Instituto Juan March
de Estudios e Investigaciones

45 CENTRO DE REUNIONES
INTERNACIONALES SOBRE BIOLOGÍA

Workshop on

NK Cell Receptors and Recognition of the Major Histocompatibility Complex Antigens

Organized by

J. Strominger, L. Moretta and M. López-Botet

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INTRODUCTION

M. López-Botet, L. Moretta and J. Strominger

In recent years it has become clear that specific recognition of MHC class I molecules on target cells may repress NK cell functions. Accordingly, cytotoxicity may take place when target cells lose MHC class I expression, when NK cells lack the appropriate inhibitory receptors or, alternatively, if changes in the structure of the MHC/peptide complex impair recognition. This currently accepted model implies the existence of other receptors and ligands capable of triggering effector functions (cytolysis); thus, NK cells are subjected to a subtle balance between activatory and inhibitory signals. A number of cell surface molecules potentially involved in NK cell recognition mechanisms have been identified in humans and rodents. Multiple combinations of these receptors may be co-expressed by individual NK clones/subsets. Surprisingly, the inhibitory receptors so far identified on human and rodent NK cells belong to different protein families, although they have similar functions; this was a central theme at this meeting.

Different gene products of the Ly49 family of C-type lectin receptors specifically interact with murine H-2 molecules, inhibiting cytotoxicity. On the other hand, members of another family of C-type lectin receptors (NKR-P1) have been proposed to trigger cytolysis upon recognition of certain negatively charged oligosaccharides, and are thus candidates for the group of activatory receptors.

By contrast, the human inhibitory NK receptors that have been characterized (p58/GL183+, p58/EB6+, p58/HP-3E4+ and p70/NKB1) belong to an Ig-related multigene family. A number of different cDNA sequences, including the group of NKATs, have been isolated. Remarkably, transfer of individual receptors conferred to NK clones the capacity to specifically recognize the predicted HLA class I alleles on target cells, leading to inhibition of lysis. Moreover, specific binding of soluble chimeric receptors to MHC class I-transfectants was directly demonstrated. EB6 or GL183 may display activatory functions in NK clones from some individuals. These triggering receptors display a lower Mr (50 kDa), have a shorter intracytoplasmic tail lacking ITAMs, and contain a charged residue (Lys) in the transmembrane region. Regarding nomenclature, NKIR

(natural killer cell inhibitory receptors) and NKAR (natural killer cell activatory receptors), related to the terms KIR and KAR used by one group, appear to be the most appropriate terms proposed.

The question as to whether any human lectin-like receptor is involved in MHC recognition was raised. The putative role of CD94, another member of the C-type lectin superfamily, as an inhibitory receptor for certain HLA alleles was extensively discussed. No consistent pattern of specificity was detected in functional assays; thus, a direct demonstration of the CD94-HLA interaction would be required to clarify the issue.

We are thus left with a remarkable enigma: did two different protein families evolve in mouse and man to serve the same function, or will we find that human NK cells also express inhibitory receptors of the C-type lectin superfamily and murine NK cells those of the Ig superfamily ?

The attention is presently focused on: a) The magnitude of receptor diversity. b) The molecular basis for NK receptor specific interaction with MHC class I/peptide complexes, and the relative importance of N-linked carbohydrates and bound peptides. c) The nature of other receptor-ligand interactions triggering effector functions. d) How do MHC molecules influence the expression of NK receptors during ontogeny. e) The biochemistry of the balanced activatory and inhibitory signaling pathways. f) The role of NK receptors in T cells.

All these aspects were addressed during the Workshop. In spite of the important advances in the field some observations cannot be satisfactorily explained based on the available knowledge, and a number of crucial issues remain unanswered. The design of more refined experimental systems where the individual receptors may be functionally analyzed, together with the development of additional reagents, shared by investigators, deserve a major effort.

The Juan March Institute has provided an unprecedented forum for the open discussion of the recent advances in this dynamic field of immunological research, promoting an essential interaction among participants.

FIRST SESSION

Chairperson: David H. Raulet

ADAPTATION OF THE MURINE NK REPERTOIRE TO SELF MHC CLASS I

Jonas Sundbäck, Mats Olsson, Maria Johansson, Lars Franksson, Margareta Waldenström, Petter Höglund, Charles Sentman, Klas Kärre. Microbiology and Tumor Biology Center, Karolinska Institute, Stockholm.

The ability of NK cells to detect "missing self" is determined by the MHC class I phenotype of the host. This is illustrated in a brief review of the altered NK repertoire of MHC class I (H-2D^{d}) transgenic B6 mice and β_2 microglobulin deficient mice, including a model where specific NK tolerance appears in mice with mosaic class I expression. NK cells maturing in an environment containing H-2^{b}/ D^{d+} as well as H-2^{b}/ D^{d-} cells do not discriminate between such cells and eliminate the latter, but they retain the ability to discriminate between normal H-2^{b} cells and cells deficient for TAP or β_2m.

The effector inhibition model for missing self recognition postulates MHC class I specific NK receptors that turn off the NK lytic program, and there is now strong evidence that Ly 49a molecules act as such receptors, capable of mediating inhibitory signals after recognition of H-2D^{d} (Yokoyama, Karlhofer, Seaman et al). The observations on Ly 49a, a C type lectin like receptor, nevertheless impose a paradox in relation to NK cells and "missing self" recognition: Ly 49A molecules cells are downregulated on NK cells in H-2D^{d} expressing mice, where they ought to be useful, but expressed at high levels in mice lacking H-2D^{d}, where they would be expected to be harmful. We propose a model based on "receptor calibration by avidity" to account for how NK cells adapt to self MHC. The key elements of this model are 1) Each NK cell can express several MHC class I receptors that transmit negative signals to inhibit killing 2) Host expression of the MHC class I ligand for an NK receptor leads to the development or selection of NK cells with reduced (but not deleted) expression of that receptor. This makes the receptor "useful", since NK cells carrying the receptor are more difficult to inhibit with low levels of ligand, and hence become more sensitive to detect "missing self". This is in contrast to the T-cell system, where high receptor expression is thought to correlate with higher sensitivity to detect the aberrant cells. 3) NK receptors for nonself MHC class I molecules may be functional and expressed at high levels; these are "useless" but not "harmful" as long as each NK cell carries at least one receptor for a self MHC class I molecule. The receptor calibration model may explain why allogeneic MHC molecules are sometimes more protective than syngeneic when NK subsets or clones are tested, while the autologous MHC combination would always be optimal for protection against the whole population of NK cells.

If MHC mediated regulation of Ly-49A represents a general phenomenon in the process by which NK cells adapt to self, it should apply also to other receptors in the family. We have investigated whether Ly-49C detected by the mab SW/5E6 is subject to similar regulation by host MHC. Ly49-C expression was regulated by host MHC differentially in mice of A/Sn (A) and C57Bl/6 (B) origin. Analysis of A x B recombinant inbred strains suggested four phenotypes of Ly-49C expressing NK cells, controlled by two independent but interacting loci, one segregating with the NK complex and the other one with the MHC. In congenic strains, the A type Ly-49C had a *low* basic expression level, and was further downregulated in the presence of the H-2^{b} haplotype (*very low*). The B type Ly-49C had a *high* basic expression level, and was downregulated in the presence of H-2^{b} (*medium*).

On the basis of these results, we hypothesized that receptors encoded by the Ly49C gene in the NK complex have different MHC specificity in A/Sn and C57BL mice because they represent allelic forms of the gene. The allelic products would both bind the antibody 5E6, but differ in critical positions leading to different affinity to H-2^b and H-2^k class I molecules and differential downregulation by these in vivo. To test this hypothesis, cDNA's for 5E6 were RT-PCR cloned from the A/Sn and C57BL/6 strains. The sequences differed by 6 nucleotides leading to 4 amino acid changes, one in the cytoplasmic tail, two in the stalk region and one in the globular, presumed ligand binding domain. Both cDNA's could be expressed as 5E6 binding membrane molecules in EL-4 cells upon transfection in an expression vector driven by the SV40 promotor. The Ly49C expression pattern in A x B F1 mice suggested that NK cells express either the A-form or the B form, consistent with the idea of allelic exclusion.

The expression pattern of Ly49C in MHC recombinant strains suggested control by the H-2K region. Further evidence for the involvment of H-2K products in regulation of NK cells were obtained in studies where RMA-S sublines could be protected from NK lysis by addition of MHC class I binding peptides before the cytotoxicity assay. Some, but not all H-$2K^b$ binding peptides were protective in this assay, suggesting that NK cells in some situations of MHC mediated protection may recognize only a subset of the relevant class I allelic product.

The results will be discussed in relation to "instruction" versus "selection" based models for determination of the NK repertoire. Although clonal selection is used for other cells in the immune system, processes based on instruction should not be excluded, since they may be more economical and in addition allow greater flexibility for adjustment of the NK repertoire.

MHC CLASS I-DEPENDENT AND INDEPENDENT SPECIFICITY OF MURINE NK CELLS. W.M. Yokoyama, A. Idris, S.-J. Kim, I. Mehta, A. Reichlin, H. Smith, and M. Orihuela, Howard Hughes Medical Institute, Mt. Sinai Medical Center, New York, NY 10029

NK cells can discriminate between tumor and infected cells that they kill and normal cells that they spare. This appears to involve a recognition process that is beginning to be unraveled at the molecular level. In the mouse, a primary determinant of NK cell specificity for tumor targets is the NK cell receptor Ly-49A, expressed by a subset of NK cells. The engagement of Ly-49A by H-2D^d on the target appears to trigger an inhibitory signal that abrogates killing. Ly-49A belongs to a family of highly related molecules, expressed on overlapping subsets, that may have similar function. Yet there exists another mechanism for NK cell specificity because mouse NK cell clones, derived from p53-deficient mice, do not display MHC class I specificity or express Ly-49 molecules, despite differences in tumor specificity. Delineation of the molecular basis for NK cell specificity and target susceptibility will require detailed examination of MHC class I-dependent and independent mechanisms.

