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ABSTRACT

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Natural killer (NK) cells are a subpopulation of lymphocytes that play an important role against tumor metastasis and various viral and bacterial infections. NK cell functions are controlled by a balance between positive and negative signals through various receptors. We have identified, cloned and characterized the 2B4 (CD244) receptor in mice and human. 2B4 is involved in killing cancer cells and virus-infected cells by NK cells. 2B4 is a counter-receptor for CD48 and recent findings show that 2B4-CD48 interactions play an important role in NK, T and B cell functions. In humans, two isoforms of 2B4, h2B4-A and h2B4-B, are expressed that differ in the extracellular domain. In the present investigation, we have studied the functions of h2B4-A and h2B4-B. Our data demonstrate that these two isoforms differ in their binding affinity for CD48, resulting in differential cytolytic function as well as cytokine production by NK cells. Thus, differential expression of 2B4 isoforms by NK cells may regulate immune responses mediated through 2B4-CD48 interactions.

THE FUNCTIONAL ROLE OF HUMAN

2B4 (CD244) ISOFORMS IN

NATURAL KILLER CELLS

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THESIS

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effort; for when I was unsuccessful we failed together but when I succeeded we thrived together. Although he may not have been in the same city physically, he was always by my side supporting me along the way.

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CHAPTER I

INTRODUCTION TO THE STUDY

Natural Killer cells (NK cells) are a type of cytolytic lymphocyte that recognize and kill virally infected cells, parasites and certain tumor cells. It is known that NK cells participate in both the innate and adaptive immune system and is supported by the wide array of stimulatory and inhibitory receptors that they bear (1). The mechanisms that control NK cell activation and cytotoxicity are believed to be determined by a delicate balance between stimulatory and inhibitory signals received from surface receptors. In fact, receptor-ligand interaction and its subsequent signaling events is what controls NK cell effector responses. This includes NK cell cytotoxicity as well as production of the cytokine interferon-gamma (IFN-γ) which has a role in the regulation of immune and inflammatory responses and potentiates the function of type I IFNs. Having a majority of activating signals initiates lysis of the target cell, which is achieved by the release of cytotoxic granules containing perforin and granzymes. The numerous receptors carrying out these functions is what NK cells use to discriminate between target and non-target cells (1). This idea is what constituted the present model of NK cell activation known as the "missing self" hypothesis developed by Klas Kärre of the Karolinska Institute. This was the first breakthrough in understanding specific recognition of target cells by NK cells, when it was appreciated that loss of MHC class I expression leads to sensitivity to

lysis by NK cells (2). The down-regulation of MHC molecules frequently observed in tumors and in virus-infected cells can lead to sensitivity to NK cells. The loss of inhibitory signals due to a reduction in HLA class I on target cells usually results in NK cell activation because the ligands of the many activation receptors (e.g., NKp46, NKp44, NKp30, 2B4, etc.) are widely expressed, particularly on hematopoietic cells (3).

The CD2 family of receptors belongs to the immunoglobulin (Ig) superfamily and is currently comprised of 11 members including 2B4 (CD244), BLAME (B lymphocyte activator macrophage expressed), CD2 (lymphocyte function-associated antigen-2, LFA-2), CD48, CD58 (LFA-3), CD84, CD84 H1 (CD84 homolog-1), CD150 (signaling lymphocyte activation molecule, SLAM), CD299, CS1 (CD2 subset 1), and NTB-A (NK-T-B-antigen)(Colonna book). Members of this family are usually type I transmembrane proteins with a single extracellular N-terminal variable (V)-set Ig domain and a single constant (C)-2-set Ig domain with conserved patterns of disulfide bonds (1). A few exceptions to this general structure exist; such as CD48, which is GPI linked, and CD229, which has an additional pair of V and C2 Ig domains.

One such member of the CD2 subset of the Ig superfamily, 2B4 (CD244), is an activating receptor expressed on all human NK cells, a subpopulation of T cells, basophils and monocytes (4, 7-8). Similar to other members of the family, 2B4 is comprised of a V-like and C2-like domain in the extracellular region and four immuno-receptor based tyrosine switch motifs (ITSM) in the intra-cytoplasmic domain with the amino acid sequence TxYxxV/I (5). In both mice and humans, CD48 has been identified as the ligand for 2B4 (8, 12-13). Engagement of 2B4 via interaction with specific

antibodies or CD48 induces cytokine secretion (IFN-gamma) and enhances non-major histocompatibility complex (MHC)-restricted killing by NK cells (7,12,14-15). This allows the ability of NK cells to kill cells which have either lost class I expression or have altered peptide-class I complexes, due to their inability to transmit an inhibitory signal to NK cells (16). Because CD48 is a common marker expressed by cells of the hematopoietic system, this suggests that 2B4 may play a role in immune regulation. In fact, 2B4 was recently reported to function as a co-receptor in human NK cell activation (5).

Identification of CD48 as the high affinity ligand of 2B4 implicated a broader role of 2B4 in immune regulation (13,17). Isolation of human 2B4 has allowed exploration into the role of 2B4 in various human immune diseases. For example, in X-linked lymphoproliferative disease (XLPD), NK cells cannot be activated via surface 2B4 (18-21). The molecular adaptor protein SLAM-associated protein (SAP)/src homology 2 domain-containing adaptor molecule (SH2DIA) associates with the cytoplasmic tail of 2B4 and SLAM (22, 23). Defective signaling via 2B4 and SLAM may contribute to the pathogenesis of XLPD due to mutations in SAP (4, 24). These patients often succumb to EBV infection and specifically EBV-induced B cell lymphoma. A case report described a patient with acute myeloid leukemia, in whom large granular lymphocytes with a CD3+/CD56+ phenotype killed leukemia cells in a 2B4-dependent manner (24). Addition of an anti-2B4 monoclonal antibody in killing assays enhanced cytotoxicity of human NK cells (25, 17). These studies further reiterate that 2B4 is an activating receptor on human NK cells and that human 2B4 monoclonal antibody triggers the

receptor rather than masking it (28). Therefore, 2B4 may have a strong role in the regulation of the immune system and disease.

Mouse NK cells express two isoforms of 2B4 (m2B4-L and m2B4-S), which are produced by alternative splicing (9). The two isoforms differ solely in their cytoplasmic domains and they transduce opposing signals (10). Transfection of RNK-16 cells with cDNAs revealed that m2B4-L is an inhibitory receptor whereas m2B4-S is a stimulatory receptor (11). In humans, two isoforms of the 2B4 receptor have been found to exist, h2B4-A and h2B4-B, and are both expressed on NK cells. The differences in the human isoforms (Fig. 1.1) arise by differential splicing that result in the addition of five amino acids at the intersection of the immunoglobulin V and C2 domains in the extracellular region of h2B4-B, while the cytoplasmic domain is identical (11). Due to their differences in the extracellular domain, the two splice variant isoforms may differ in their binding specificities or affinities. Previous studies had demonstrated that mutational analysis of human 2B4 in the V domain disrupted 2B4-CD48 interaction and impaired the functional activation of human NK cells (26). Therefore, the existence of the five-amino acid addition in the h2B4-B splice variant may constitute a difference in relative binding affinity to CD48 as well as cytolytic function as compared to h2B4-A.

Given the importance of h2B4-A and h2B4-B in regulating immune responses, a clearer understanding of the capabilities of each in directing NK cell cytotoxicity is critical. It has been investigated in this study that the two isoforms may have different binding affinities for the same ligand, resulting in differential cytokine release and

cytotoxicity. Here, we study the functional role of human 2B4 isoforms h2B4-A and h2B4-B.

FIGURE 1.1. **Comparison of the predicted amino acid sequences of h2B4-A and h2B4-B.** The difference between the amino acid sequences is shown in the shaded box. The immunoreceptor- tyrosine-based switch motifs (ITSM) in the cytoplasmic domain are shaded. The regions in the sequence are represented as SS = signal sequence, V = variable domain, C2 = constant region, TM = transmembrane region.

