unlabeled cells) controls. Cells were gated for TH lymphocytes using forward- and side-scatter properties and CD3+CD4+ double positivity. Data were analyzed with FlowJo 5.7 (Tree Star, Inc., Ashland, OR) to confirm the TH1 and TH2 isolations as well as to determine the frequency of NKG2A marker positivity among cells in the isolated TH fractions. Statistical significance of percent positive cells and mean fluorescence intensity between cell types was tested with SPSS 13 (SPSS) using paired T-tests of log-transformed data.

Results

Negative Isolation of TH1 and TH2 Lymphocytes and Fraction Purity

Validation of the negative isolation cocktails was performed both by flow cytometry and RT-PCR on a select group of samples. Flow cytometry showed that TH1 and TH2 cell isolates were depleted of cells positive for markers contained in the isolation cocktails. (Figure 2) Further, the TH1 and TH2 cell enrichments were characterized using RT-PCR as in Chakir, et al. [29]. This method utilizes the ratio of T-bet:GATA-3 mRNAs to determine an estimate of the TH1:TH2 ratio within each isolate. The mean T-bet:GATA-3 ratio for TH1 lymphocytes was 9.3 ± 6.28 ($P_{TH1} = 90.3\%$) and for TH2 cells was 0.19 ± 0.06 ($P_{TH2} = 84.1\%$). The mean T-bet:GATA-3 ratio for TH2 cells was statistically significantly different from that for a group of non-fractionated CD4+ TH-enriched control cells (2.39 ± 1.28 , $p = 0.04$) (Figure 1). The TH1 cells were not statistically different from the non-fractionated TH cells or the TH2 cells.

TH1 and TH2 mRNA Expression Profiles

mRNA expression profiles were completed on each sample of TH1 and TH2 cells from three of the eleven subjects using Affymetrix U133A microarrays in order to determine differential gene expression between the cell types. Comparative analyses using both dChip- and MAS5-calculated expression values were performed in GeneSpring®, generating expression values that were filtered for present calls and a p-value of 0.05.

A list of 46 TH1:TH2 cell differentially expressed transcripts was generated by these analyses (Table 1). Of these, two contained NKG* or KLR* in their names: 1) Combined NKG2A and NKG2C [TH1:TH2 Relative expression ratio (Range) = 7.69 (6.16 to 20.99), $p = 0.02$ by dChip] and 2) KLRK1 [1.46 (1.44 to 1.53), $p = 0.04$ by dChip] (Table 2). NKG2A, NKG2C, and KLRK1 have proximate chromosomal location (chromosome 12p13.2–13.1) and all similarly bind HLA-E. However, NKG2A and NKG2C were chosen as targets for RT-PCR confirmation because the single probe set representing both NKG2A and NKG2C showed the greatest difference in relative expression between the cell types and these markers were found previously on CD4+ TH cells [17].

NKG2 Isoform mRNA Expression

We extended the microarray data by quantitative RT-PCR using primers specific for NKG2A and NKG2C, as well as a primer set recognizing both isoforms. This latter primer set corresponds to the region of the transcript also queried by Affymetrix Probe Set (PS) 206785_s_at. A paired analysis of TH1 and TH2 cells from the same three microarray-studied subjects was performed. Wells showing detectable primer-dimers by gel electrophoresis were

excluded. Standard curves allowed the calculation of relative expression levels (TH1:TH2) as follows: NKG2A = 0.24 ± 0.18 (~4-fold enrichment in TH2) and NKG2C = 2.83 ± 1.31 (~3-fold enrichment in TH1). The primer set corresponding to Affymetrix Probe Set (PS) 206785_s_at showed a ratio of 0.60 ± 0.35 (~2-fold enrichment in TH2). (Table 2)

Differential NKG2 Protein Expression after Activation of TH2 Cells

To validate the RT-PCR finding of NKG2A predominance among TH2 cells, flow cytometry was carried out for both TH1 and TH2 cells before and after cell activation in samples from the remaining eight subjects. Samples were gated on CD3+CD4+ cells during analysis. However, no cell isolates showed evidence of CD8+ cell contamination. (Figure 2)