VINAY KUMAR
Department of Pathology. University of Texas
Southwestern Medical School. 6000 Harry HGines Blvd.
NB6. 402
DALLAS, TX. 75235-9072 (USA)

Ontogeny of NKRP-1 and Ly49 molecules: functional implications

The functions of murine NK cells seem to be regulated by the opposing effects of activation/triggering molecules and inhibitory or negative signaling molecules. To the former category belong members of the NKRP-1 gene family (NK-1.1, 10A7, 3A4) and 2B4, a member of Ig superfamily. The natural ligands of the activating molecules have not been identified. The negative signaling molecules belong to the multimember Ly49 family.

Adult splenic NK cells can lyse allogeneic T cell blasts in vitro and in this assay the Ly49A molecules deliver negative signals by interaction with H-2^d, and H-2^k and H-2^f class I molecules. The inhibitory MHC ligands for Ly49C molecules seem to depend on the H-2 type of the host. Thus Ly49C molecule expressed on NK cells of H-2^b or H-$2^{d/b}$ mice seems to receive a negative signal upon interaction with H-$2K^b$ but not H-2^d T cell blasts. Whereas in H-2^d mice the Ly49C molecule seems to interact with both H-2^b and (self) H-2^d.

To understand the regulation of these NK cell receptors, 14 to 16 day fetal liver cells were cultured in IL-2 and NK-1.1+, CD3- cell lines were obtained. These cells expressed all of the NKRP-1 gene products, as well as 2B4, but did not express or transcribe Ly49A, Ly49G.2 and Ly49C. They killed the prototypic NK sensitive target YAC-1 quite efficiently, were able to secrete IFN-γ, but unlike adult splenic NK cells they were not able to lyse normal allogeneic T cell blasts. Given the absence of Ly49 molecules that deliver negative signals to NK cells upon interaction with MHC class I molecules, the inability of NK-1.1+, CD3-, 2B4+, Ly49A-, Ly49C-, Ly49G.2- cells to lyse allogeneic cells was unexpected. These data suggest that either fetal NK1.1+ cells express other undefined negative signaling molecules, or that some members of the Ly49 family are required for positive allorecognition. Preliminary studies indicate that expression of Ly49A and Ly49C on NK-1.1+ cells does not occur until 8 to 9 days after birth. Further studies to correlate expression of Ly49 family molecules and acquisition of allospecific killing are being pursued.

Role of the MHC class I molecules in NK cell development

Margarita Salcedo[1], Petter Höglund[1], Luc Van Kaer[2], and Hans-Gustaf Ljunggren[1]

[1]Microbiology and Tumor Biology Center, Karolinska Institute, S-104 01 Stockholm, Sweden and [2]Howard Hughes Medical Institute, Department of Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, TN 37232, USA

We have addressed the role of individual MHC class I subunits as well as MHC class I bound peptides in the development of NK cell specificities. Targeted disruption of genes affecting MHC class I ($H\text{-}2^b$) cell surface expression influences development of NK cells (1-4). Such mutations include TAP1, b2m and combined TAP1/b2m deficiencies. NK cells in such mice are normal in numbers, readily kill YAC-1 cells, but are tolerant towards endogenous Con A blast targets and bone marrow grafts.

Although TAP1, b2m, and TAP1/b2m mutant mice superficially appear similar, expression of class I molecules differs between the three types of mice: Mice devoid of b2m express low numbers of class I heavy chains. Mice devoid of TAP1 express low numbers of class I heavy chains complexed with b2m, either totally devoid of peptide, or occupied by peptides that are delivered to the class I molecules independent of the TAP complex. Mice devoid of both TAP1 and b2m lack serologically detectable levels of class I molecules (5,6).

Initial comparative analysis of NK cell specificities in such mice revealed that NK cells that develop in b2m mutant mice kill target cells from TAP1/b2m mutant mice. These results indicate that NK cells can be selected and acquire a specificity when selected on free class I heavy chains devoid of b2m, and that free class I heavy chains can mediate protection from NK cell mediated lysis. Furthermore, the data indicate that even very low levels of MHC class I expression may be able to shape the NK cell repertoire.

To address the role of MHC class I bound peptides, we have studied the specificity of NK cells that develop in mice harboring bm mutant class I alleles (7). Such bm mutant class I alleles are presumed to be very similar to the corresponding wild type alleles

with regard to 3D-structure while they harbor a different peptide repertoire in the antigen binding cleft. Preliminary data did not reveal any differences with respect to development of NK cell specificity imposed by a bm mutant peptide repertoire compared to that of the wild type.

Based on these and other results we will present a model encompassing the role of individual MHC class I subunits in shaping the NK cell repertoire in vivo.

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Recognition of class I MHC by the Ly-49 multigene family.

Fumio Takei, Jack Brennan, Gwendolyn Mahon, Wilfred Jefferies, and Dixie Mager
Departments of Pathology, Medical Genetics, and Immunology and Microbiology, University of British Columbia, and the Terry Fox Laboratory, B.C. Cancer Agency, Vancouver, B.C., Canada

Ly-49 is a family of type II transmembrane proteins containing carbohydrate recognition domains (CRD). One member of this family, Ly-49A, has been shown to bind certain class I MHC molecules and transduce a negative signal to NK cells. To determine the binding of class I MHC to other members of the Ly-49 multigene family, we isolated cDNA clones encoding five members of the Ly-49 multigene family and transfected them into COS cells. Flow cytometric analysis of the transfected cells with various NK cell antibodies established that three mAb, YE1/48, YE1/32, and A1 specifically recognize Ly-49A whereas Ly-49C is recognized by the mAb 5E6, which was previously reported to react with NK cell subsets responsible for hybrid resistance. Three color flow cytometric analysis of NK cells from C57BL/6 mice using NK1.1, YE1/48, and 5E6 mAb showed that $NK1.1^+$ cells can be divided into single positive, double positive, and double negative subsets. The binding of Ly-49 to class I MHC was determined by the adhesion of various cell lines with defined MHC to Ly-49-transfected COS cells. Ly-49A bound $H\text{-}2^d$ and $H\text{-}2^k$ cell lines whereas Ly-49C bound all cell lines tested except $H\text{-}2^a$ cell lines. The cell adhesion to Ly-49C-transfected COS cells was inhibited by appropriate antibodies to class I MHC or Ly-49C. The cell adhesion was also inhibited by fucoidan, λ-carrageenan, and dextran sulfate. Furthermore, the treatment of cell lines with fucosidase, but not neuraminidase, significantly reduced their adhesion to Ly-49C-transfected COS cells. These results indicate that at least two of the Ly-49 multigene family members bind different repertoires of class I MHC, and that the interaction between Ly-49 and class I MHC seems to be dependent on carbohydrates on class I MHC antigens. To further define the interaction between Ly-49 and class I MHC, the CRD of Ly-49A and C were swapped and the binding of the chimeric Ly-49 to various cell lines was determined. The results indicated that CRD is necessary but not sufficient for specific recognition of class I MHC. The precise specificities of class I MHC recognition by Ly-49C and other members of the Ly-49 multigene family as well as the domains within Ly-49 required for these interactions are currently being studied.

SECOND SESSION

Chairperson: Klas Kärre

Expression of mouse Ly-49A on the rat NK cell line RNK-16 inhibits lysis of targets expressing H-2D^d. Jay C. Ryan, Mary Nakamura, Eréne Niemi, Brian Daniels, and William E. Seaman, VA Medical Center and University of California, San Francisco, USA.

Mouse NK cells that express Ly-49A cannot effectively lyse targets that express H-2D^d. This inhibitory effect is mediated by a direct interaction of Ly-49A with H-2D^d, but the mechanisms by which Ly-49A inhibits natural killing are not known, and studies have been hampered by the lack of an NK cell line that expresses Ly-49A. We therefore transfected the rat NK cell line RNK-16 with the cDNA for Ly-49A by electroporation. Ten independent lines were derived, with varying levels of surface Ly-49A. Killing by these RNK-16.Ly-49A cells of P388D1 tumor cells (H-2^d) was inversely related to their level of expression of Ly-49A, and lysis of this target was restored in the presence of 50μg/ml F(ab')$_2$ anti-Ly-49A. Like Ly-49A$^+$ mouse NK cells, RNK-16.Ly-49A cells retained the ability to kill B-16 melanoma cells (H-2^b) and YAC-1 (H-2$^{k/d}$) targets.

In order to examine mechanisms by which Ly-49A prevents cytotoxicity by NK cells, we loaded RNK-16.Ly-49A cells with ^{3}H-myosinositol, and examined polyphosphoinositide turnover in these cells following exposure to target cells. The RNK-16.Ly-49A cells demonstrated undiminished polyphosphoinositide turnover in response to YAC-1 targets but, in contrast to wild-type RNK-16 cells, they did not generate water-soluble polyphosphoinositides in response to P388D1 cells. Further studies transmembrane signaling RNK-16.Ly-49A cells are in progress and will be discussed.

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Formation of a self-specific, tolerant repertoire of NK cells expressing Ly49 inhibitory receptors specific for class I molecules.

David H. Raulet, Werner Held and Jeffrey Dorfman, University of California, Berkeley, California, USA.