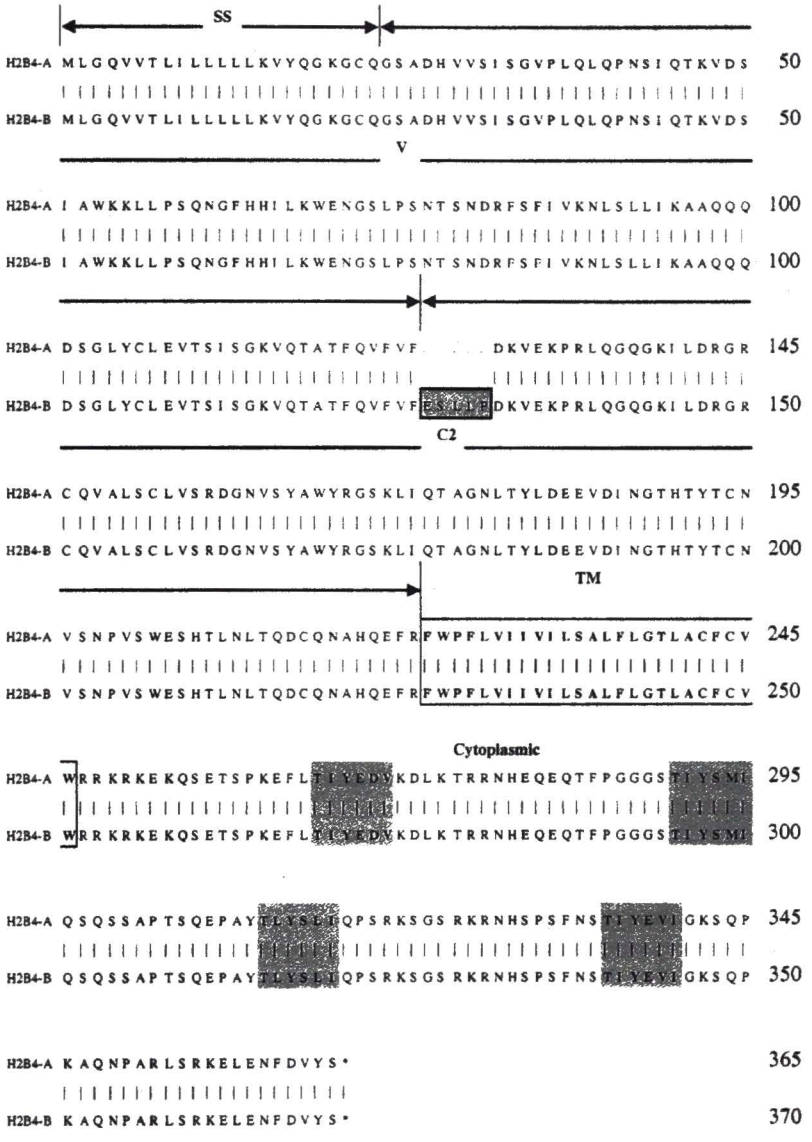
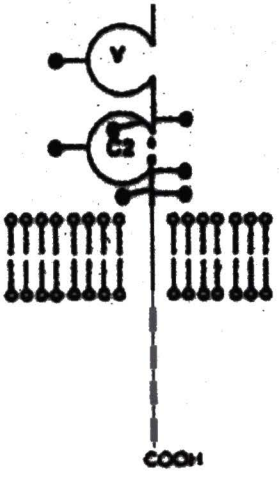
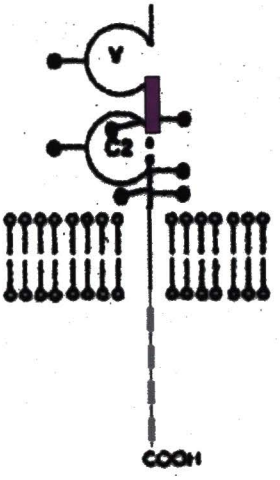

SS
H2B4-A MLGQVVTLILLLLLKVYQGKGCQGSADHVVSISGVPLQLQPNSIQTKVDS 50
H2B4-B MLGQVVTLILLLLLKVYQGKGCQGSADHVVSISGVPLQLQPNSIQTKVDS 50
V
H2B4-A IAWKKLLPSQNGFHHILKWENGSLPSNTSNDRFSFIVKNLSLLIKAAQQQ 100
H2B4-B IAWKKLLPSQNGFHHILKWENGSLPSNTSNDRFSFIVKNLSLLIKAAQQQ 100
H2B4-A DSGLYCLEVTSISGKVQTATFQVFVF DKVEKPRLQGQGKILDRGR 145
H2B4-B DSGLYCLEVTSISGKVQTATFQVFVF DKVEKPRLQGQGKILDRGR 150
C2
H2B4-A CQVALSCLVSRDGNVSYAWYRGSKLIQTAGNLTYLDEEVDINGTHTYTCN 195
H2B4-B CQVALSCLVSRDGNVSYAWYRGSKLIQTAGNLTYLDEEVDINGTHTYTCN 200
TM
H2B4-A VSNPVSWESHTLNLTQDCQNAHQEFRFWPFLVIIVILSALFLGTLACFCV 245
H2B4-B VSNPVSWESHTLNLTQDCQNAHQEFRFWPFLVIIVILSALFLGTLACFCV 250
Cytoplasmic
H2B4-A WRRKRKEKQSETSPKEFL KDLKTRRNHEQEQTFPGGGS 295
H2B4-B WRRKRKEKQSETSPKEFL KDLKTRRNHEQEQTFPGGGS 300
H2B4-A QSQSSAPTSQEPAY QPSRKSGSRKRNHSPSFNS GKSQP 345
H2B4-B QSQSSAPTSQEPAY QPSRKSGSRKRNHSPSFNS GKSQP 350
H2B4-A KAQNPARLSRKELENFDVYS* 365
H2B4-B KAQNPARLSRKELENFDVYS* 370

FIGURE 1.2. **Schematic representations of human 2B4-A and 2B4-B.** V represents Ig-variable-like domain and C2 represents Ig-constant-like domain. The colored region shows the addition of 5 amino acids between the V and C2 domains in h2B4-B.



h2B4-A



h2B4-B

CHAPTER II

BINDING AFFINITY STUDIES OF H2B4-A AND H2B4-B ISOFORMS TO CD48

SUMMARY

Two isoforms of 2B4 have been identified on human NK cells. Since the newly discovered splice variant h2B4-B is identical in its cytoplasmic domain but differs in its extracellular domain, this study was conducted to determine whether the two isoforms differ in their binding affinity to the natural ligand of h2B4-A, CD48. In order to answer this question, the extracellular domain of both h2B4-A and h2B4-B was cloned into a pCD5lneg1 vector using sequence specific primers. Each of these vectors was then transiently transfected into a B16 mouse melanoma cell line in order to produce soluble recombinant fusion proteins for both h2B4-A and h2B4-B. The fusion proteins contain the extracellular domain of either h2B4-A and h2B4-B fused with the Fc portion of human IgG. The fusion proteins were then used to determine differences in binding affinity to CD48. The results indicated that h2B4-A was able to bind to CD48 at a significantly greater binding affinity than did h2B4-B.

MATERIALS AND METHODS

Cell lines

The NK92 (human lymphoma), B16F10 (mouse melanoma) and P815 (mouse mastocytoma) cell lines were cultured in culture media (RPMI 1640, supplemented with 10% FBS, 2mM glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 10mM HEPES and 10 mM non-essential amino acids). Cells were maintained at 37°C in a humidified 5% CO_2/95% air incubator.

cDNA cloning, Vector construction and Transfections

A cDNA library of h2B4-A and h2B4-B was previously created in the lab from mRNA of pooled human NK cells derived from human peripheral blood mononuclear cells. Using sequence specific primers, full-length h2B4-A and h2B4-B was amplified and then cloned into pCI-neo. The extracellular domain of h2B4-A and h2B4-B was then amplified from h2B4 cDNA introducing *NheI* and *BamHI* restriction sites and was subcloned into the pCD5lneg1 vector (Fig. 2.1). The pCD5lneg1 vector contains the CH2 and CH3 regions of the human IgG1. The plasmid DNA was then isolated using Maxi-prep plasmid isolation. The plasmid was digested with *Nhe*I and *Bam*HI for 2 hours at 37°C to check for presence of the correct insert. The purified DNA was then sent for sequencing by *Seqwright* (Houston, TX). Transient transfection of 1μg of the

pCD5lneg1-h2B4-Fc was performed using the Fugene6 transfection reagent into B16 cells to produce secreted fusion proteins of h2B4-A and h2B4-B (Fig. 2.2).

Western Blot

After determination of fusion protein concentration, 20μg of fusion protein, which was either reduced with DTT (dithiothreitol; Cleland's reagent) or in native form, was separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes (Millipore, Bedford, MA). Membranes were blocked for 1 hour with 3% BSA/TBST and then probed with horseradish peroxide conjugate secondary antibody (goat anti-human IgG) using chemiluminescence (Fig 2.4).

Flow Cytometry

One million CD48 positive NK92 cells were incubated with 18μg of fusion protein for 1 hour. A CD48 negative cell line, P815, was used as a negative control and was also incubated with 18μg of fusion protein for 1 hour. Since both cell lines are $Fc\gamma R^+$, samples were incubated with 4μg of Fc blocker for 30 minutes prior to fusion protein incubation. Samples were then incubated with FITC conjugated secondary antibody against human IgG for 40 minutes. They were then resuspended in PBS-BSA and used for subsequent flow cytometric analysis (Coulter, FC500 Flow Cytometer) (Fig 2.5).

RESULTS

Generation of h2B4-A-Fc and h2B4-B-Fc fusion proteins

Plasmids encoding fusion proteins comprising the extracellular domain of h2B4-A and h2B4-B and the Fc region on human IgG1 was constructed as detailed in section 2. The plasmids were introduced into B16 mouse melanoma cell line by Fugene-6 transfection reagent. The h2B4 part of the fusion protein was confirmed by Western blot analysis (Fig. 2.4). The h2B4-A-Fc and h2B4-B-Fc fusion proteins give a ~66 kDa band on reducing and ~150 kDa band on non-reducing gel indicating that the protein is expressed as a dimer, as expected.

h2B4-A has a greater binding affinity to CD48 than h2B4-B

We have used the soluble h2B4-A-Fc and h2B4-B-Fc fusion proteins to compare the relative binding affinity to CD48. Receptor-ligand interaction was assessed by $CD48^{+}$ NK92 reactivity with the fusion proteins and was analyzed by flow cytometry. Binding is presented as specific mean fluorescence intensity (MFI) of FITC-conjugated anti-human IgG. As seen in Fig. 2.5, the h2B4-A-Fc fusion protein showed high levels of binding affinity with NK92-CD48, whereas h2B4-B-Fc fusion protein showed little binding (Fig. 2.5). The flow cytometric studies suggest that h2B4-A-Fc is specific for CD48, while h2B4-B-Fc may bind to CD48 with lower affinity.

FIGURE 2.1. **Diagram of the pCD5lneg1vector**. The extracellular domain of both h2B4-A and h2B4-B was subcloned into *Bam*HI and *Nhe*I enzyme sites. The CD5 vector also contains the Fc portion of human IgG1.