TH2-enriched lymphocytes showed Mean $\pm$ SEM NKG2A+ surface expression of $2.89 \pm 0.78\%$, CD56+ of $1.43 \pm 0.91\%$, NKG2C+ of $0.56 \pm 0.14\%$, and NKG2A+CD56+ cells of $0.16 \pm 0.09\%$. Once activated, the TH2 lymphocytes showed a significant increase in the number of cells positive for NKG2A, CD56, and for both. TH2 cells expressing NKG2A tripled to $10.76 \pm 4.01\%$ ($p=0.05$); CD56 expression showed a 23-fold increase to $35 \pm 14.54\%$ ($p=0.03$); and NKG2A+CD56+ double-positive cells increased significantly to $3.04 \pm 1.38\%$ ($p=0.04$). NKG2C expression did not change significantly: $0.72 \pm 0.39\%$ ($p=NS$). (Table 3 and Figure 3)

Before activation, the TH1-enriched lymphocytes showed NKG2A+ of $2.56 \pm 0.52\%$, CD56+ of $3.15 \pm 1.69\%$, NKG2C+ of $1.12 \pm 0.42\%$, and NKG2A+CD56+ cells of $0.86 \pm 0.5\%$. Activated TH1 lymphocytes showed no statistically significant change in mean surface expression of NKG2A+ ($4.66 \pm 1.59\%$; $p=0.08$), CD56+ ($7.27 \pm 4.77\%$; $p=NS$), NKG2C+ ($1.59 \pm 0.52\%$; $p=NS$), and NKG2A+CD56+ ($1.38 \pm 1.08\%$; $p=NS$) (Table 3 and Figure 2).

Although there were increases in IFN- γ expression in both activated TH1 and TH2 cell samples, these changes from the non-activated state were not significant. However, there were significant increases in intracellular IL-4 in both activated samples, especially TH2 cells. (Table 3 and Figure 3).

Discussion

Studies that examine TH1 and TH2 lymphocytes are fundamental to answering questions about the balance of these cells and their cytokines in diseases such as asthma and many autoimmune states. However, TH cells polarized in culture are likely not ideal for these studies because they may not display the same in vitro phenotype as do in vivo TH1 and TH2 cells. Therefore, we have reported the first ex vivo analyses of negatively isolated TH1 and TH2 cells by microarray, RT-PCR, and flow cytometry. We focused data analysis and validation on NKG2A to determine if it has differential expression between TH1 and TH2 cells.

Using a novel antibody cocktail we developed for the negative isolation of human TH1 and TH2 cells, we obtained 90.3% enrichment for TH1 cells and 84.1% enrichment for TH2 cells. These results were validated with the methods of Chakir, et al. [29] using the T-bet:GATA-3 ratios of each sample by RT-PCR. By flow cytometry, we found a three-fold increase in NKG2A surface protein expression following activation on TH2 cells, though not on TH1 cells. The NKG2C receptor surface expression on TH2 cells did not change noticeably under the same conditions.

The antibody combinations used for the negative isolations were chosen based on differential prevalence of surface markers on each cell type reported by Matsui, et al. and in other published studies [33,34,35,36,37,38,39,40,41,42,43,44,45,46]. We recognized a priori this would not achieve 100% purity, but would allow for sufficient purity and yields to conduct these experiments. Additionally, some cells would not be expected to express any of the markers in

the isolation cocktails and therefore would appear in both cell type enrichments. These cells could have comprised up to 15% of the total CD3+CD4+ cell counts [33] and therefore could impact the results, but their presence in both samples would bias results towards the null.

Additionally, a small percentage (1–5%) of the isolated cells was CD3+CD4–. These were not evaluated more closely but could represent NKT cells as well as other lymphocyte subsets. These cells could have skewed the microarray and PCR results to some degree as NKT cells, in particular, highly express NKG2A and NKG2C. However, in the flow cytometry studies we conducted, we gated on CD3+CD4+ cells only, so it is not possible for NKT cells to have affected those results. This could explain the discrepancy between the RT-PCR and flow cytometry results for quiescent cells.

As well, there were some CD56+ cells among the non-activated cells of both types which could represent actual contamination or mild ex vivo activation during cell processing. In either event, the longitudinal nature of the study examined intra-individual differences and this small percentage of CD56+ cells would not have had an effect on the results. Despite each of these limitations, the use of human ex vivo cells made this an attractive approach.

In light of the earlier finding that activated non-fractionated CD4+ TH lymphocytes express NKG2A [17], our work indicates that the TH2 fraction is responsible for this. Given a previous study that found NKG2A, NKG2C, or NKG2E on murine Th1 cells [18], one might have expected the human TH1 cells to preferentially express this receptor. However, as we did find low levels of NKG2A and NKG2C expression on TH1 cells, our results are not incongruous with that prior study.