Murine NK cells express clonally distributed receptors encoded by genes in the Ly49 family, which confer specificity for allelic variants of class I MHC proteins. Engagement of these receptors by class I molecules on target cells globally inhibits NK cytolysis [1].The Ly49 family comprises approximately eight genes, which encode type II transmembrane receptors with homology to type C lectins [2]. Several Ly49 receptors with known specificity have been identified with monoclonal antibodies, including Ly49A (specific for D^d and D^k), Ly49G2 (specific for D^d and L^d) and Ly49C (specific for H-2^b and H-2^d class I molecules). Distinct but overlapping subsets of NK cells express these three receptors. We have investigated the mechanisms that generate the repertoire of Ly49 receptors. We have observed that the Ly49A and Ly49C genes undergo allelic exclusion, in that most individual NK cells in an Ly49 heterozygote express one or another but not both of the two Ly49 alleles [3]. Furthermore, we have observed that MHC genes influence the repertoire of Ly49 specificities. This is most clearly revealed in the case of cells expressing two or three Ly49 receptors simultaneously. These cells are substantially more frequent in β2-microglobulin-(class I)-deficient mice than in class I^+ mice. The frequencies of cells expressing any one receptor also appears to be slightly higher in class I-deficient mice. Furthermore, the frequencies of cells expressing any one or two self-specific Ly49 receptors was reduced significantly compared to the frequencies of these cells in mice expressing an irrelevant MHC haplotype. These results may bear on mechanisms that establish a useful, self-tolerant repertoire among NK cells. That NK cells are adapted to the individuals MHC molecules, resulting in self tolerance, was previously suggested by analysis of class I transgenic [4] and deficient [5, 6] [7] mice, in which alteration of the class I complement of the animal alters the reactivity of resident NK cells accordingly. Furthermore, recent data suggest that the NK cell subset which expresses the D^d-specific Ly49A receptor, is nevertheless rendered unresponsive to (tolerant of) H-2^b cells when the NK cells develop in an H-2^b mouse ([8], and our unpublished data). We provide evidence that these NK cells are not anergic, nor are they inhibited through the Ly49A receptor, suggesting that the cells express additional H-2^b-specific inhibitory receptors. Two models are proposed to account for the effects of MHC on the responsiveness and repertoire of NK cells. In one model, an initially stochastic repertoire of NK cell specificities is subject to selection for expression of self-MHC specific receptors, and against (albeit incompletely) cells expressing multiple self-MHC-specific receptors. In the other model, NK cell progenitors stochastically and sequentially activate Ly49 receptors, until a sufficient reaction with self-MHC class I molecules occurs, resulting in the termination of new receptor expression.

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Structure and Function of NK and T Cell Forms of NKR-P1

W.H. Chambers, C.S. Brissette-Storkus, T. Kenniston, and P.M. Appasamy
University of Pittsburgh Cancer Institute and Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213

NKR-P1s represent multigene families of Type II glycoproteins belonging to Group V of the C-type lectin superfamily, in rats and mice. Three highly homologous genes encoding NKR-P1s have been identified in mouse NK cells, and at least three members of the NKR-P1 family have been identified in rat NK cells. At present, only one human NKR-P1 homologue has been reported in NK cells. Sequence homology among the various rat and mouse NKR-P1s is around 70%, and human NKR-P1A shares approximately 50% homology with rat NKR-P1A. It has been reported that certain tumor target cells express multiple oligosaccharide moieties which are bound by NKR-P1 and result in activation of NK cell lytic function. Much of the available data suggests that NKR-P1 is involved in providing positive signals for activation of NK lytic function for some targets, but it is clear that it is not absolutely essential for lysis of targets as NKR-P1$^-$ clones of rat NK cells efficiently mediate lysis of most susceptible targets. While NKR-P1s have predominantly been characterized from NK cells, they are also expressed on small subsets of T cells (i.e. NKR-P1^{dim}/TCR$\alpha\beta^+$ and NKR-P1^{dim}TCR$\gamma\delta^+$ in rats), in particular, those with non-MHC restricted cytolytic activity in rats and mice. As NKR-P1(s) from the T cell subsets have not been characterized, we performed a variety of biochemical and molecular analyses on NKR-P1 expressed on NKR-P1^{dim}/TCR$\alpha\beta^+$ rat T cells. ECL-Western blot analyses indicated that anti-NKR-P1 recognized a single molecular species of ~60 kD from NK cells and NKR-P1^{dim}/TCR$\alpha\beta^+$ cells. Northern blot analyses using RNA from highly purified populations of NK cells and NKR-P1^{dim}/TCR$\alpha\beta^+$ cells suggested that both populations expressed multiple forms of message encoding NKR-P1, with a predominant form at ~1.0 kB. Using various sets of primers corresponding to conserved regions among the known mouse and rat NK cell forms of NKR-P1s, we generated PCR products which were then cloned and sequenced. At least one unique form of NKR-P1 was identified using RNA from the NKR-P1^{dim}/TCR$\alpha\beta^+$ subset of T cells. Interestingly, whereas most defined murine forms of NKR-P1s have a high level homology (85-90%) in the carbohydrate recognition domain (CRD), this novel form has only about 50% homolgous in the CRD to the other members of this family.

THE HARNESSING OF EFFECTOR FUNCTIONS OF NK CELLS THROUGH CARBOHYDRATE-PROTEIN INTERACTIONS

Ten Feizi, The MRC Glycosciences Laboratory, Northwick Park Hospital, Harrow, UK.

A major aim of our current research is to elucidate specificities of oligosaccharide-protein interactions in inflammation and host defence, and thus to discover ways of boosting or inhibiting these interactions as part of prophylactic and therapeutic measures in infections, inflammatory disorders and malignant disease. This is an extension of earlier work with monoclonal antibodies which revealed programmed changes in the display of oligosaccharides at the surface of haematogenous and other cell types during development and differentiation, and often predictable changes in malignant transformation. Our thesis has been that such carbohydrate differentiation antigens may have important roles as ligands in macromolecular interactions which determine the way cells migrate or respond to various microenvironments (1-3). This concept has received much support from developments in vascular biology with the discoveries that cell adhesion molecules (the selectins) which have crucial roles at the initial stages of leucocyte recruitment and extravasation in inflammation are calcium-dependent (C-type) lectins that act by binding to carbohydrate differentiation antigens of leucocytes (4,5).

To facilitate the detection and chemical characterization of saccharide ligands, we introduced the 'neoglycolipid technology', a micromethod analogous to the 'blotting' methods for nucleic acids and proteins, and applicable to oligosaccharides after their release from proteins (6). Neoglycolipids are generated by conjugation of oligosaccharides to an aminophospholipid by microscale reductive amination procedures. The versatility is in that the technology is applicable to mixtures of oligosaccharides released from proteins, and to desired structurally defined or chemically synthesized oligosaccharides. Moreover, the technology incorporates a microsequencing strategy as the neoglycolipids have unique ionization properties in mass spectrometry. This approach has proven to be a powerful means of singling out and elucidating structures of ligands for carbohydrate-binding proteins and evaluating the influence of carrier proteins and lipids in presentation and recognition of saccharide ligands (5).

A novel application of neoglycolipids has been in elucidating an important mechanism in the effector function of NK cells (7). Working with the rat NK system, we have shown that NK-sensitive tumour cell-lines bear specific oligosaccharides on their surface which serve as ligands that are bound strongly by the C-type lectin, NKR-P1, on NK cells (8,9) thereby triggering their complete cytotoxic cascade (7). The critical elements of these ligands consist only of one or two monosaccharides with a negative charge, making it relatively easy to synthesize drugs based on their structure. Indeed, we have shown that if such small oligosaccharides, in the form of neoglycolipids on liposomes, are embedded as clusters in the surface of NK-resistant tumour cells, these cells can be rendered NK-sensitive (7). This new principle is potentially a powerful means of harnessing NK cells for the purging of undesirable cells such cancer cells and those infected with viruses, *in vitro* and (in conjunction with targeting reagents) *in vivo.*

NK cell functions can also be negatively modulated by the oligosaccharide ligands of NKR-P1: we have observed inhibition of killing in the presence of low density ligands on liposomes, and in the presence of the soluble, monomeric ligands. Thus, there are potential novel therapeutic applications also for the damping down of overactive NK cells in disorders of immunity.

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"ALLOREACTIVE NK CELLS IN THE RAT. COMPLEX GENETICS OF MHC CONTROL."

Bent Rolstad, Christian Naper, Guro Løvik, *Kurt Wonigeit, and John Torgils Vaage,
Department of Anatomy, Institute of Basic Medical Sciences, University of Oslo, Norway and *Klinik für Abdominal und Transplantationschirurgie, Medizinische Hochschule, Hannover, Germany.

Allogeneic Lymphocyte Cytotoxicity (ALC) is the rapid rejection, within 24h, of lymphocyte allografts taking place in nonsensitized rats [1]. Salient features of this rejection are: 1) it is independent of T cells or antibodies [1,2] 2) perturbation of the NK system profoundly affects the rejection [2,3], 3) it shows immunogenetic specificity to MHC-controlled alloantigens [4] 4) like the mouse Hh system the MHC affects target susceptibility in a complex manner, but unlike it parental homozygous parental rats sometimes reject F1 lymphocytes demonstrating nonrecessive inheritance of target structures. [1,5] 5) Purified unstimulated or IL-2 activated NK cells (CD3-, NKR-P1+) exhibit the same patterns of lysis of allogeneic lymphocytes or Con A blasts in vitro as found in ALC, further indicating that NK cells are the main effector cells of ALC [5,6].

We have investigated the role of MHC and its subregions in controlling target susceptiblity, using this in vitro assay for NK alloreactivity and a panel of MHC congenic and intra MHC recombinant rats. The rat MHC (RT1) shows several similarities with mouse H-2 in that a central class II/III region is flanked on each side by class I genes. Unlike H-2 most, if not all the class I genes restricting T cells to environmental and minor histocompatilbility antigens is located within the RT1.A cluster on the left hand side of RT1 homologous to H-2K [7,8]. A functional homologue of H-2D is lacking in the rat, but instead, in the right flank of RT1 (RT1.C region) 60-70 class I genes have been identified, some of which are polymorphic and expressed at the cell surface in low concentration [9,10]. Their funcion is unknown, and they have therefore been regarded as nonclassical class I antigens.