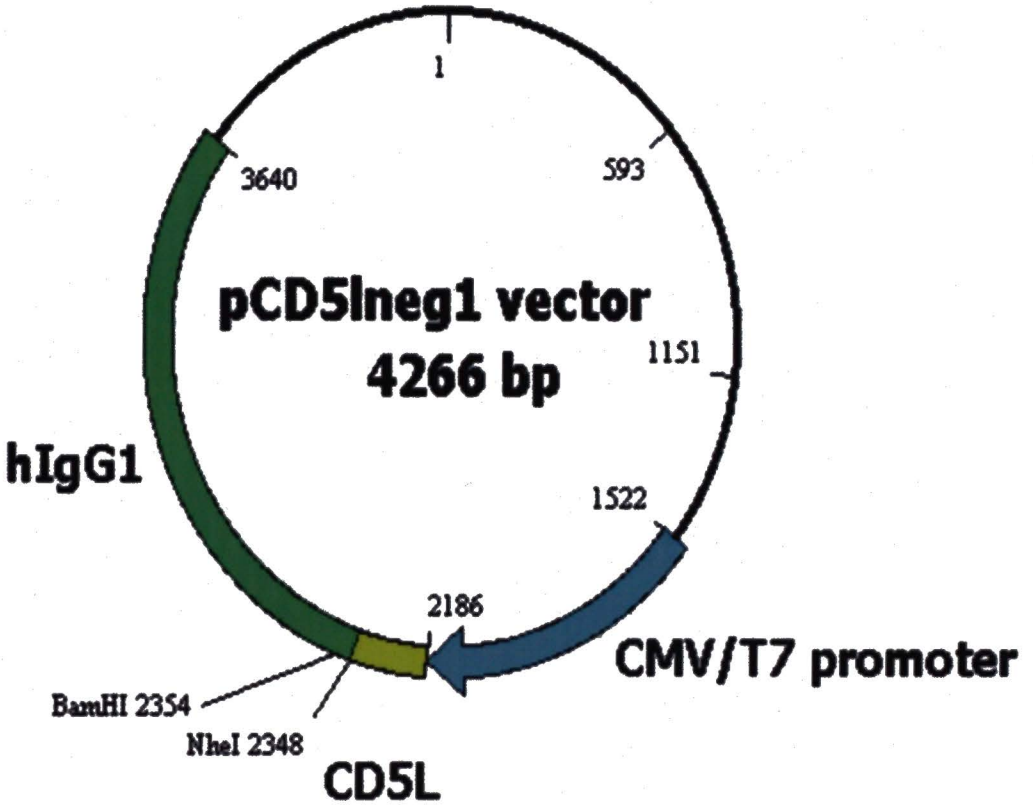
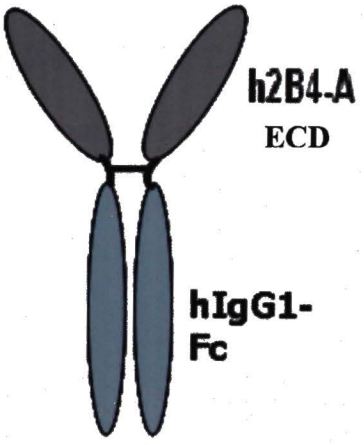
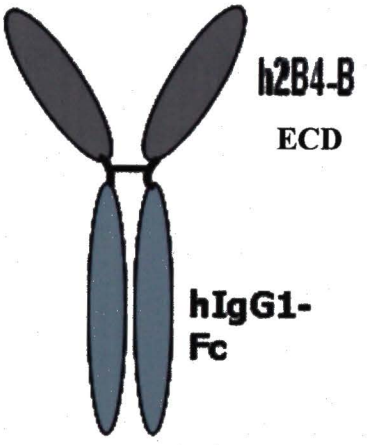

1
593
1151
1522
2186
3640
pCD5Ineg1 vector
4266 bp
hIgG1
CMV/T7 promoter
BamHI 2354
NheI 2348
CD5L

FIGURE 2.2. **Diagram of h2B4-A and h2B4-B soluble recombinant fusion proteins.** Fusion protein is divalent, containing two h2B4-A or h2B4-B extracellular domains in the arm regions and one Fc region. ECD = extracellular domain.

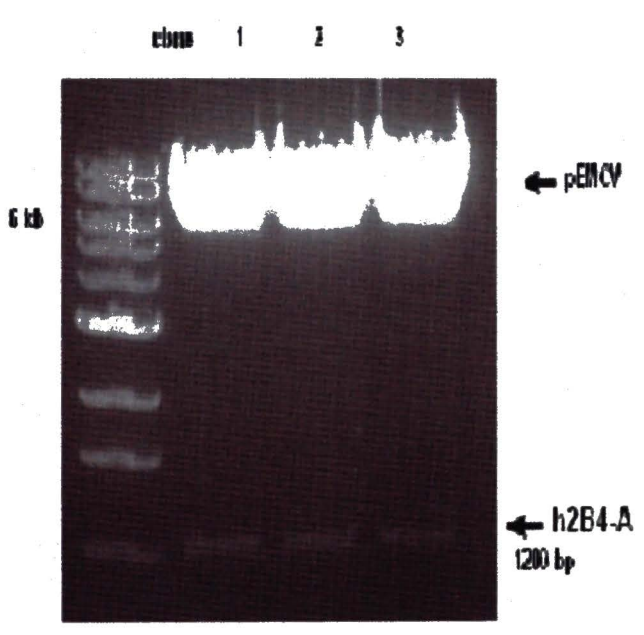


h2B4-A -hIgG1 Fc fusion protein



h2B4-B -hIgG1 Fc fusion protein

FIGURE 2.3. **Restriction digest of pCD5lneg1(5 kb) with *Bam*HI and *Nhe*I to check for insertion of h2B4-A or h2B4-B extracellular domain (600 bp).** Digest was performed for 2 hours at 37°C. Each lane represents different clones and digestion was performed in 3 clones of the h2B4-A-CD5 and h2B4-B-CD5 constructs. DNA was then sent for sequencing (Seqwright).


clone 1 2 3
6 kb
pEHCV
h2B4-A
1200 bp

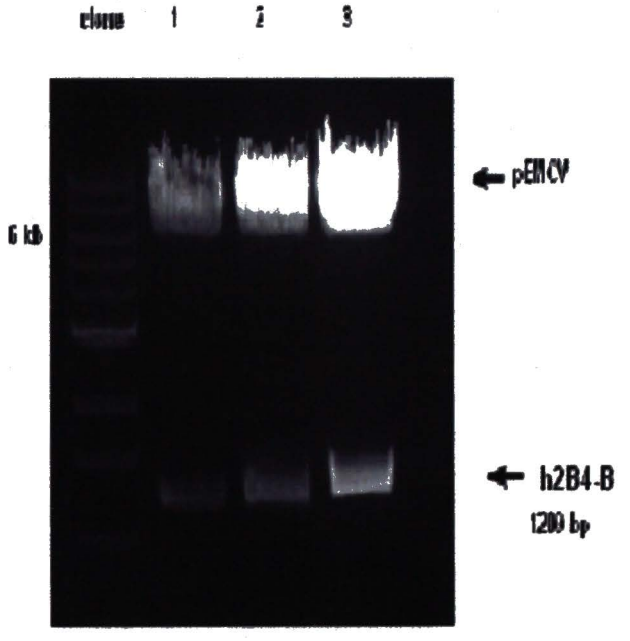

clone 1 2 3
6 kb
pEHCV
h2B4-B
1200 bp

FIGURE 2.4. **Western blot of h2B4-A and h2B4-B fusion proteins**. The h2B4-A-Fc and h2B4-B-Fc plasmid was transiently transfected into B16 mouse melanoma cells using Fugene-6 transfection reagent. The collected protein was separated on SDS-PAGE under reduced (R) and non-reduced (NR) conditions. Molecular masses are indicated in kDa.

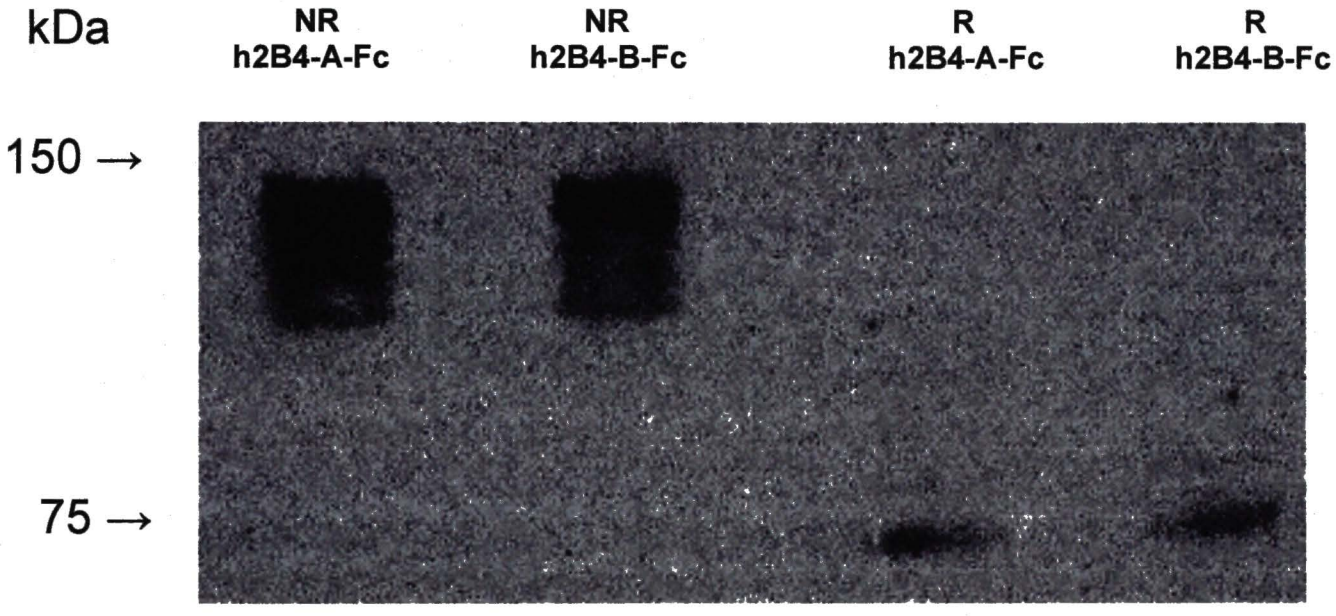
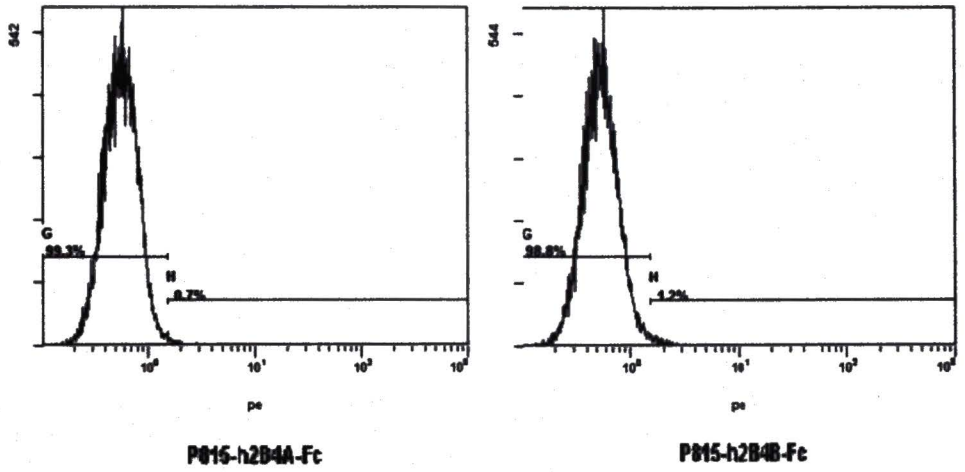