Of note, we also found marked inter-individual variability in the level of expression of the markers of interest before and after activation. This phenomenon has been described for NKG2 receptors in rhesus monkeys previously [47] and could reflect the dynamic nature of these receptors as their surface expression switches from one type to another.

It is clear from our data that NKG2A receptors, which are MHC class I-responsive via HLA-E, undergo increased surface expression on TH2 lymphocytes following activation. However, this is a departure from standard as the TH2 phenotype customarily promotes a humoral immune response to foreign antigen recognition by the MHC class II complex. TH1 cells are usually responsible for instigating a cellular immune response to foreign antigen presentation by MHC class I to the T-cell receptor (TCR) complex. As such, this is the first report of TH2 cell capability for MHC class I recognition.

There are published data that provide context for our findings. Marusina, et al. showed that GATA-3 is a key transcription factor in the regulation of NKG2A expression [48]. In fact, GATA-3 up-regulation also initiates TH2-specific cell polarization via IL-4 induction of STAT6 [49]. GATA-3 acts at the CNS-I region of the Th2 cytokine coding locus to activate transcription of IL-4 and IL-13 [50,51,52,53,54,55,56]. At a certain point, GATA-3 auto-activates its own transcription leading to further transcription of Th2 cytokines and the commitment of naïve T Helper cells to the TH2 phenotype [57,58]. Further, Romero, et al. showed that the potent TH2 cytokine, IL-10, is an important positive contributor to NKG2A expression on activated CD4+ TH cells [17].

The discovery of NKG2A, an inhibitory receptor for MHC class I peptides via HLA-E, on TH2 cells raises questions as to its function. It seems plausible that the NKG2A receptor could act as an inhibitory mechanism for modulation of TH2 cell function. If NKG2A behavior on TH2 cells is analogous to that on CD8+ T lymphocytes, one would expect concomitant binding of NKG2A to HLA-E along with the CD4+-TCR to MHC class II. This parallel receptor-ligand binding would hypothetically result in transmission of an inhibitory signal from NKG2A along

with the activating signal from the TCR. In this way, NKG2A would serve to inhibit typical TH2 effector functions by dampening the activating signal from the TCR. For example, the co-regulation of CD56 with NKG2A identified in our TH2 samples indicates that NKG2A receptor surface expression correlates with degree of cell activation. This suggests that in the situation of viral infections where lymphocytes are activated but MHC class I production is often decreased [59,60], NKG2A inhibition of TH2 effector function would be diminished despite an increased level of surface expression. This hypothesis would lead one to expect a relatively robust TH2 response compared to that of activated TH1 cells, in light of their lack of NKG2A expression.

In fact, a successful attempt to regulate inflammation via the NKG2A receptor was recently reported by Kawamura, et al. In their study, anti-NKG2A monoclonal antibodies were shown to restrict donor T cell expansion and improve signs of murine acute graft-versus-host disease [61]. However, it has not been shown whether these results can be extrapolated to humans and other diseases where TH1/TH2 cell and cytokine balance is important.

Our identification of NKG2A receptors on activated human TH2 lymphocytes is the next step in this extrapolation. Given the context provided by recent reports of NKG2A on CD4+ TH cells, our findings raise important functional questions about its role in TH1/TH2 cell and cytokine balance. The future study of TH1 and TH2 cells in autoimmune states and acute and chronic TH-related diseases will benefit from clarifying the functional significance of activated TH2 cell NKG2A receptors in humans.

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Figure 1.

Comparison of ratios of mRNA for T-bet to GATA-3, and T-bet and GATA-3 to TBP, in TH1 and TH2 lymphocyte subsets and non-fractionated CD4+ T lymphocytes by quantitative RT-PCR. (*p=0.04)

Figure 2.

Flow cytometry of a representative sample of lymphocytes before and after enrichment for TH1 and TH2 lymphocytes. All analyses were gated by forward- and side-scatter properties to select for lymphocytes. TH1 and TH2 cell analyses were also gated on CD3+CD4+ cells. Gates showing percent positive cells are derived from cell type and marker-specific isotype controls.

Figure 3.

Flow cytometry comparison for a representative pair of TH1 and TH2 lymphocytes before and after activation examining NKG2A, intracellular IFN- γ , and intracellular IL-4 positivity. These analyses were gated to select for CD3+CD4+ cells only.