With a panel of MHC congenic and intra-MHC recombinant rat strains derived from five different RT1 haplotypes, we have shown that:

a) "self" MHC exert a strong influence on the ability of NK cells to lyse MHC incompatible cells from several rat strains, i.e. on the NK allorecognition repertoire [11]

b) polyclonal NK cells from a certain rat strain (PVG) recognize and discriminate between four different allogeneic MHC haplotypes [6]

c) a new function has been assigned to the RT1.C region in controlling several of the target MHC specificities to alloreactive NK cells [6]

d) NK cells specifically can recognize single MHC mismatches within the RT1.C region only, and that deletion of a class I encoding subregion within the RT1.C (Im1 mutant rat strain) leads to loss of an NK allospecificity [6].

Collectively these data are most easily interpreted according to a model at variance with the missing self hypothesis for NK allorecognition. We have previously suggested that some rat NK cells have a recognition system for allogeneic class I molecules with a limited number of specificities and with the mode recognition: kill [10]. By comparison with NK allorecognition systems in other species we assume that class I antigens are also central in rat NK cell recognition of normal allogeneic lymphocytes. The simplest interpretation of our system would therefore be that some rat NK cells positively recognize certain foreign MHC class I molecules and kill target cells expressing them.

More recent experiments have complicated this issue, since in a wider panel of RT1 recombinants and in F1 hybrids between them we now show that:

a) depending on the MHC haplotype combination of effector and target cells both nonrecessive and recessive inheritance patterns of target susceptibility can be seen, and also F1-hybrid-anti-parental responses [11]

b) in a certain combination of RT1 haplotypes target susceptibility to alloreactive NK cells maps to the classical RT1.A rather than the nonclassical RT1.C region.
c) the expression of this particular RT1.A controlled determinant is dependent on the allele combination between the A and B/D-C region, since in other recombinant rats with the same allele in the RT1.A region but different ones in the B/D-C region target susceptibility again maps to the B/D-C region.
d) Specificity studies indicate that this was due to a gene interaction between the two class I regions RT1.A and C.

Collectively our data indicate:
1) that the NK recognition response to autologous and allogeneic MCH molecules may be different; i.e. while "self" MHC may be inhibitory, at least some allogeneic MHC molecules may trigger cytotoxicity.
2) that a new function has been assigned to the nonclassical class I (RT1.C) region in controlling expression of NK target structures
3) that the rat NK target structure on normal allogeneic lymphoblasts is determined by the MHC at more than one level, and may also be dependent on interactions between different subregions of the MHC.
By inference with the role of peptides selected for presentation by class I in determining NK target determinant in other species, we hypothesize that the NK target structure recognized by rat alloreactive NK cells can be determined by the MHC at two levels; by the presence of the polymorphic class I molecules themselves and by the selection of peptides available for presentation by class I.
A receptor-ligand interaction in this system has so far awaited identification, but recently we have identified an NK surface molecule defining a subset associated with most of the alloreactivity and whose expression is controlled by the haplotype within the RT1.C region. Data on this issue will be presented.

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THE INVOLVEMENT OF ADHESION-, ACTIVATION-, MHC CLASS I-, AND CD45-MOLECULES IN RAT NK CELL-MEDIATED LYSIS.

K.M. Giezeman-Smits[1], A.M.M. Eggermont[2], G.J. Fleuren[1], P.J.K. Kuppen[3].
[1]Department of Pathology, University of Leiden; [2]Department of Surgery, University Hospital Rotterdam-Daniel den Hoed Cancer Center, Rotterdam; [3]Department of Surgery, University of Leiden; The Netherlands.

The precise mechanism by which natural killer (NK) cells lyse target cells is not fully understood. Using the monoclonal antibodies (mAb) WT.3 (anti-CD18), OX18 (anti-MHC class I), 3.2.3. (anti-NKR-P1) and the by us newly developed mAb ANK5, ANK61, and ANK74 we investigated in a syngeneic rat model the involvement of several different types of structures on both, NK cells and target cells in NK cell-mediated lysis. NK activity was measured with ^{51}Cr release assays. MAb WT.3 (anti-CD18) inhibited completely the NK cell-mediated lysis of various tumor cell lines, indicating that the CD18-integrins are essential for target cell binding. Preincubation of tumor cells and normal cells as target cells with OX18 (anti-MHC class I) strongly enhanced the NK cell-mediated lysis. The lysis of OX18-preincubated target cells was again completely blocked by WT.3. We concluded from these data that the CD18-integrins are a prerequisite for lysis by NK cells and that masking of MHC class I on target cells by the anti-MHC class I antibody OX18 is sufficient for NK cells to recognize them as non-self, resulting in lysis. The ability of ANK5, ANK61 and 3.2.3 (as positive control) to induce redirected killing of the Fcγ receptor (FcγR)-positive tumor cell line P815 was analyzed. The FcγR-negative tumor cell line YAC-1 served as negative control. The lysis level of P815 by NK cells was significantly enhanced in the presence of the mAb ANK5 and ANK61. None of the mAb had any effect on the lysis of YAC-1 by NK cells. These results show that the mAb ANK5 and ANK61 bind to activation structures on NK cells which trigger NK cells to target cell lysis. Additionally, we showed that the activation of NK cell-mediated lysis by mAb ANK5 and ANK61 was inhibited by mAb WT.3, demonstrating that activation is dependent on binding via adhesion structures. The effect of mAb ANK74 on NK cell-mediated lysis was tested using MHC class I-negative tumor cells and MHC class I-positive tumor cells preincubated with and without OX18. The MHC class I-negative tumor cells were lysed at high levels and this lysis level was not affected by mAb ANK74. The MHC class I-positive tumor cells were lysed at low levels, but were lysed at high levels after OX18-preincubation. MAb ANK74 inhibited the OX18-mediated enhanced lysis of target cells completely. Immunoprecipitation studies showed that mAb ANK74 recognized the leucocyte common antigen, CD45. These data indicate that CD45 is involved in MHC-associated target cell recognition and perhaps provides the inhibitory signals involved in NK cell-mediated lysis. In conclusion, we hypothesize, based on our observations, that NK cell-mediated lysis in rats depends on: CD18-associated integrins, which provide binding between NK cells and target cells; activation structures, which are capable of activating the lytic machinery (e.g. the 3.2.3-, ANK5-, ANK61-antigens); and inhibition structures (a putative MHC class I-receptor, possibly in combination with CD45) which bind to 'self' MHC class I on target cells, resulting in downregulation of the lytic machinery.

THIRD SESSION

Chairperson: Alessandro Moretta

RECEPTORS FOR HLA CLASS-I MOLECULES IN HUMAN NATURAL KILLER CELLS

Lorenzo Moretta*^, Roberto Biassoni*, Massimo Vitale*, Cristina Bottino*, Maria Cristina Mingari*°, and Alessandro Moretta+•.

*Istituto Scientifico Tumori e Centro Biotecnologie Avanzate, Viale Benedetto XV N.10, 16132 Genova; ^Istituto di Patologia Generale, Università di Genova; °Istituto di Oncologia Clinica e Sperimentale, Univ. di Genova; +Istituto di Istologia ed Embriologia Generale, Univ. di Genova; •Dipartimento di Scienze Biomediche e Biotecnologie, Università di Brescia

NK cells have been tought to represent a functionally homogeneous cell population responsible for "non specific" functions. However, more recent data have provided clear evidence that NK cells express clonally-distributed receptors for HLA-class I molecules. Interaction between these receptors and their ligands expressed on target cells prevents target cell lysis (inhibitory receptors). Originally, two homologous receptors, each recognizing a given group of HLA-C alleles (sharing aminoacid positions 77 and 80 in the peptide binding groove) have been identified by the EB6 and GL183 mAbs. Genes coding for p58 molecules have recently been cloned. Different from murine Ly49 (shown to represent the class I-specific murine NK receptor), p58 are type-I transmembrane proteins containing 2 Ig-like domains in their extracytoplasmic portion. Studies are in progress to characterize additional members of the p58-related gene family for their ligand/specificity and function. Some NK clones express additional receptors specific for two groups of HLA-B alleles characterized by the Bw4 or the Bw6 supertypic specificity. Antibodies to these receptors revealed two different molecules represented by NKB1-p70 and CD94 molecules, respectively. Although only one type of class I-specific inhibitory receptor may be expressed by single NK cells, in some instances, different receptors can be coexpressed at the single cell level. Importantly, in addition to the typical inhibitory receptors expressed by all individuals, activatory receptors displaying the same antibody specificity, but differences on their molecular mass, have been identified in some individuals. Thus, MHC class I recognition may result not only in NK cell inhibition but also in activation. Remarkably, the expression of activatory receptors is always accompanied by the coexpression of inhibitory receptors characterized by different class I allele specificity. HLA class-I-specific receptors have also been detected in a small subset of T cells displaying NK-like activity. Importantly, they can regulate the TCR-mediated T cell activation. The molecular mechanisms involved in this regulation are being investigated.

Recognition of MHC class I by human NK and T cells

Lewis L. Lanier, DNAX Research Institute of Molecular and Cellular Biology, 901 California Avenue, Palo Alto, CA 94304 U.S.A.

Human NK cell clones kill the HLA class I-deficient Epstein Barr virus-transformed human B lymphoblastoid cell lines 721.221 and C1R. Transfection with certain alleles of HLA-A, -B, or -C can protect 721.221 or C1R from lysis. In collaboration with Peter Parham's laboratory (Stanford University), we have demonstrated that an individual NK cell clone can recognize multiple alleles of HLA-A,-B, and -C (1). Analysis of an extensive panel of NK clones generated from a single individual revealed a complex pattern of HLA recognition. While the NK clones were able to recognize certain HLA-C molecules, the same NK clones also recognized HLA-A and HLA-B gene products (1). Recently, we have cloned cDNA encoding a receptor, designated NKB1, responsible to recognition of HLA-B molecules bearing the Bw4 serological determinant (2-4). The NKB1 receptor is expressed on T lymphocytes, as well as NK cells, and regulates TcR-dependent activation (5). In this presentation, we will review our studies of HLA class I molecule recognition by NK and T cell clones. Results from our studies suggest that NK and T cell cytolytic function represents a balance between stimulatory and inhibitory signals.