kDa
NR
h2B4-A-Fc
NR
h2B4-B-Fc
R
h2B4-A-Fc
R
h2B4-B-Fc
150 →
75 →

FIGURE 2.5. **h2B4-A-Fc has a greater relative binding affinity with CD48 than h2B4-B-Fc**. Binding is presented as specific mean fluorescence intensity (MFI) of FITC-conjugated anti-human IgG. Flow cytometry shows h2B4-A-Fc with $CD48^+$ NK92 has a MFI of 20.2 while h2B4-B-Fc with $CD48^+$ NK92 has an MFI of 3.6. A, P815, a CD48 negative cell line, was used as a negative control and was incubated with h2B4-A and h2B4-B fusion protein. B, $CD48^+$ NK92 cells were incubated with h2B4-A and h2B4-B fusion protein.

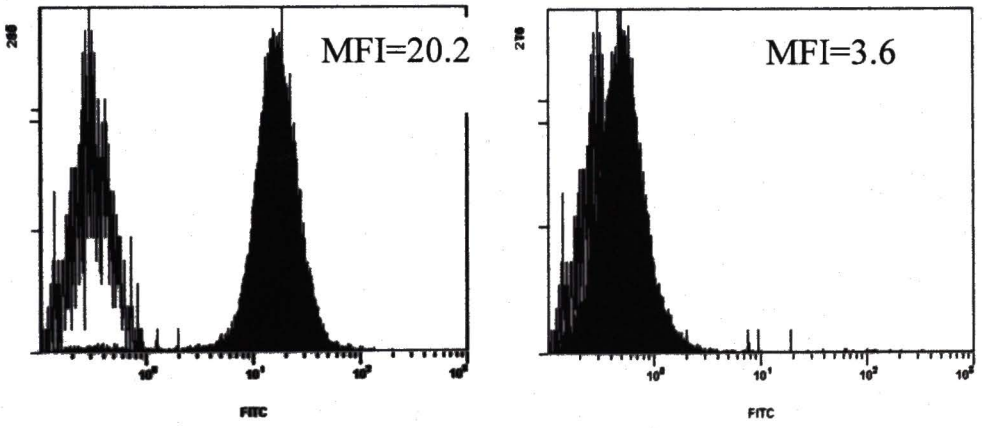
A



P815-h2B4A-Fc

P815-h2B4B-Fc

B



NK92-h2B4A-Fc

NK92-h2B4B-Fc

CONCLUSIONS

In humans, CD48 is the high affinity receptor of h2B4-A. Previous studies had determined that the h2B4-A-CD48 interaction site involves charged amino acids in the V domain of h2B4-A (26). We have shown in our study, that h2B4-B binds to CD48 with a much lower relative binding affinity than h2B4-A. This lower binding affinity of h2B4-B occurs even though the 5 amino acid addition is present at a site that is different than the h2B4-A-CD48 binding site. Therefore, the additional 5 amino acids may cause a change in the three-dimensional configuration of the h2B4-B protein rendering it unable to fit in to the CD48 binding site.

CHAPTER III

FUNCTIONAL ANALYSIS OF H2B4-A AND H2B4-B UPON INTERACTION WITH CD48

SUMMARY

The purpose of this study was to determine the functions of each isoform using the h2B4-A and h2B4-B fusion proteins as explained in Chapter II. Functional differences between h2B4-A and h2B4-B were investigated by performing redirected cytotoxicity assays and ELISA using these soluble fusion proteins. NK cells are known to release Interferon-γ (IFN- γ) upon activation. Therefore, in order to assess whether the difference in binding to CD48 by the isoforms results in a differential cytolytic function, we measured the amount of IFN- γ release upon interaction. The results indicated that upon CD48 interaction h2B4-A inhibits IFN- γ production, while h2B4-B interaction results in IFN- γ release no different than basal level. Cytotoxicity assays were also conducted by cross-linking each fusion protein to Fc-R on K562 to CD48 on NK92 cells. This assay determined that incubation h2B4-A fusion protein increased the killing activity of NK92 when compared with the reduction in cytotoxicity when in the presence of h2B4-B-Fc. Therefore, due to the fact that h2B4-A has a greater binding affinity to CD48 than h2B4-B, this may result in a greater NK cytotoxicity upon interaction with CD48.

MATERIAL AND METHODS

Cell lines

NK92 (human NK cell line) and K562 cells (human erythroleukemic cell line) were cultured in culture media (RPMI 1640, supplemented with 10% FBS, 2mM glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 10mM HEPES and 10 mM non-essential amino acids). Cells were maintained at 37°C in a humidified 5% CO_2/95% air incubator.

ELISA

Human IFN-γ sandwich ELISA was performed using the Peprotech 900-K27 kit protocol. Affinity purified anti-hIFN-γ capture antibody was diluted with PBS to a concentration of 1μg/mL and 100μL was added to each well. The plate was sealed and incubated overnight at room temperature. h2B4-A and h2B4-B fusion protein (10μg/mL) were incubated with 500,000 NK92 cells that were treated with 4μg of Fc blocker for 30 minutes and 50,000 K562 (human leukemia cell line) cells. In addition to the fusion proteins, 200ng/mL 22B5 (control mAb) and media alone (B16/optimem) were incubated with NK92 and K562 and used as negative controls, while C1.7 (anti-2B4 mAb) was used as a positive control. Each reaction was conducted in triplicate. Samples were incubated overnight in the 37°C humidified 5% CO_2/95% air incubator. Biotinylated antigen-affinity purified rabbit anti-hIFN-γ detection antibody, at a concentration of 0.25

μg/mL, was added per well (100μL) and incubated at room temperature for 2 hours. Avidin-HRP conjugated antibody was diluted 1:2000 and 100μL was added to each well. The plate was incubated at room temperature for 30 minutes. 100μL of ABTS Liquid substrate was added to each well and color development was monitored by the ELISA plate reader at 405nm with wavelength correction set at 650nm.

Cytotoxicity assay

Target cells (K562) were labeled by incubating 1 million cells with 2 MBq of $Na^{51}CrO_3$ (NEN Research Products, Boston, MA) for 90 minutes at 37°C under 5% CO_2 in the air incubator. NK92 that were treated with 4μg of Fc blocker for 30 minutes, were incubated with h2B4-A and h2B4-B fusion protein (100μg) and were cross-linked to the target cells via FcγR. After incubation for 4 hours at 37°C, the cells were pelleted at 250g for 5 minutes, 100ul of the supernatant was removed and the radioactivity was measured using a scintillation counter. Percent specific target cell lysis was calculated for each E:T (effector to target) ratio as 100% × (sample ^{51}Cr release – spontaneous ^{51}Cr release)/(maximal ^{51}Cr release – spontaneous ^{51}Cr release).

RESULTS

h2B4-A-CD48 interaction produces less cytokine release than h2B4-B

NK cells are known to release IFN- γ upon activation. Therefore, in order to assess whether the difference in binding to CD48 by the isoforms results in a difference in the cytokine release, we measured the amount of IFN- γ release upon fusion protein interaction with CD48. h2B4-A-Fc and h2B4-B-Fc fusion proteins (100μg) were incubated with NK92 effector cells prior to incubation with target K562 cells. The activation of the NK92 cells, as indirectly measured by IFN- γ release, when incubated with h2B4-B-Fc presented no difference as compared to negative controls. However, h2B4-A-Fc incubation and interaction with CD48 resulted in an inhibition of IFN- γ production as compared to negative controls 22B5 (control mAb) and media alone (B16/optimem)(Fig. 3.1). Overall, there seems to be an inhibition in IFN- γ release with h2B4-A-CD48 interaction, whereas using a monoclonal antibody against 2B4 (C1.7, positive control) increases production of IFN- γ.

h2B4-A-Fc fusion protein increased NK cytotoxicity when interacting with CD48 as compared to h2B4-B-Fc fusion protein

h2B4-A has been previously shown to activate NK cell-mediated cytoxicity via signaling through CD48 (1). However, to compare whether there exists a difference between the two isoforms we performed a redirected cytotoxicity assay by cross-linking the fusion protein to Fc receptor ($Fc\gamma R^+$) on K562 to CD48 on NK92 (Fig 3.2). h2B4-A-Fc fusion protein interaction with CD48 on NK92 resulted in an increase in cytotoxicity while h2B4-B-Fc fusion protein does not induce NK cytotoxicity as compared to negative control (incubation with mCD48-Fc fusion protein).