Relative expression of differentially-expressed transcripts and T-bet and GATA-3 as determined by microarray analysis for quiescent TH1 and TH2 cells

Affymetrix Probe Set	GenBank ID	Corresponding Transcript (s)	Mean TH1 Expression	TH1 Range	Mean TH2 Expression	TH2 Range	TH1:TH2 Expression Ratio	p-value	dChip or MAS5
206785_s_at	NM_002260	NKG2A and NKG2C	3.052	(1.398 to 4.052)	0.397	(0.193 to 0.602)	7.69	0.02	D
1405_i_at	M21121	killer cell lectin-like receptor subfamily C, member 2	2.403	(1.234 to 3.309)	0.573	(0.379 to 0.766)	4.19	0.02	D
212599_at	AK025298	chemokine (C-C motif) ligand 5	2.112	(1.092 to 4.101)	0.663	(0.533 to 0.908)	3.19	0.02	D
206978_at	NM_000647	autism susceptibility candidate 2	2.761	(0.895 to 6.219)	0.874	(0.709 to 1.105)	3.16	0.01	D
213906_at	AW592266	chemokine (C-C motif) receptor 2	2.040	(1.241 to 3.212)	0.696	(0.625 to 0.759)	2.93	0.03	D
206666_at	NM_002104	v-myb myeloblastosis viral oncogene homolog (avian)-like 1	1.829	(0.967 to 2.828)	0.761	(0.58 to 1.033)	2.40	0.04	D
201143_s_at	BC002513	granzyme K (serine protease, granzyme 3; tryptase II)	1.691	(0.846 to 2.353)	0.768	(0.545 to 1.154)	2.20	0.05	D
206618_at	NM_003855	eukaryotic translation initiation factor 2, subunit 1 alpha, 35kDa	1.746	(0.796 to 3.38)	0.836	(0.306 to 1.265)	2.09	0.04	D
203329_at	NM_002845	interleukin 18 receptor 1	1.999	(0.784 to 3.997)	1.041	(0.714 to 1.665)	1.92	0.02	D
215177_s_at	AV733308	protein tyrosine phosphatase, receptor type, M	2.064	(0.795 to 3.334)	1.087	(0.553 to 1.581)	1.90	0.02	D
205821_at	NM_007360	integrin, alpha 6	1.322	(1.006 to 1.525)	0.905	(0.816 to 0.994)	1.46	0.04	D
211085_s_at	Z25430	killer cell lectin-like receptor subfamily K, member 1	1.524	(0.9 to 2.572)	1.108	(0.412 to 2.447)	1.38	0.05	D
213757_at	AA393940	serine/threonine kinase 4	1.597	(0.863 to 2.949)	1.242	(0.963 to 1.741)	1.29	0.02	D
217604_at	A1086530	eukaryotic translation initiation factor 5A	1.338	(0.665 to 2.356)	1.114	(0.893 to 1.444)	1.20	0.04	D
218397_at	NM_018062	Transcribed sequence with moderate similarity to protein ref:NP_055301.1 (H sapiens)	1.447	(0.84 to 2.488)	1.309	(0.696 to 2.243)	1.11	0.04	D
205033_s_at	NM_004084	neuronal thread protein	1.402	(0.493 to 3.184)	1.298	(0.623 to 1.894)	1.08	0.01	D
207339_s_at	NM_002341	Fanconi anemia, complementation group L	1.017	(0.971 to 1.102)	0.980	(0.875 to 1.04)	1.04	0.04	M
202973_x_at	NM_014883	defensin, alpha 1, myeloid-related sequence	1.420	(0.452 to 2.961)	1.422	(0.562 to 2.552)	1.00	0.01	D
201593_s_at	AV716798	lymphotoxin beta (TNF superfamily, member 3)	1.457	(0.866 to 2.371)	1.462	(0.628 to 3.016)	1.00	0.04	D
209771_x_at	AA761181	likely ortholog of mouse immediate early response, erythropoietin 4	1.439	(0.485 to 2.393)	1.473	(1.381 to 1.564)	0.98	0.04	D
202917_s_at	NM_002964	nz09g03.s1 NCI CGAP GCB 1	1.139	(0.351 to 2.538)	1.201	(0.51 to 1.62)	0.95	0.02	D
209534_x_at	BF222823	Homo sapiens cDNA clone IMAGE:1287316 3' similar to gb:M57627	1.432	(0.856 to 2.486)	1.522	(0.813 to 2.707)	0.94	0.05	D
208322_s_at	NM_003033	INTERLEUKIN-10 PRECURSOR (HUMAN);, mRNA sequence.	1.000	(0.98 to 1.02)	1.094	(0.808 to 1.272)	0.91	0.01	M
217047_s_at	AK027138	S100 calcium binding protein A8 (calgranulin A)	1.414	(0.528 to 2.897)	1.554	(0.61 to 2.867)	0.91	0.01	D
222024_s_at	AK022014	A kinase (PRKA) anchor protein 13	1.349	(0.861 to 2.3)	1.513	(0.688 to 2.735)	0.89	0.05	D
AFFX-r2-Hs18SrRNA-5_at	AFFX-r2-Hs18SrRNA-5	sialyltransferase 4A (beta-galactoside alpha-2,3-sialyltransferase)	1.122	(0.547 to 1.698)	1.357	(0.363 to 2.255)	0.83	0.04	D
202648_at	BC000023	family with sequence similarity 13, member A1	0.932	(0.825 to 1.039)	1.143	(1.051 to 1.235)	0.82	0.03	M
201996_s_at	A1524033	A kinase (PRKA) anchor protein 13	1.506	(0.927 to 2.519)	1.936	(0.698 to 4.309)	0.78	0.01	D
		ribosomal protein S19							
		SMART/HDAC1 associated repressor protein							