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Threonine 80 on HLA-B27 confers protection to lysis by NK clones of NK4 specificity.

José Peña*, Isabel Luque*, M. Dolores Galiani*, Rafael González*, Fernando García#, José A. López de Castro# and Rafael Solana*.

**Departamento de Inmunología. Facultad de Medicina. Hospital Reina Sofía. Universidad de Córdoba. 14004 Córdoba. (Spain); and #Centro de Biología Molecular "Severo Ochoa". Consejo Superior de Investigaciones Científicas. Universidad Autónoma de Madrid. Cantoblanco. 28049 Madrid. (Spain).*

NK cells are able to lyse hematopoietic target cells lacking expression of MHC class I molecules on their surface, while the presence of these molecules provides protection from NK lysis (1-2). MHC-dependent inhibition of NK lysis is related to the structural polymorphism of the HLA Class I molecule, although its molecular basis remains unclear. The use of NK clones has defined three NK specificities (NK1, NK2 and NK3) according to their capacity to recognize Class I polymorphic motifs (3-4). This effect is mediated by NK inhibitory receptors, some of which have been cloned and identified (5-6).

In an attempt to further analyze how NK cells recognize HLA-B27, we have studied whether the inhibitory effect induced by HLA-B*2705 on some NK clones is extensive to other HLA-B27 subtypes. To analyze this, we selected clones that were specifically inhibited by HLA-B*2705 to test their cytotoxicity towards C1R transfected with the B*2701 through B*2706 subtypes. C1R transfectants express equivalent surface amounts of HLA-B27, as demonstrated by labeling with anti HLA class I and anti HLA-B27 mAbs. The results indicate that whereas B*2701, B*2703, B*2704 and B*2706 also induced strong protection to lysis by these particular NK clones, HLA-B*2702 was unable to induce protection. From this we can conclude that whereas B*2701, B*2703, B*2704 and B*2706 induced strong protection to lysis by these particular NK clones, equivalent to the protection induced by B*2705, the B*2702 subtype was unable to induce protection.

A feature which differentiates B*2702 from all other subtypes, is the presence of Ile at position 80 in B*2702, whereas the remaining HLA-B27 subtypes analyzed have Thr in this position (8). Thus our results suggested that Thr80 of HLA-B27 is of particular relevance in the induction of resistance to lysis by these NK clones,termed NK4 specificities.

To confirm the role of Thr80 in HLA-B27 recognition by NK cells, C1R cells transfected with the B*2705 gene specifically mutated in positions 74, 80 and 81 to mimic the changes in B*2702 at single positions, were used as target cells. The results show that the protection conferred by B*2705 to lysis by these NK clones was reverted when Thr80 was changed to Ile80, whereas mutations at positions 74 (D->Y) or 81 (R->A) did not affect B*2705 induced protection to lysis. In addition, C1R transfectants expressing the double mutant N77I80 were lysed less efficiently than untransfected C1R or I80 transfectants by two of these clones, suggesting that the N77

change modulates the effect of residue 80 on lysis by these NK clones. The fact that residue 80 is involved in the structure of pocket E and F of the peptide binding groove (9), suggests that the peptides bound to MHC molecules could participate in the specificity of NK recognition. Although other results analyzing the effect of single mutations in NK recognition (10-11) and in particular the results of Malnati et al.(12),further support this possibility, this issue still remains controversial.

We have also studied the expression of the NKAT gene family on the group of NK4 clones. The results show that these clones specifically express NKTA4 but not NKAT3, NKAT1 or NKAT2 genes, suggesting that the molecule encoded by NKAT4 is the NK inhibitory receptor responsible to confer the NK4 specificity to these clones.

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INHIBITORY AND ACTIVATORY NK RECEPTORS FOR HLA-CLASS I MOLECULES

A. Moretta*^, S. Sivori^ and M. Vitale^ and C. Bottino^.
*Departement of Biomedical Sciences and Biotechnology, University of Brescia; ^ I.S.T. and C.B.A., Genova, ITALY.

Human NK clones specifically recognize HLA class I alleles via clonally distributed receptors. These receptors were first identified by mAbs GL183 and EB6 as a 58 kD molecular family specifically directed to distinct groups of HLA.C alleles.
More recently the existence of distinct receptors for HLA.B alleles has been defined. Each of the receptor molecules for HLA.C or HLA.B alleles may be coexpressed at the clonal level with one or another of the other receptors and with different sets of activatory molecules.
Thus the function of individual NK clones would be the result of the cocktail of inhibitory (HLA-specific) and activatory (HLA-non specific?) receptors. In all istances however the balance between activatory and inhibitory receptors is characterized by the downregulation of the activatory signals by the inhibitory ones.
We will present data on different groups of activatory and inhibitory receptors that cooperate in the shaping of the cytolitic function of different alloreactive and non-alloreactive groups of NK clones.

Modulation of Activation-Associated Signal Transduction Pathways in NK Cells by HLA Class I Recognition. Nicholas M. Valiante*, Jennifer Gumperz*, Annalisa D'Andrea#, Joseph H. Phillips#, Lewis L. Lanier# and Peter R. Parham*. *Department Of Structural Biology, Stanford University, Stanford, CA 94305, U.S.A.; #Department of Human Immunology, DNAX Research Institute, Palo Alto, CA. 94306, U.S.A.

Expression of major histocompatibility complex (MHC) class I molecules protects potential targets from cytolysis by natural killer (NK) effector cells. Protection results from specific interactions between class I allotypes possessing particular polymorphic determinants and clonally distributed receptors of NK cells. It has been proposed that recognition of MHC class I allotypes by NK cells inhibits activation of cytolysis through the delivery of a negative signal to the NK effector cell. Experimental evidence, although limited, is consistent with this hypothesis. To understand further the mechanisms that govern the class I induced non-responsiveness of NK cells , we are comparing pathways of signal transduction in NK cell clones interacting with target cells distinguished in their sensitivity to lysis due to difference in class I allotype or level of expression. Using the class I deficient B cell line 721.221, and a panel of derivative transfectants expressing individual HLA class I alleles as target cells, we have investigated the transmission and propagation of intracellular signals in $CD56^+$ human NK cell clones following their interaction with targets that are either maximally sensitive or maximally resistant to lysis. Following recognition of class I, we find alterations in the early activation of signaling pathways dependent upon protein tyrosine kinases (PTK). The particular pathways affected and the potential mechanisms responsible for inhibiting NK cell activation are under continued investigation and will be discussed further.

Recognition of MHC class I B2705 peptide-induced conformation by B*2705 specific NK clones

Mauro S. Malnati+, Marta Peruzzi§, Kenneth C. Parker*, John E. Coligan* and Eric O. Long§
+ Laboratory of Human Virology, HSR San Raffaele, Milan Italy
§ Laboratory of Immunogenetics, NIH-NIAID, Rockville USA
* Laboratory of Molecular Structure, NIH-NIAID, Rockville USA

Fully assembled class I B*2705 molecules reconstituted by exogenous addition of a B27 specific peptide (FRYNGLIHR) in TAP deficient cells block their lysis by a particular subgroup of B27 specific NK clones ($CD3^-$ $CD56^+$ $CD16^+$).
We have assessed the role of each aminoacid in the protective peptide by the use of single alanine substitutions. In addition we have further characterized the specificity of this particular subgroup of NK clones by testing 14 B*2705-natural peptides.
The results obtained clearly demonstrate that NK cells recognize peptide-induced conformational change rather than directly recognize a specific peptide-MHC complex. Recognition is indeed specific since that only a particular class of B*2705 peptides with common features confer the appropriate protective conformation. The lenght of the protective peptides does not appear to be critical.

SINGLE AMINOACID SUBSTITUTIONS CAN INFLUENCE THE NK-MEDIATED RECOGNITION OF HLA-C MOLECULES. ROLE OF SERINE-77 AND LYSINE-80 IN THE TARGET CELL PROTECTION FROM LYSIS MEDIATED BY "GROUP 2" OR "GROUP 1" NK CLONES

R. Biassoni*, M. Falco*, A. Cambiaggi*, P. Costa*, S. Verdiani*, D. Pende*, R. Conte*, C. Di Donato*, P. Parham° and L. Moretta*^. *IST, CBA, Genova, Italy; ^Ist. Pat. Gen., Univ. Genova, Italy; ° Depts. Struct. Biol. and Microb. & Immunol., Stanford Univ, Sch Med Stanford, CA, U.S.A.

NK cells have been shown to express a clonally distributed ability to recognize HLA class I alleles. The previously defined NK clones belonging to "group 1" recognize HLA-Cw*0401 (Cw4) and other HLA-C alleles sharing Asn at position 77 and Lys at position 80. Conversely, the "group 2" NK clones recognize HLA-Cw*0302 (Cw3) and other HLA-C alleles characterized by Ser at position 77 and Asn at position 80. We assessed directly the involvement of these two residues in the capacity of NK cell clones to discriminate between the two groups of HLA-C alleles. To this end, Cw3 and Cw4 alleles were subjected to site-directed mutagenesis. Substitution of the aminoacids typical of the Cw3 allele (Ser-77 and Asn-80) with those present in Cw4 (Asn-77 and Lys-80), resulted in a Cw3 mutant which was no longer recognized by "group 2" NK cell clones but was specifically recognized by "group 1" clones. Analysis of Cw3 or Cw4 molecules containing single amino acid substitutions indicates roles for Lys-80 in recognition mediated by "group 1" clones and for Ser-77 in recognition mediated by "group 2" clones. These results demonstrate that NK-mediated specific recognition of HLA-C allotypes is affected by single natural amino acid substitutions at position 77 and 80 of the heavy chain.