Figure 3.1. **h2B4-A interaction with CD48 on NK92 cells inhibits IFN-γ release while h2B4-B interaction with CD48 produces no effects as compared to negative controls.** NK92 cells were incubated media alone (B16/optimem, negative control), C1.7 (anti-2B4 mAb, positive control), 22B5 (control mAb, negative control), h2B4-A-Fc or h2B4-B-Fc at 45 min prior to the introduction of K562 cells. *, Denotes statistical significance between h2B4-A-Fc-NK92 (10μg/ul) and B16/optimem ($p<0.001$) as well as h2B4-A-Fc-NK92 (10μg/ul) and h2B4-B-Fc-NK92 (10μg/ul) ($p<0.001$).

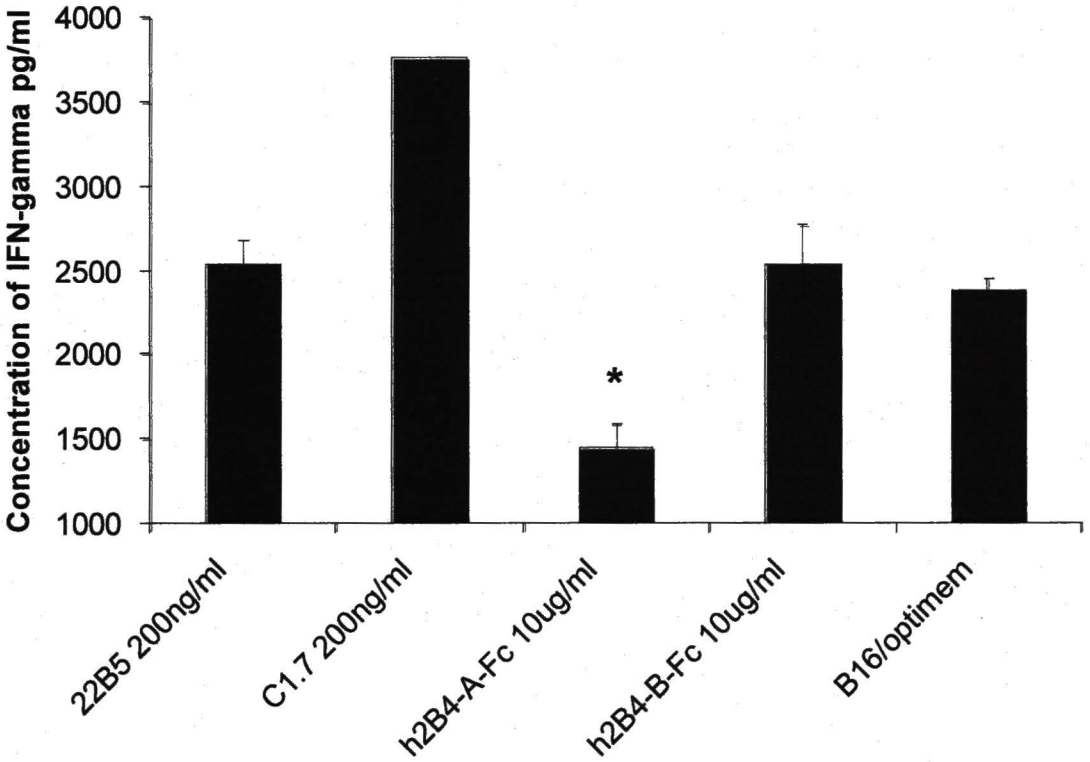

Concentration of IFN-gamma pg/ml
4000
3500
3000
2500
2000
1500
1000
*
22B5 200ng/ml
C1.7 200ng/ml
h2B4-A-Fc 10ug/ml
h2B4-B-Fc 10ug/ml
B16/optimem

Figure 3.2. **Model of redirected lysis**. NK92 cells that are $CD48^{+}$ were used as effector cells in a standard chromium release assay against the human FcR^{+} target K562. NK92 that were treated with 4μg of Fc blocker for 30 minutes, were incubated with h2B4-A and h2B4-B fusion protein (100μg) and were cross-linked to the target cells via FcγR.

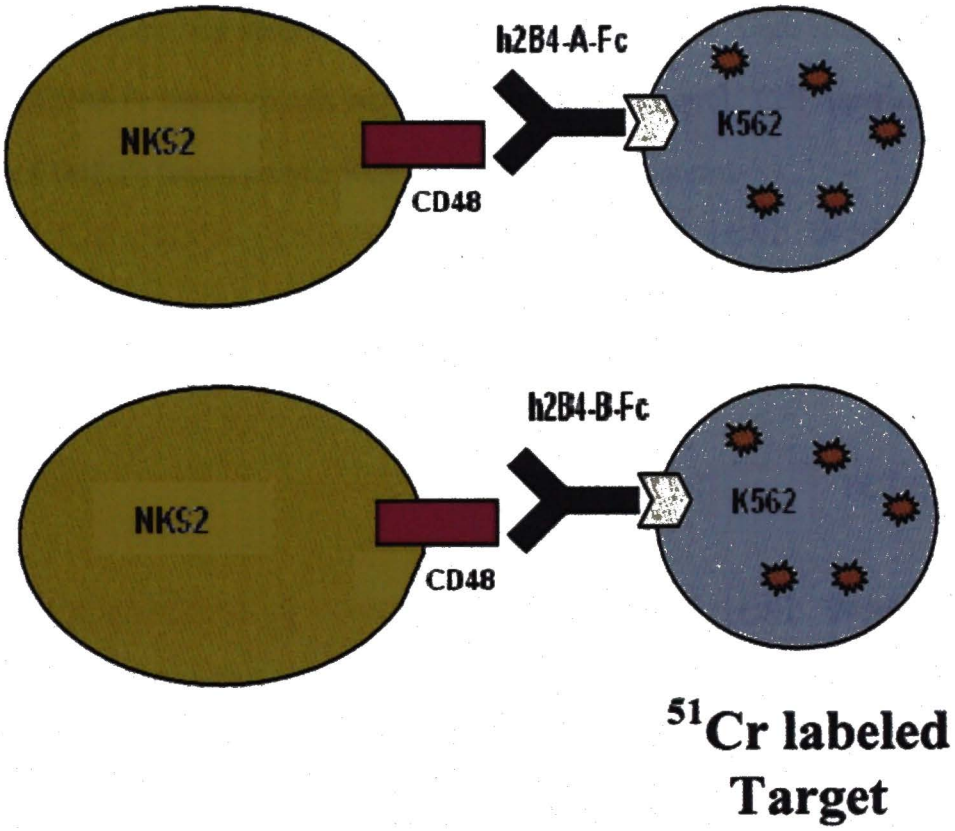
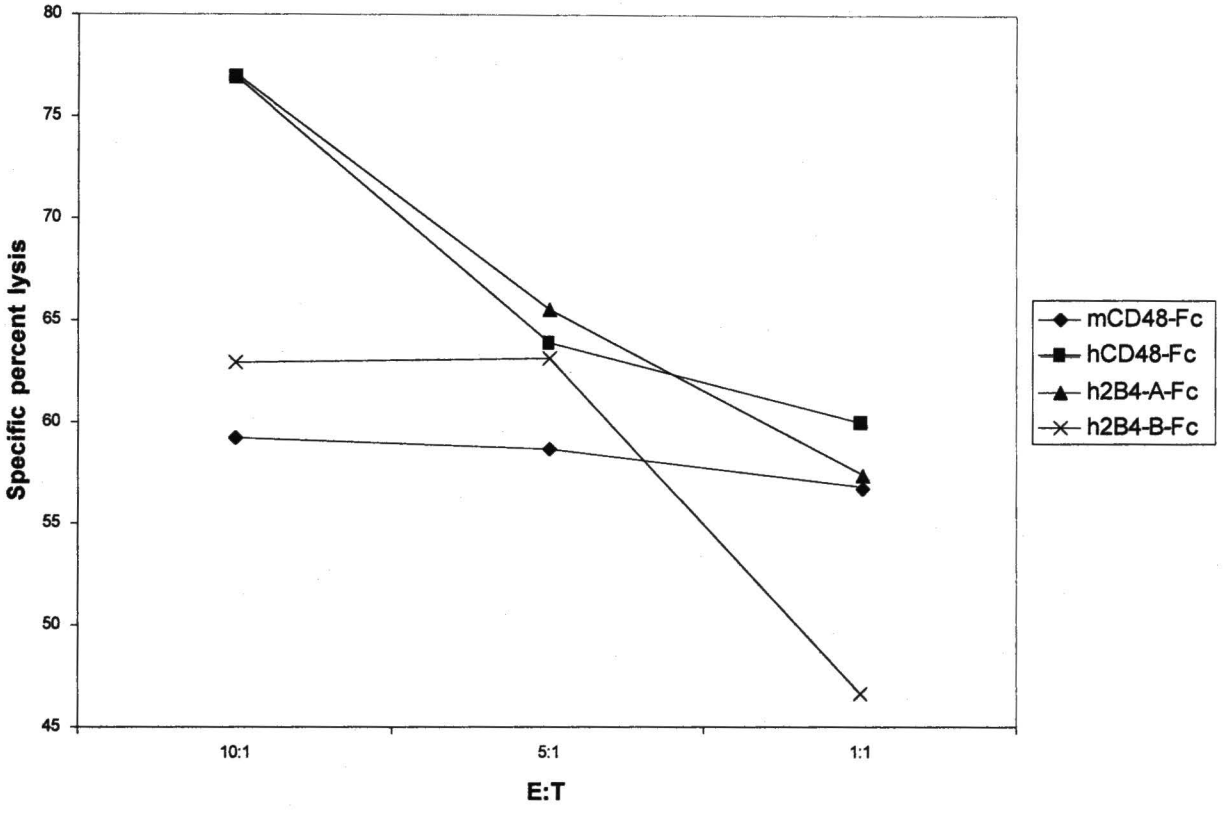