Affymetrix Probe Set	GenBank ID	Corresponding Transcript (s)	Mean TH1 Expression	TH1 Range	Mean TH2 Expression	TH2 Range	TH1:TH2 Expression Ratio	p-value	dChip or MAS5
210254_at	<u>L35848</u>	MS4A3 membrane-spanning 4-domains, subfamily A, member 3	1.784	(0.805 to 2.764)	2.302	(1.195 to 3.409)	0.77	0.03	D
205671_s_at	<u>NM_002120</u>	HLA-DOB hematopoietic cell-specific major histocompatibility complex,	1.796	(0.95 to 3.388)	2.415	(0.946 to 3.883)	0.74	0.01	D
205673_s_at	<u>NM_024087</u>	ASB9 class II, DO beta ankyrin repeat and SOCS box-containing 9	0.990	(0.294 to 2.055)	1.348	(0.913 to 2.043)	0.73	0.05	D
213975_s_at	<u>AV711904</u>	LYZ AV711904 DCA Homo sapiens cDNA clone DCAAIE08 5', mRNA sequence.	0.780	(0.335 to 1.448)	1.094	(0.798 to 1.281)	0.71	0.04	M
201392_s_at	<u>BG031974</u>	IGF2R insulin-like growth factor 2 receptor	1.293	(0.649 to 2.027)	1.837	(0.391 to 4.325)	0.70	0.02	D
AFFX-HUMGAPDH/	<u>AFFX-HUMGAPDH/</u>	GAPD	0.736	(0.335 to 0.986)	1.055	(1.014 to 1.085)	0.70	0.05	M
M33197_M_at	<u>M33197_M</u>								
200818_at	<u>NM_001697</u>	ATP5O ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit (oligomycin sensitivity conferring protein)	0.667	(0.246 to 1.106)	1.096	(0.894 to 1.2)	0.61	0.05	M
AFFX-HUMRGE/	<u>AFFX-HUMRGE/</u>								
M1 0098_5_at	<u>M1 0098_5</u>		0.817	(0.269 to 1.66)	1.360	(0.859 to 2.081)	0.60	0.05	D
209710_at	<u>AL563460</u>	GATA2	1.014	(0.904 to 1.125)	1.790	(0.88 to 3.393)	0.57	0.03	D
210607_at	<u>U03858</u>	FLT3LG fms-related tyrosine kinase 3 ligand	0.977	(0.797 to 1.158)	1.733	(1.217 to 2.249)	0.56	0.03	M
AFFX-r2-	<u>AFFX-r2-</u>		0.742	(0.227 to 1.136)	1.404	(0.849 to 1.741)	0.53	0.02	M
Hs18SrRNA-M_x_at	<u>Hs18SrRNA-M</u>								
206059_at	<u>NM_003430</u>	ZNF91 zinc finger protein 91 (HPF7, HTF10)	0.967	(0.651 to 1.326)	1.897	(0.932 to 3.69)	0.51	0.03	D
213998_s_at	<u>AW188131</u>	DDX17 DEAD (Asp-Glu-Ala-Asp) box polypeptide 17	0.836	(0.328 to 1.238)	1.691	(0.703 to 3.311)	0.49	0.03	D
AFFX-HUMRGE/	<u>AFFX-HUMRGE/</u>								
M1 0098_M_at	<u>M1 0098_M</u>	GPR34	0.667	(0.234 to 1.024)	1.472	(0.976 to 1.852)	0.45	0.05	M
217836_s_at	<u>NM_018253</u>	YAP YY1 associated protein	0.676	(0.268 to 1.089)	1.506	(0.911 to 2.106)	0.45	0.02	M
200003_s_at	<u>NM_000991</u>	RPL28 ribosomal protein L28	0.587	(0.21 to 1.037)	1.411	(0.963 to 1.682)	0.42	0.01	M
221506_s_at	<u>BG258639</u>	TNPO2 transportin 2 (importin 3, karyopherin beta 2b)	0.918	(0.754 to 1.005)	2.380	(1.084 to 3.676)	0.39	0.03	D
205159_at	<u>AV756141</u>	CSF2RB colony stimulating factor 2 receptor, beta, low-affinity (granulocyte-macrophage)	1.157	(0.714 to 1.627)	3.280	(0.713 to 8.255)	0.35	0.00	D
220684_at	<u>NM_013351</u>	T-bet	2.130	(1.78 to 2.48)	1.016	(1.02 to 1.02)	2.10	0.19	D
209603_at	<u>AI796169</u>	GATA3	1.198	(0.8 to 1.62)	0.920	(0.59 to 1.64)	1.30	0.60	D
209604_s_at	<u>BC003070</u>	GATA3	1.226	(0.91 to 1.79)	1.151	(0.99 to 1.45)	1.07	0.63	D
209602_s_at	<u>AI796169</u>	GATA3	0.928	(0.77 to 1.1)	1.033	(0.93 to 1.1)	0.90	0.46	D