FOURTH SESSION

Chairperson: Lewis L. Lanier

KILLER CELL INHIBITORY RECEPTORS SPECIFIC FOR HLA-C AND HLA-B IDENTIFIED BY DIRECT BINDING AND BY FUNCTIONAL TRANSFER

Eric O. Long, Nicolai Wagtmann, Sumati Rajagopalan, Christine C. Winter and Marta Peruzzi
Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 12441 Parklawn Drive, Rockville, Maryland 20852, USA

An inhibitory signal is delivered to Natural Killer (NK) cells (1) and a subset of cytotoxic T cells (2-4) upon recognition of HLA class I molecules on target cells (5-7). We demonstrate that distinct killer cell inhibitory receptors (KIR) bind directly and specifically to HLA-C alleles. Furthermore, transfer of individual KIR into NK clones reconstituted target cell recognition, leading to inhibition of lysis. Using such functional reconstitution, a related KIR that confers specificity for some HLA-B alleles was also identified. These KIR share conserved tyrosine phosphorylation motifs in their cytoplasmic tails (8). Thus, a single receptor in NK cells provides both specificity for HLA class I on target cells and the inhibitory signal that prevents lysis (9).

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NK Associated Transcripts Encode Ig-SF Molec ıles Involved in HLA-C and HLA-B Recognition b y Human Natural Killer Cells

Marco Colonna & Jacqueline Samaridis

Basel Institute for Immunology, Basel CH-4005, Switzerla ıd.

Cytotoxicity by Natural Killer (NK) cells is inhibited by l Iajor Histocompatibility Complex (MHC) class I molecules on target ells. This inhibition is mediated by clonally distributed NK rece] tors, with different MHC specificities. Recently, it has been descrit :d a new family of cDNAs, designated NKATs (NK assoc ated transcripts), which are selectively expressed on NK cells. 'hey encode closely related transmembrane proteins, characterize(by an extracellular region with two or three Ig-superfamily dom ins, and by a cytoplasmic domain with an unusual immune rec(ptor tyrosine based activation motif (ITAM). The distribution of ı ese cDNAs is clonotypic and the expression of certain NKATs corre ates precisely with NK cell inhibition by particular class I all les. Transfection of one cDNA determines cell surface expression of the p58 molecule identified by mAb HP-3E4, which has l een previously proposed as a receptor for HLA-C. Thus, NKAT cD NAs encode receptors for class I molecules on NK cells.

THE Ig-RELATED KILLER CELL INHIBITORY RECEPTOR BINDS ZINC AND REQUIRES ZINC FOR RECOGNITION OF HLA-C ON TARGET CELLS

Sumati Rajagopalan, Christine C. Winter, Nicolai Wagtmann, and
Eric O. Long
Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, 12441 Parklawn Drive, Rockville, Maryland 20852, USA

Members of the Ig superfamily are predominantly receptors that mediate interactions between cells, or provide signals to cells when binding specific ligands. Here we describe an Ig-related receptor that requires zinc for its function. Killer cell inhibitory receptors (KIR) belonging to the Ig superfamily mediate inhibition of NK cells upon recognition of HLA-C molecules on target cells (1). The unusually high abundance of histidine residues in the first extracellular domain of KIR suggested that this receptor may bind zinc. Two distinct KIR molecules that mediate recognition of HLA-Cw4 and -Cw8, respectively, bound specifically to zinc affinity columns. Furthermore, addition of the zinc chelator 1,10-phenanthroline during chromium release assays reversed the inhibition of killing mediated by NK clones specific for HLA-Cw4 or HLA-Cw8, demonstrating that zinc is necessary for the inhibitory function of KIR (2). Such functionally relevant zinc binding has not been described for other members of the Ig superfamily and may represent a novel regulatory mechanism for antigen receptor-ligand interactions.

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Relationship between non-MHC-restricted T cells and allogeneic NK cells: expression of HLA-C molecules confers target cell resistance to lysis by both effector populations.

Christine S. Falk, Alexander Steinle, Barbara Maget and Dolores J. Schendel. Institute of Immunology, University Munich, Goethestr. 31, D-80336 Munich, Germany.

An involvement of HLA class I molecules has recently been demonstrated for the regulation of lysis by NK cells induced through allostimulation. In contrast to cytotoxic T cells (CTL), these allogeneic NK cells could be inhibited by particular HLA-B or C molecules expressed on the surface of the target cells. In some cases the specificity of inhibition can be correlated with members of a family of receptors expressed on some subpopulations of NK cells. Depending on the expression of receptor variants lysis by $CD3^-$ NK cells could be inhibited by the presence of particular HLA-B or -C molecules on the target cells. We have found that HLA-C mediated inhibition of lysis is not limited to $CD3^-$ NK cells: $CD3^+/CD56^-$ non-MHC-restricted T cells characterized by their lysis of class I deficient target cells could be inhibited by a cluster of HLA-C alleles (Cw1, Cw3 and Cw7), thus exhibiting an NK2-like specificity. Starting with an allogeneic mixed lymphocyte culture (MLC) of mixed phenotype ($CD4^+$,$CD8^+$ and $CD56^+$ cells), purification of subpopulations of $CD3^+/CD56^-$ non-MHC-restricted T cells and $CD3^-/CD56^+$ NK cells resulted in lines showing identical lysis patterns, related to the NK2 specificity: Resistance to lysis was found when target cells expressed Cw1, Cw3 or Cw7, using both a panel of B-LCL and class I transfectants. In contrast, susceptible cells were either deficient in class I expression or expressed Cw2, Cw4 or Cw6 alleles. Blocking of the HLA-C molecules on the target cells with class I specific mAb restored lysis by both NK and by non-MHC-restricted T cells. To investigate the influence of peptides in this HLA-class I mediated inhibition we tested HLA-B and -C transfectants of the TAP deletion mutant T2 for resistance or susceptibility to lysis by these non-MHC-restricted T cells and NK cells. The results suggested that peptides might play only a minor role since the TAP-deficient HLA-Cw7 transfectant could inhibit lysis by both populations. In summary, T cells and HLA molecules show a bidirectional relationship: classical $CD8^+$ CTL are specifically restricted in their recognition of peptides presented by MHC class I molecules leading to lysis of a target cell; in contrast, non-MHC-restricted T cells are able to lyse a variety of target cells even when deficient for class I but they can be inhibited by defined HLA-C molecules; the pattern of inhibition seems to be related to the NK-specificity of allogeneic NK cells.

Paul Fisch

Recognition by human NK cell clones and γ/δ T cell clones is differentially controlled by HLA class I antigens expressed with human β2m as compared to murine β2m.

Human Vγ9/Vδ2 T cell clones recognize Daudi cells in a TCR dependent fashion. Daudi cells lack expression of MHC class I antigens due to lack of β2-microglobulin (β2m). However, recognition of Daudi by human Vγ9/Vδ2 T cell clones is not caused by absent HLA class I since *(a)* transfection of β2m into Daudi cells does not reduce the proliferative response and lymphokine secretion of human γ/δ T cell clones to Daudi (Fisch et al. Science 250:1269,1990 and unpublished results) and *(b)* three malignant hematopoietic cell lines other than Daudi expressing HLA class I can be recognized by the Vγ9/Vδ2 encoded TCR (Fisch et al. unpublished results). The molecular mechanisms responsible for recognition of these cell lines by human γ/δ T cells remain unclear, but the data suggest that similar to NK cells, γ/δ T cells could play a role in the immunosurveillance against tumors, possibly by recognizing antigens other than those recognized by conventional α/β T cells or NK cells.

Since human Vγ9/Vδ2 T cell clones display high level of lysis of Daudi cells, we examined, how expression of HLA class I antigens on Daudi cells modulates cytotoxicity by human Vγ9/Vδ2 T cell clones and NK cell clones. Expression of HLA class I upon transfection of Daudi with murine β2m (m-β2m) markedly reduced lysis by all 20 γ/δ T cell clones and 12 NK cell clones studied. In contrast, expression of HLA class I following transfection of Daudi with human β2m (h-β2m) had a differential effect on the recognition by NK and Vγ9/Vδ2 T cell clones. All 12 NK clones and 14 out of 20 Vγ9/Vδ2 T cell clones showed reduced lysis of h-β2m transfected Daudi cells as compared to the parental cell line. In contrast, the other 6 Vγ9/Vδ2 T cell clones displayed unchanged or even increased "recognition" of h-β2m Daudi cells, as compared to the parental HLA deficient cell line (although the same clones showed reduced lysis of m-β2m transfected Daudi cells). All Daudi cell lines, the parental HLA class I deficient cells, as well as the m-β2m and h-β2m Daudi cells induced proliferation and GM-CSF secretion by CD4+ Vγ9/Vδ2 T cell clones at equal levels, suggesting that there was no general difference in the recognition of the "Daudi ligand" via the Vγ9/Vδ2 TCR. Reactivity with mAb W6/32 confirmed that the HLA class I molecules on the surface of the HLA class I+ Daudi cells were associated with h-β2m, even for the m-β2m Daudi variant, since soluble h-β2m derived from human serum exchanges with m-β2m at the cell surface, if the m-β2m Daudi cells are grown in human serum (the W6/32 mAb does not recognize m-β2m associated HLA class I molecules; i.e. if the m-β2m Daudi cells are grown under serum free conditions).

The most likely explanation for these results is that human γ/δ T cells express receptors for HLA class I antigens that can modify TCR dependent recognition of the "Daudi ligand". These receptors may to be influenced by HLA class I bound peptides that are transported to the cell surface upon association of the peptides with the HLA class I α-chains and β2m in the endoplasmatic reticulum (ER). Since m-β2m changes the peptide affinity and perhaps peptide specificity of HLA class I molecules, as compared to h-β2m, this may explain, why a subset of clones differentially recognizes HLA class I molecules that have travelled to the cell surface in association with h-β2m, as compared to m-β2m.

FIFTH SESSION

Chairperson: Paul J. Leibson

Reagents for the Study of the NKG2 Family of Lectin-like Receptors.

Jeffrey P. Houchins§, Joseph H. Phillips†, and Lewis Lanier†, §Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN 55455 USA and †Department of Human Immunology, DNAX Research Institute, Palo Alto, CA 94304 USA

We have been attempting to develop reagents for the study of the NKG2 family of transmembrane lectin-like molecules that are expressed preferentially on human NK cells. Expression constructs were prepared that direct the synthesis of recombinant NKG2-A and NKG2-D proteins in bacteria or COS cells. The purified recombinant proteins were used to immunize mice.