h2B4-A-Fc
NKS2
CD48
K562
h2B4-B-Fc
NKS2
CD48
K562
51Cr labeled
Target

FIGURE 3.3. **h2B4-A-Fc interaction with CD48 results in a greater cytotoxicity as compared to h2B4-B-Fc**. NK92 cells that are $CD48^+$ were used as effector cells in a standard chromium release assay against the human FcR^+ target K562. Only NK92 with h2B4-A-Fc had increased killing activity against K562 via CD48 signaling. 2B4-B-Fc shows reduced activity upon interaction with CD48 on NK92. Cells stimulated with human CD48-Fc fusion protein were used as a positive control. Cell stimulated with mouse CD48-Fc fusion protein were used as a negative control.


80
75
70
65
60
55
50
45
Specific percent lysis
10:1
5:1
1:1
E:T
mCD48-Fc
hCD48-Fc
h2B4-A-Fc
h2B4-B-Fc

CONCLUSIONS

Receptor-ligand interactions on the cell membrane determine NK cell function and are therefore important in immune cell function and tumor cytotoxicity. We had seen earlier in the study that h2B4-A has a greater relative binding affinity with CD48 than h2B4-B. Here we have seen that upon CD48 interaction, h2B4-A results in a greater tumor killing activity than h2B4-B. This suggests that since h2B4-B cannot interact with the binding site on CD48 with the same affinity as h2B4-A, there could be reduced NK cell activation resulting in a level of cytoxicity no different than basal level (mCD48-Fc incubation). Whereas, h2B4-A-CD48 interaction and association with the binding site on CD48 results in a greater NK killing activity.

While it has been determined that in humans, activation of NK cells through h2B4-A via CD48 interaction increases IFN- γ production (1,4,7,24), this is the first investigation to study activation of NK cells through CD48 via h2B4-A and h2B4-B interaction. Our results indicate that h2B4-A-CD48 interaction causes an inhibition in IFN-γ secretion while h2B4-B-CD48 interaction does not. This suggests that interaction of CD48 with either of the isoforms may be a method to control immune activation through regulation of IFN- γ secretion.

CHAPTER IV

DIFFERENCE IN EXPRESSION OF H2B4-A AND H2B4-B

SUMMARY

It has been shown that h2B4-A mRNA is expressed on various human cell lines such as HL-60 (human myelocytic leukemia cell line), YT (NK cell line; acute lymphoblastic lymphoma), Jurkat (immature T cell line) and DB (B cell line). However, it was discovered that h2B4-B is not expressed on these same cell lines. Importantly, both primary human NK and T cells express h2B4-A and h2B4-B forms. NK92 was determined by RT-PCR to express both isoforms. Using freshly isolated NK cells from 6 healthy individuals, it was also shown that in humans there are varying levels of h2B4-A and h2B4-B expression.

MATERIALS AND METHODS

Cell lines

NK92 (human NK cell line), HL-60 (human myelocytic leukemia cell line), YT (NK cell line; acute lymphoblastic lymphoma), Jurkat (immature T cell line) and DB (B cell line) were cultured in culture media (RPMI 1640, supplemented with 10% FBS, 2mM glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 10mM HEPES and 10 mM non-essential amino acids). Cells were maintained at 37°C in a humidified 5% CO_2/95% air incubator.

Human NK cell isolation

Isolation of NK cells from PBMC was performed by depletion of non-NK cells. Mononuclear cells from peripheral blood (PBMC) were obtained by density gradient centrifugation over ficoll paque (Sigma Chem., St. Louis, MO). To deplete T cells, B cells and myeloid cells from PBMC, these cells were magnetically labeled using a cocktail of hapten-conjugated CD3, CD14, CD19, CD36 and anti-IgE antibodies. Cells were labeled with hapten antibody cocktail and FcR blocking reagent. After removing the unbound antibodies by washing, the cells were incubated with anti-hapten microbead (Miltenyi Biotec, Auburn, CA) and then magnetically labeled cells were depleted by retaining them on a magnetic column (MACS; Miltenyi Biotec, Auburn, CA). Purity of NK cells was evaluated by labeling cells with PE conjugated anti-CD56 mAb.

Reverse Transcriptase (RT)-PCR

Total RNA was isolated with the RNAstat 60 reagent according to the manufacturer's protocol (Teltest Inc, Friendswood, Tex.) and first strand cDNA was synthesized from 5μg of total RNA using superscript II (Invitrogen) reverse transcriptase and random primers in a volume of 20ul. PCR was performed using primers specific for h2B4-A and h2B4-B sequences (Integrated DNA Technologies, Coralville, Iowa). Following amplification, portions of the PCR reactions were electrophoresed through a 1.2% agarose gel.

RESULTS

h2B4-B is expressed on freshly isolated NK cells.

The mRNA expression of h2B4-A and h2B4-B on HL60, YT, Jurkat, DB cell lines and primary NK and T cells was studied by RT-PCR. The results show that only h2B4-A is expressed on the various cell lines while both h2B4-A and h2B4-B is expressed on primary freshly isolated NK and T cells (Fig. 5.1).

h2B4-B is expressed on the NK92 cell line.

As determined by RT-PCR and using sequence specific primers for h2B4-B, NK92 expresses both isoforms of 2B4 (Fig. 5.2).

h2B4-A and h2B4-B are expressed at varying levels in freshly isolated NK cells from healthy individuals.

h2B4-A and h2B4-B mRNA expression was studied in 6 different individuals by RT-PCR after NK cell isolation from healthy human volunteers. It was shown that the levels of expression of both isoforms vary from individual to individual (Fig. 5.3).

FIGURE 5.1. **RT-PCR analysis of h2B4-A and h2B4-B expression in various cell lines and primary NK and T cells**. PCR was performed using common PCR primers for h2B4-A and h2B4-B (A) and primers specific for h2B4-B (B) in HL-60 (human myelocytic leukemia cell line), YT (NK cell line; acute lymphoblastic lymphoma), Jurkat (immature T cell line) and DB (B cell line) as well as primary NK and T cells isolated from PBMC in healthy human volunteers. (C) β-actin.

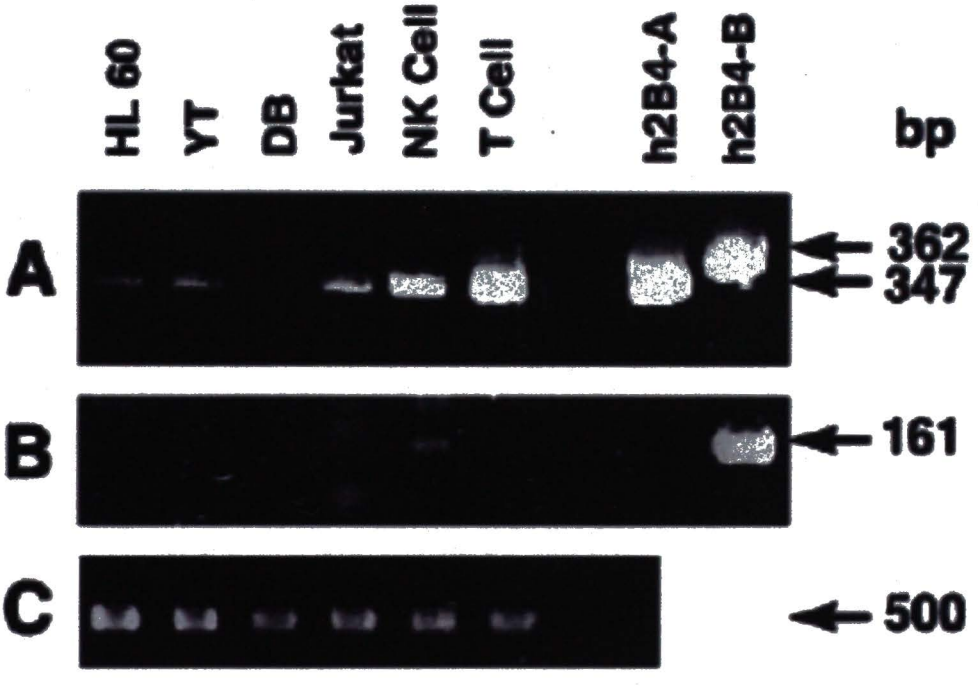

HL 60
YT
DB
Jurkat
NK Cell
T Cell
h2B4-A
h2B4-B
bp
A
362
347
B
161
C
500

FIGURE 5.2. **RT-PCR analysis of h2B4-A and h2B4-B expression in NK92.** PCR was performed using common PCR primers for h2B4-A and h2B4-B (A) and primers specific for h2B4-B (B). (C) GAPDH.