Table 2

Relative Expression of C-type Lectin Genes in TH1 with Respect to TH2 Lymphocytes by Microarray and Quantitative RT-PCR

Affymetrix Probe Set	GenBank ID	Corresponding Transcript(s)	Microarray		RT-PCR		
			Mean TH1:TH2 Fold Change (Range)	p- value	Mean±SEM TH1:TH2 Fold Change	p-values	
206785_s_at	<u>NM_002260</u>	NKG2A	-	-	0.24±0.18	NS	0.07
		NKG2C	-	-	2.83±1.31	0.09	Ref
		Probe Set: NKG2A +NKG2C	7.69 (6.16 to 20.99)	0.02	0.60±0.35	Ref	NS
205821_at	<u>NM_007360</u>	KLRK1	1.46 (1.44 to 1.53)	0.04	-	-	

NS=Not significant, Ref=Reference sample

Table 3
Surface Antigen and Intracellular Cytokine Expression by Cell Type and Activation State

Cell Type	Activation Status	NKG2A+	CD56+	NKG2C+	NKG2A + CD56+	IFN- γ +	IL-4+
TH1	Activated	4.66	7.27	1.59	1.38	7.91	9.78
		± 1.59 (5.35)	± 4.77 (11.69)	± 0.52 (2.18)	± 1.08	± 4.91 (4.92)	± 3.82 (7.64)
	Non-Activated	± 1.21	± 6.46	± 0.16	$\pm 0.86 \pm 0.5$	± 2.22	± 3.43
		2.56	3.15	1.12		0.25	1.12 ± 0.04 (2.2)
TH2	Activated	± 0.52 (6.14)	± 1.69 (29.45)	± 0.42 (2.23)		± 0.18 (1.83)	± 0.22
		± 1.04	± 11.03	± 0.21		± 0.3	
	Non-Activated	0.08 (NS)	NS (0.03)	NS (NS)	NS	0.09 (NS)	0.03 (0.09)
		10.76	35	0.72	3.04	8.30	41.55
	Activated	± 4.01 (6.28)	± 14.54 (17.64)	± 0.39 (3.14)	± 1.38	± 4.67 (4.39)	± 13.68 (13.51)
		± 1.45	± 8.37	± 0.98		± 1.01	± 4.68
	Non-Activated	2.89	1.43 ± 0.91 (9.5)	0.56	0.16	0.16	1.86
		± 0.78 (5.99)	± 4.64	± 0.14 (2.66)	± 0.09	± 0.12 (1.89)	± 1.33 (2.51)
	Activated	± 0.97		± 0.21		± 0.35	± 0.23
		0.05 (NS)	0.03 (0.07)	NS (NS)	0.04	0.07 (0.01)	0.01 (0.03)

Results represent the mean percentage of positive cells $\pm$ standard error. Mean fluorescence intensity (MFI $\pm$ SEM) is shown in parentheses. P-values are given for activated versus non-activated samples within a cell type. P-values in parentheses are for MFI comparison. NS = not significant.