One mouse monoclonal antibody was obtained that reacts only with NKG2-A, and a second antibody also cross-reacts with the closely related molecules NKG2-C and NKG2-E. On western blots prepared from NK cells, these antibodies detect a diffuse band that runs at approximately 45 kDa under reducing conditions and about 90 kDa in non-reducing conditions. Both antibodies specifically recognize recombinant NKG2-A proteins in ELISA, and both stain the surface of COS that has been transfected with NKG2-A. Although NKG2-A expression in NK cells has been detected by the staining of western blots, the antibodies fail to stain the surface of NK cells. Western blots prepared from cloned NK cells reveal that NKG2-A is clonally expressed. Efforts are continuing to correlate functional activity with expression of this molecule.

Antibodies have also been prepared that recognize recombinant NKG2-D proteins on ELISA and western blots. These antibodies also stain the surface of NKG2-D-transfected COS. However, no endogenous expression of NKG2-D has been detected in NK cells.

Recombinant lectin domains secreted by transfected COS have been used in attempts to identify cells that express target ligands for NKG2-A and D. The proteins were coated onto several types of beads in order to generate a multivalent reagent for use in cell binding assays. EBV-transformed B cells expressing numerous different MHC haplotypes were examined, but no binding was observed.

CHARACTERIZATION AND TARGET CELL BINDING OF THE NKG2-C RECEPTOR

Erhard Hofer, Markus Düchler and Fritz H. Bach

Laboratory of Transplantation Immunology at VIRCC, Department of Dermatology, University of Vienna, Brunnerstrasse 59, A-1230 Vienna, Austria

NKG2-C is a member of the NKG2 family of genes and proteins, which are preferentially expressed on human NK cells. These potential NK cell receptors belong to a superfamily of type II integral membrane proteins with a C-type lectin domain (1,2). Among the NK cell-related members of the superfamily are the rodent NKR-P1 and Ly49 families as well as the recently isolated human NKR-P1 and CD94 proteins. Since the NKG2 family has sofar been only defined by cDNA cloning, we have characterized the expressed form of the NKG2-C protein and tested the binding of a soluble NKG2-C-receptor to a panel of target and non-target cells of NK cell killing (3,4). We provide data that NKG2-C is expressed as a glycosylated 36 kD integral type II membrane protein by in vitro translation and recombinant expression. An identical 36 kD NKG2 protein can be detected on the surface of the human NK cell clone B22 as shown by flow cytometry and immunoprecipitation. Further, a recombinant soluble NKG2-C-immunoglobulin fusion protein binds specifically to K562 cells, which are target cells for NK cell killing, to RPMI 8866 cells, which are feeder cells for NK cells, and to a lower extent to Daudi cells, which are LAK targets. Several other hematopoietic cell lines tested do not show any binding. This suggests that NKG2-C preferentially binds to cells which are known to interact with NK cells. Moreover, the binding structures on the surface of K562 cells rapidly disappear concomitant with a loss in susceptibility to killing when the cells are induced to differentiate. The loss of binding is not due to an extended change in overall glycosylation, since surface carbohydrates as detected by binding of wheat germ agglutinin and concanavalin A do not change following induction. In addition, the specific carbohydrate structures Lewis X and Lewis Y, which are implicated in the binding of some proteins with lectin domains, were found unchanged. Also the mucin-like CD43 molecule is not reduced following induction of the cells. These data suggest the presence of specific target molecules for NKG2-C on K562 cells, which are removed from the surface when K562 cells initiate differentiation and lose proliferative capacity. In respect of binding to target cells which are highly susceptible to lysis by NK cells, NKG2-C resembles the rodent NKR-P1 molecules. We propose that NKG2-C mediates a specific interaction of NK cells and their target cells with importance for NK cell functions.

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STRUCTURE AND FUNCTION OF THE CD94 NK CELL LECTIN-LIKE RECEPTOR

M. López-Botet, J. Pérez-Villar, A. Rodríguez and M. Carretero.
Servicio de Inmunología. Hospital de la Princesa. Madrid (Spain).

The CD94 (Kp43) surface molecule is selectively expressed by NK cells and subsets of CD8+ $\alpha\beta$+ and $\gamma\delta$+ T lymphocytes. Several lines of evidence indicate that signaling via CD94 regulates NK cell function. We initially reported that soluble $F(ab')_2$ fragments of anti CD94 mAbs induced LFA-1-dependent homotypic adhesion, TNF production and cytotoxicity against human T cell blasts (autologous and allogeneic). More recently we observed that CD94-specific mAbs may alternatively trigger, inhibit or unaffect NK cell functions (redirected lysis and TNF production) in different subsets of NK clones, revealing a complex functional ambivalence of this receptor. The pattern of response of NK clones correlated with their surface phenotype, as $CD94^{bright}$ cells appeared preferentially inhibited in redirected lysis, whereas activated clones were included in the $CD94^{dull}$ subset. On an indirect experimental basis, it has been proposed that this receptor may participate in repressing the cytotoxicity of a subset of $CD94^{bright}$ clones, upon specific recognition of certain HLA-B alleles (Bw6+).

We have explored the effect of anti CD94 stimulation on integrin function. IL2-activated NK cell populations or clones were incubated either with soluble $F(ab')_2$ CD94, CD16, CD7 mAbs or with PMA; subsequently, we quantitated their attachment to different solid phase-coupled integrin ligands and the effect of integrin-specific mAs was determined in parallel. Our results indicate that anti CD94 or CD16 mAbs rapidly (1 h) increased cell adhesion to: Fibronectin, type I collagen, laminin, VCAM-1 and ICAM-1. The responsiveness of different NK clones to anti CD94 in this assay was also variable, and dependent on tyrosine kinase activity. Remarkably, the enhanced avidity for integrin ligands was observed in $CD94^{bright}$ NK cells, that conversely were inhibited in redirected lysis by the specific mAb, thus indicating that qualitative changes in ligand interaction may determine CD94-dependent activation vs inhibition of different NK cell functions.

CD94 (Mr 70 kDa) is a dimer with two disulfide-linked subunits (Mr 43 kDa). The electrophoretic mobility of CD94 immunoprecipitates was slightly different in clones activated in the redirected lysis assay (Mr 40 kDa), indicating a structural heterogeneity of CD94, that was further supported by comparative SDS-PAGE analysis of deglycosylated and V8-digested immunoprecipitates. In collaboration with another group (L.L. Lanier, DNAX, Palo Alto. CA), several cDNAs encoding for CD94 have been cloned. They correspond to a type II membrane protein encoded by a single-copy gene of the C-type lectin superfamily located in chromosome 12. All of them display an identical ORF predicting a 180-aminoacid polypeptide with a short cytoplasmic domain (7 aa). SDS-PAGE analyses of the deglycosylated molecule immunoprecipitated from CD94 stable transfectants yields a single band (22 kDa), which does not correspond to the major product detected under the same conditions from NK cells (26 kDa). We currently address the possibility the transcriptional and/or mRNA processing events may generate different isoforms of CD94, eventually providing structural clues to understand the functional complexity of this receptor.

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MHC ALTERATION IN TUMOURS. BIOLOGICAL IMPLICATIONS.

Federico Garrido. Dept. Análisis Clínicos & Inmunología.

Hospital Virgen de las Nieves, 18014 GRANADA, SPAIN.

Alteration of MHC Class I molecules is a frequent finding in different human cancers. Different phenotypes have been defined: HLA total losses, HLA A and B locus specific losses, HLA haplotype losses and HLA individual allelic losses. These altered MHC phenotypes could represent a mechanism by which tumour cells evade T cell immune responses favoring invasion and metastasis. Alternatively the HLA downregulation could activate other immune effector mechanisms such as NK cells. Our laboratory is actively involved in defining new phenotypes and the exact frequency of these alterations. In recent studies we have shown that some tumour types can present HLA downregulation in more than 80% of the samples indicating that this phenomenon is more frequent than previously detected. Many different mechanisms can produce HLA altered phenotypes in tumour cells. Probably any of the steps require for sintezising an active HLA β2m-peptide complex can be the target for a particular mechanism producing an altered phenotype. Our laboratory is also studing and defining new mechanisms that can produce HLA class I losses. Data will be presented from experimental and human tumours to answer questions such as: The nature of MHC alterations, The timing, The mechanisms, The frequency and the possible clinical implications.

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MOLECULAR CHARACTERIZATION OF HUMAN CD94: A TYPE II MEMBRANE GLYCOPROTEIN RELATED TO THE C-TYPE LECTIN SUPERFAMILY.

A. Rodríguez[1], C. Chang[2], M. Carretero[1], J.H. Phillips[2], L. Lanier[2] and M. López-Botet[1]. [1]Servicio de Inmunología, Hospital de la Princesa, Universidad Autónoma de Madrid. Spain, and [2]Department of Human Immunology, DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, CA. USA.

We originally characterized the Kp43 (CD94) surface antigen, whose expression is restricted to human NK cells and a minor T lymphocyte subset. There is evidence that signalling via CD94 regulates NK cell-mediated cytotoxicity and TNF production.

Several cDNAs encoding for CD94 were cloned from an NK cell-specific cDNA library in the pJFE14 expression vector using the method described by Aruffo and Seed. All of them display and identical ORF encoding a 180 amino acid polypeptide (predicted Mr ~20 kD). Its structure corresponds to a type II membrane protein with a 26 amino acid transmembrane domain located near the NH_2-terminus. The extracellular domain comprises 147 amino acids and contains 2 potential sites for N-linked glycosylation. Remarkably, the cytoplasmic domain of the polypeptide includes only 7 amino acids (1).