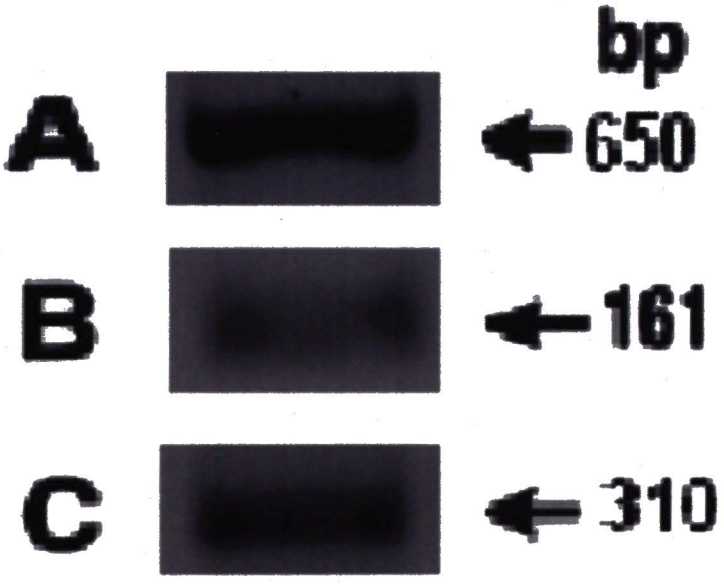
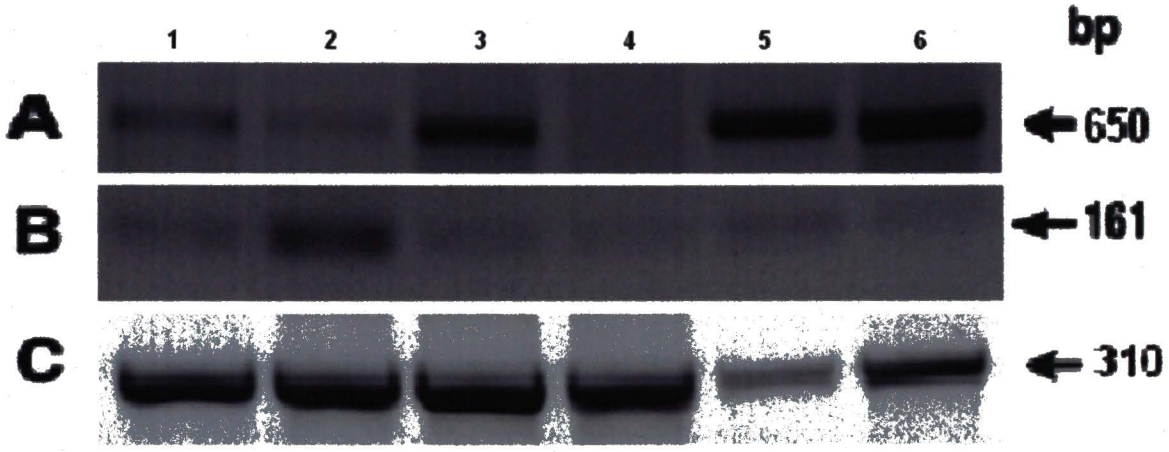

bp
A
650
B
161
C
310

FIGURE 5.3. **RT-PCR analysis of h2B4-A and h2B4-B expression in peripheral blood mononuclear cells (PBMC) of 6 healthy individuals**. Each lane represents a different healthy individual. PCR was performed using common PCR primers with h2B4-A and h2B4-B (A) and primers specific for h2B4-B (B). (C) GAPDH


1
2
3
4
5
6
bp
A
650
B
161
C
310

CONCLUSIONS

Using two sets of primers, our current results indicate that both h2B4-A and h2B4-B mRNA transcripts are present in NK cells as well as T cells that are freshly isolated and pooled from normal individuals. Upon further investigation, we determined that between individuals, the mRNA transcripts are expressed at various levels in PBMC of healthy volunteers. Moreover, between the 6 individuals, some expressed more of h2B4-A while some showed a higher expression of h2B4-B. It could be speculated that this difference in expression could be due to regulation of expression in each individual at either the transcriptional level or control of alternative splice variation. To further understand this variation in mRNA expression, it is essential to study expression of the two isoforms at the protein level. It is also necessary to study the differences in NK cell activity between the 6 individuals with varying h2B4-A and h2B4-B expression. It is also unknown whether the differences between individuals could be due to heterogeneity in the population in terms of race, age and gender. Given the fact that the two receptors isoforms are functionally distinct, further pursuit of this issue may be warranted.

CHAPTER V

DIFFERENTIAL EXPRESSION OF H2B4-A AND H2B4-B EXPRESSION UPON STIMULATION WITH A MONOCLONAL ANTIBODY AGAINST 2B4 (C1.7)

SUMMARY

Since NK92 was determined to be the only known cell line that has both 2B4-A and 2B4-B expression, these cells were used to study the effect on expression after 2B4 stimulation. The NK cell stimulator, C1.7 (anti-2B4 mAb), was incubated with NK92 over a time period of 8 hours at either 1 or 2 hour intervals. The results showed that C1.7 stimulation caused a downregulation of h2B4-A from 1 hour to 4 hours and an upregulation for h2B4-B from 4 hours to 8 hours. Thus, both isoforms are expressed in opposing manners during C1.7 activation.

MATERIALS AND METHODS

Cell lines

NK92 (human NK cell line) were cultured in culture media (RPMI 1640, supplemented with 10% FBS, 2mM glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 10mM HEPES and 10 mM non-essential amino acids). Cells were maintained at 37°C in a humidified 5% CO_2/95% air incubator.

C1.7 Stimulation

NK92 cells were stimulated in culture using 5μg of C1.7 (anti-2B4 mAb) at different time points. This included 0, 1, 2, 4, 6 and 8 hour timepoints.

Reverse Transcriptase (RT)-PCR

Total RNA was isolated with the RNAstat 60 reagent according to the manufacturer's protocol (Teltest Inc, Friendswood, Tex.) and first strand cDNA was synthesized from 5μg of total RNA using superscript II (Invitrogen) reverse transcriptase and random primers in a volume of 20ul. PCR was performed using primers specific for h2B4-A and h2B4-B sequences (Integrated DNA Technologies, Coralville, Iowa). Following amplification, portions of the PCR reactions were electrophoresed through a 1.2% agarose gel.

RESULTS

h2B4-A and h2B4-B is regulated upon C1.7 activation

The effect of C1.7 (anti-2B4 mAb) stimulation of NK92 cells shows that h2B4-A is downregulated by 4 hours while h2B4-B is upregulated from 4 hour to 8 hour timepoints (Fig. 6.1). Results are presented as relative units of each transcript compared to GAPDH and as a ratio of 0 hour C1.7 stimulation. Therefore, after C1.7 stimulation, when h2B4-A is downregulated, h2B4-B is upregulated. Thus, both isoforms are being expressed in opposing manners (Fig. 6.2).

Figure 6.1. **Regulation of h2B4-B mRNA expression upon C1.7 stimulation**. RT-PCR analysis of h2B4-A and h2B4-B expression in NK92 with and without C1.7 stimulation at time points: 0, 1 hour, 2 hour, 4 hour, 6 hour and 8 hour. PCR was performed using common PCR primers for h2B4-A and h2B4-B (A) and primers specific for h2B4-B (B). (C) GAPDH.

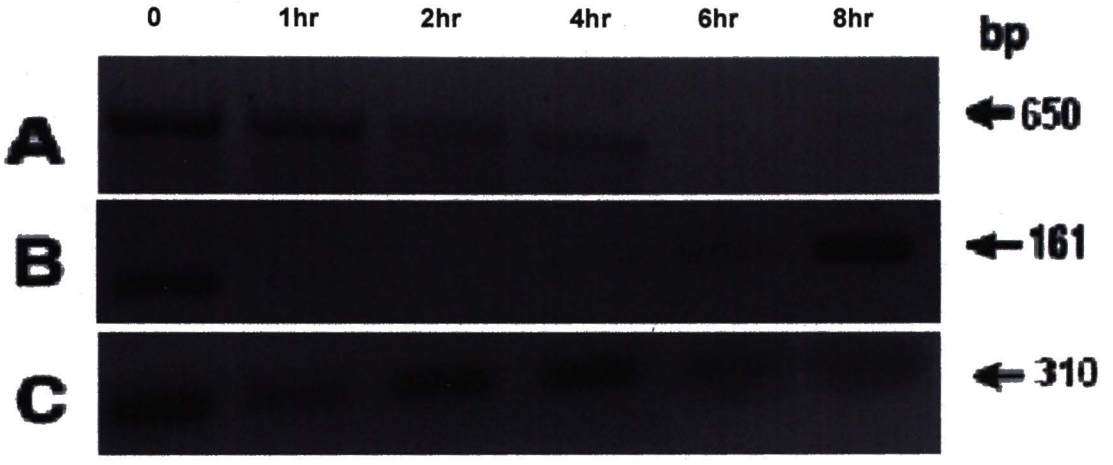
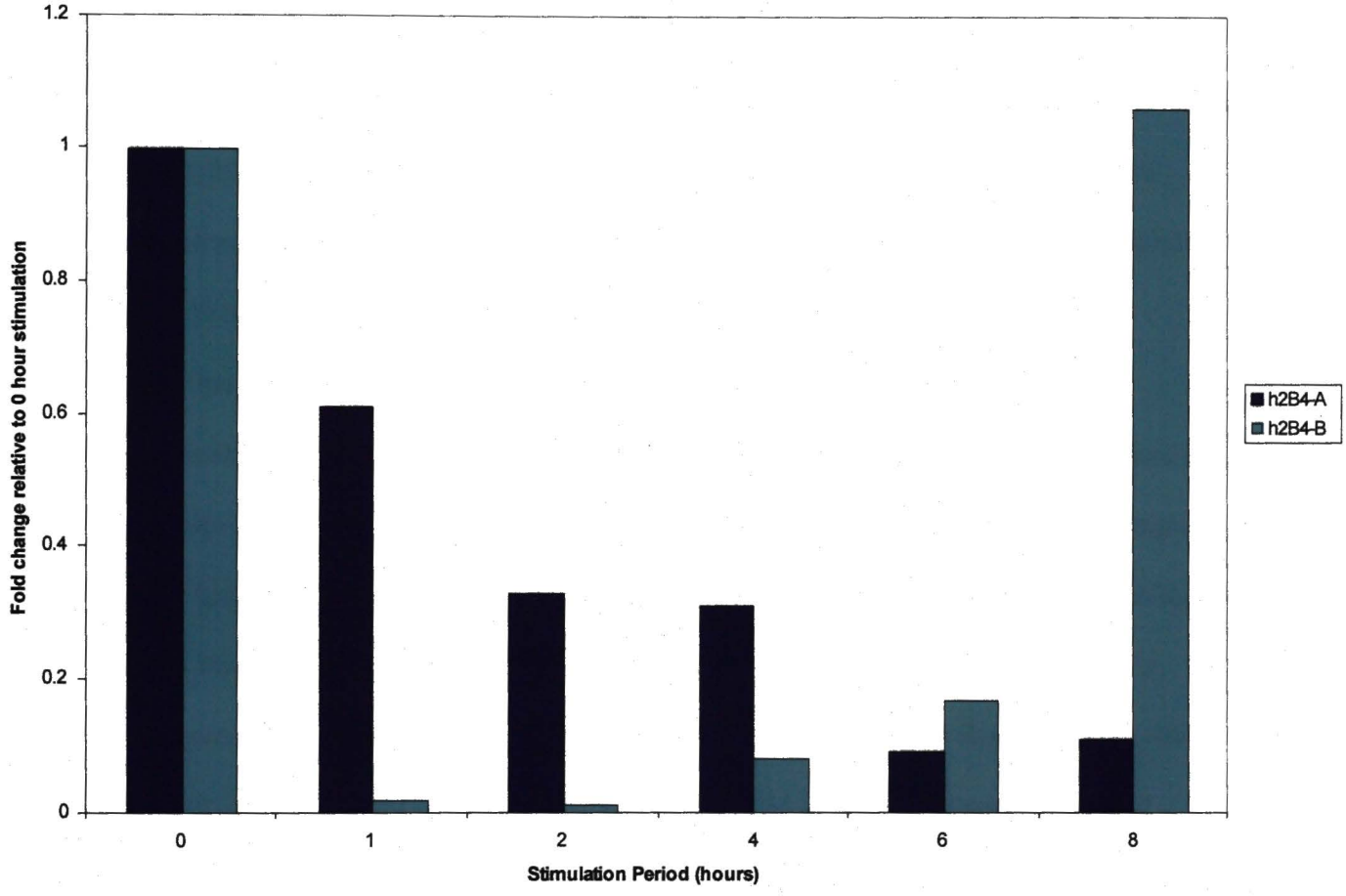

0
1hr
2hr
4hr
6hr
8hr
bp
A
B
C
650
161
310

Figure 6.2. **Densitometric analysis of band intensities revealed the regulation of mRNA levels of h2B4-A and h2B4-B upon C1.7 stimulation.** RT-PCR analysis of h2B4-A and h2B4-B expression in NK92 with and without C1.7 stimulation at time points: 0, 1 hour, 2 hour, 4 hour, 6 hour and 8 hour. Graph shows expression levels normalized to GAPDH and relative to fold change of 0 hour C1.7 stimulation.


1.2
1
0.8
0.6
0.4
0.2
0
Fold change relative to 0 hour stimulation
0
1
2
4
6
8
Stimulation Period (hours)
h2B4-A
h2B4-B

CONCLUSIONS

2B4 interaction with its ligand CD48 regulates NK cell function and thus plays a central role in various immune responses. We have shown in this study that upon NK cell stimulation, h2B4-A and h2B4-B mRNA transcripts are differentially expressed at various time points. During 1 to 4 hours of C1.7 stimulation (via interaction with h2B4-A) there exists primarily the expression of h2B4-A followed by its complete downregulation by the 8 hour period. Earlier studies had determined that this downregulation occurs due to a reduction in the promoter activity at the Ets element (20). However, the effect of C1.7 stimulation on h2B4-B expression was not yet investigated. We found in our study that at 1 to 2 hours there was no expression of h2B4-B, while at 4 to 8 hours the expression of h2B4-B was upregulated upon C1.7 stimulation. It is important to note in this study that it has been determined that the ligand binding site, CD48, and the mAb C1.7 binding site of human 2B4 involve the same amino acid residues in the V domain of 2B4 (23). Therefore, it could be suggested that the upon CD48 interaction, regulation of h2B4-A and h2B4-B mRNA expression may occur and could be a mechanism to control the co-stimulatory signal from h2B4-A and h2B4-B-CD48 interactions. As a result, C1.7 or CD48 interaction with h2B4-A and h2B4-B may regulate NK cell function by regulating mRNA expression at the transcriptional level or more plausibly by controlling alternative splice variation. The mechanism by which C1.7 may regulate the mRNA expression of both isoforms warrants further investigation.

CHAPTER VI

DISCUSSION

2B4 (CD244) is a member of the CD2 subset of the Ig superfamily. This molecule is expressed on innate immune cells, including NK cells, and on subsets of T cells. The human 2B4 molecule interacts with CD48, which is widely expressed on hematopoietic cells and has been determined to be an activating receptor on NK cells. It is known that human 2B4-A can stimulate NK cell cytotoxicity and cytokine production by interacting with cells expressing CD48. However, the detailed immunological functions and biology of the two human receptors, h2B4-A and h2B4-B, have not been studied. The purpose of this study was to determine the existence of the isoforms and the role of h2B4-A and h2B4-B on human NK cells.

In this study, we have determined that h2B4-A has a greater binding affinity to CD48 and results in a greater cytolytic function than h2B4-B. We found that soluble h2B4-A-Fc fusion protein containing the extracellular domain of h2B4-A bound to NK92-CD48^{+} with greater affinity than the h2B4-B-Fc fusion protein. Therefore, it is proposed that this difference in 15 nucleotides may result in this difference in relative binding affinities with CD48 between the isoforms. The difference could be due to the resulting change in protein folding as a result of the additional amino acids in h2B4-B. It is not yet known whether these two isoforms may recognize different ligand molecules or may just have different binding affinities for the same ligand, as suggested here.

Interestingly, we found that when h2B4-A-Fc fusion protein was incubated with $CD48^+$ NK92, there was a decreased level of IFN-γ released by NK92 relative to h2B4-B-Fc incubation. Therefore, we have found that h2B4-A-CD48 interaction results in an inhibition of IFN- γ production even more so than h2B4-B-CD48. While it has been determined that in humans, activation of NK cells through h2B4-A via CD48 interaction increases IFN- γ production (27), this is the first investigation to study activation of NK cells through CD48 via h2B4-A and h2B4-B interaction. Therefore, this inhibition in IFN- γ production upon h2B4-A interaction with CD48, may be a method to regulate activation of NK cells via CD48 and may thus play a role in immune cell regulation. It may also be possible that h2B4-B may play a role in IFN- γ production when activating through CD48 instead of h2B4-A since we observed a secretion of IFN- γ comparable to baseline secretion. In either case, the existence of the two isoforms may play a role in regulating the immune system via IFN- γ production.

The results of our study also show that h2B4-A-Fc fusion protein interaction with CD48 on NK92 resulted in a greater cytotoxicity than did h2B4-B-Fc. Hence, any possibility of interactions between h2B4-B and CD48 on target cells may lead to a decrease in basal killing level of tumor cells. It may be possible here that h2B4-B plays a role in regulating NK cell cytotoxicity by bringing the percentage of lysis of tumor cells back to basal level of killing. While h2B4-A plays an important role in increasing the cytolytic activity of NK cells.

Human 2B4-A has been shown to be expressed via RT-PCR in various cell lines including those from promyelocytic leukemic, NK, B and T cell lineages. However, it

was determined in this study that h2B4-B is only expressed in primary NK and T cells as well as NK92, a NK cell lymphoma that is IL-2 dependent. Interestingly, it was also determined that humans express varying levels of h2B4-A and h2B4-B in PBMC. This result suggested that in humans, there could be a regulation of expression levels. Therefore, we proceeded to study the effects of NK cell activation on the regulation of isoform expression. Previous studies had indicated that AP-1 and Ets transcription elements are involved in the regulation of the 2B4 gene. It was also determined in another study that stimulation of NK cells through surface h2B4-A down-regulates its own expression due to a reduction in the promoter activity at the Ets element (26). Therefore, we continued to study the effects of NK stimulation through surface 2B4 on the expression levels of both h2B4-A and h2B4-B in NK92. We determined at earlier timepoints that while h2B4-A is expressed, there is downregulation of the expression of h2B4-B. However, at the later time points when h2B4-A is downregulated, there is an upregulation of h2B4-B expression. Thus, both isoforms are expressed at opposing levels and are regulated together. This is possibly due to the fact that h2B4-A and h2B4-B are located on the same gene since the two isoforms are splice variants and may be transcriptionally regulated. It may also be likely that splice variation itself could be controlled upon NK stimulation. In either case, the regulation of h2B4-A and h2B4-B could be a mechanism to both attenuate and amplify the co-stimulatory signal from 2B4-CD48 interactions, therefore controlling the intensity of NK cell cytotoxicity.

At present, the complete functional role of the h2B4 isoforms described here is not fully understood. Studies are underway to determine the differential expression of the isoforms, including various disease states and LAK cells to better understand the role of these novel isoforms of human 2B4. However, these results might lead to the discovery of novel information to investigate the mechanism of immune disease including cancer metastasis as well as various autoimmune disorders.

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