RNA was isolated from the leukemia cell line NK-L and IL-2 dependent polyclonal NK cell populations established from two unrelated donors and examined by Northern blot analysis using the CD94 cDNA as probe. Four bands corresponding to transcripts of approximately 5.5, 3.5, 1.8 and 0.8 kb were detected whereas no CD94 transcripts were observed in the leukemia cell line RPMI-8866. Southern blot analysis of genomic DNA revealed a relatively simple restriction enzyme pattern indicating that CD94 is encoded in a single copy gene. The extracellular domain structure indicated that CD94 is a member of the C-type lectin superfamily. By analysis of a panel of human x hamster hybrid cell lines we determined that the CD94 gene is located on human chromosome 12, as reported for other members of this superfamily (hNKR-P1A, NKG2 and CD69). Homology of CD94 with hNKR-1 (26%) and NKG2 (27%-32%) is limited to the conserved structural motifs in the carbohydrate recognition domain. Human chromosome 12 is the syntenic of mouse chromosome 6 where the "NK gene complex" (NKC), comprising the NKR-P1 and Ly49 gene families, has been mapped; thus, the data further support the existence of a human NKC.

Comparative SDS-PAGE analysis of N-glycanase treated immunoprecipitates from NK cells and LL288-CD94-transfectants indicated that the major product obtained from NK-cells (~26 kD) is larger than the LL288 protein (~22 kD), suggesting the existence of different isoforms of the molecule. Although we have been unable so far to find specific cDNAs with longer open reading frames in an extensive search (RT-PCR analysis, RACE5', screening of two different cDNAs libraries), we do not exclude the expression of alternatively spliced CD94 transcripts that may explain the biochemical and functional complexity of CD94 in NK cells.

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CD94 (KP43)-INITIATED SIGNAL TRANSDUCTION: POSITIVE VERSUS NEGATIVE REGULATORY SIGNALS. *Brumbaugh, K. M., Pérez-Villar, J. J., Dick, C. J., Schoon, R. A., López-Botet, M., and Leibson, P. J.* Mayo Clinic, Rochester, MN, USA and Hospital de la Princessa, Madrid, Spain.

Little is known about signal transduction from a set of MHC class I recognizing receptors on natural killer (NK) cells whose ligation dramatically modulates NK cell-mediated killing. Recent evaluations suggest that CD94 (kp43) is a member of the C-type lectin superfamily and that certain clonal populations of NK cells (Group A) expressing CD94 are activated (cytotoxicity and cytokine secretion) after receptor ligation, whereas other clonal populations (Group B) are potently inhibited. We evaluated proximal signals that regulate these alternative responses. CD94 receptor ligation of Group A clones induced the rapid activation of intracellular protein tyrosine kinases, including the src-family member, lck, and the syk-family member, ZAP-70. The downstream regulatory consequences include PI-3 kinase activation, tyrosine phosphorylation of PLC-γ2, inositol phosphate release, and increases in intracellular free calcium concentrations. In contrast, CD94 receptor ligation on Group B clones has none of the above noted effects. Rather, CD94 crosslinking of these cells inhibits FcR-induced tyrosine phosphorylations of ZAP-70 and PLC-γ2, the formation of phosphozeta-ZAP 70 complexes, and the release of inositol phosphates. The clonal differences in CD94-initiated signaling were a stable characteristic of each of the populations. These results define distinct proximal signal events initiated after CD94 ligation, and suggest that clonotypic differences in signaling generate fundamentally different immune responses.

POSTERS

The human natural killer genetic complex: The C-type animal lectin receptor CD94 (Kp43) gene maps to the same region as CD69 gene in human chromosome 12p12-13

Authors:
Antonio Rodríguez+, Mónica Renedo, Lewis L. Lanier*, Mi uel López-Botet+ and Elena Fernández-Ruiz.
Unidad de Biología Molecular and +Servicio de Inmunolog a, Hospital de la Princesa, Universidad Autónoma, Madrid, Spain and *Department of Human Immunology, DNAX Research Inst tute of Molecular and Cell Biology, Palo Alto, USA.

Abstract
CD94 (Kp43) is an antigen of the Ca^{2+}-dependent (C- ype) animal lectin superfamily of type II transmem rane receptors. These signal transmitting receptors are implicated in the regulation of natural killer (NK) cell function and includes other NK associated genes as N RP1, NKG2 and CD69. All three genes have been assigned to uman chromosome 12.
We report here the assignment of the CD94 gene to uman chromosome 12 using PCR analysis on a panel of hams er x human somatic cell hybrid genomic DNA. Fluorescence in situ hybridization on human metaphase chromosomes coupled with GTG-banding, further localized the CD94 gene to 12p 2-13 region. The CD94 gene is, therefore, located in the proximity of the CD69 gene, the first human C-type l ctin gene regionally assigned to this chromosomal region. This human genetic region, between VWF and KRAS-2 marker , is syntenic to the distal mouse chromosome 6, where t e NK genetic complex was previously described.

SELF-REACTIVE CYTOTOXIC γδ T LYMPHOCYTES IN GRAVES' DISEASE SPECIFICALLY RECOGNIZE THYROID EPITHELIAL CELLS

Marta Catálfamo, Carme Roura-Mir, Mireia Sospedra, *Pedro Aparicio, †Sabine Costagliola, †Marian Ludgate†, Ricardo Pujol-Borrell and Dolores Jaraquemada

Unitat d'Immunologia, Hospital Universitari Germans Trias i Pujol, Universitat Autònoma de Barcelona, 08916 Badalona, Spain, *Dept. de Bioquímica e Inmunología, Universidad de Murcia, 30100 Murcia, Spain and †IRIBHN, Université Libre de Bruxelles, Hôpital Erasme, Route de Lennik 808, B-1070, Bruxelles, Belgium.

ABSTRACT

We have isolated and characterized a self-reactive cytotoxic γδ T cell line, 158RE.2, originated from the T lymphocyte population infiltrating the thyroid gland of a patient with Graves' disease. Functional data using this cell line demonstrate that γδ T cells expanded in the thyroid tissue specifically recognize a ligand expressed by thyroid epithelial cells and cell lines of endocrine epithelial origin. The TCR expressed by these γδ T cells -VγI/Vδ5 - is unusual in peripheral blood lymphocytes and its specificity is clearly different from that observed in a high percentage of γδ T cells from PBL, expressing the common TCR Vγ9Vδ2. The VγI/Vδ5 receptor is involved in the recognition of the ligand expressed by the thyroid cells but not in the NK-like activity also displayed by 158RE.2. These cells express CD8αα dimers which participate in the thyroid ligand recognition but not in the NK-like activity. The epithelial cell recognition is not restricted by classical MHC Class I or Class II molecules, although the CD8αα participation in the recognition suggests the involvement of non-classical MHC molecules. These are the first data so far of self-reacting γδ T cells in human epithelium.

TRANSCRIPTIONAL REGULATION OF THE GENE ENCODING THE HUMAN C-TYPE LECTIN RECEPTOR AIM/CD69. FUNCTIONAL CHARACTERIZATION OF ITS TNF-α RESPONSIVE ELEMENTS.

By Manuel López-Cabrera*, and Francisco Sánchez-Madrid.
From the Servicio de Inmunología and *Unidad de Biología Molecular, Hospital de la Princesa and Universidad Autónoma de Madrid. 28006 Madrid. Spain.

The human activation antigen CD69 is a member of the C-type animal lectin superfamily that functions as a signal-transmitting receptor. Although the expression of CD69 can be induced *in vitro* on cells of most hematopoietic lineages with a wide variety of stimuli, *in vivo* it is mainly expressed by T lymphocytes located in the inflammatory infiltrates of several human diseases. To elucidate the mechanisms that regulate the constitutive and inducible expression of CD69 by leukocytes, we isolated the promoter region of the CD69 gene, and carried out its funtional characterization. Sequence analysis of the 5'-flanking region of the CD69 gene revealed the presence of a potential TATA element 30bp upstream of the major transcription initiation site and several putative binding sequences for inducible transcription factors (NF-kB, AP-1, Egr-1), which might mediate the inducible expression of this gene. Transient expression of CD69 promoter-based reporter gene constructs in K562 cell line, indicated that the proximal promoter region spanning from -78 to +16 contained the *cis*-acting sequences necessary for basal and PMA-inducible transcription of CD69 gene. The removal of the upstream sequences located between -78 and -38, resulted in a decreased promoter strength and abolished the response to PMA. We also found that TNF-α is capable to induce the surface expression of CD69 molecule, as well as the promoter activity of fusion plasmids that contain 5'-flanking sequences of the CD69 gene, suggesting that this cytokine may regulate *in vivo* the expression of CD69 antigen. In addition, cotransfection experiments demonstrated that the CD69 gene promoter can be activated the NF-kB family members c-rel and relA. The deletion of the sequence spanning from -255 to -170 abolished both the response to TNF-α and the trans-activation by NF-kB. These results indicate that the NF-kB binding site located at position -223 is necessary for the TNF-α-induced expresión of the CD69 gene. Mobility shift assays showed the two NF-kB motifs located in the proximal promoter region (-223 and -160) bind variuos NF-kB-related complexes including the heterodimers p50/relA and p50/c-Rel, and homodimers of P50 (KBF1) and relA. Our findings help to understand the regulated synthesis of CD69 *in vivo* and suggest that TNF-α has a key role in the expression of this molecule a the sites of chronic inflammation.

REVERSAL OF IL-2-ACTIVATED NK CELL KILLING OF TUMOR, AND ALLOGENEIC, BUT NOT SYNGENEIC TARGETS BY PERTUSSIS TOXIN (PT) OR CHOLERA TOXIN (CT). A. A. Maghazachi, J. T. Vaage, C. Naper, K.-M. Torgersen and B. Rolstad. Department of Anatomy, Univ. of Oslo, Norway.

In the present communication, we examined the role of G proteins in rat IL-2-activated NK cell recognition of various neoplastic and normal target cells. Effector cells were generated from the strains PVG, PVG.R8 and PVG.1U with different MHC on the PVG background. The tumor cultured cell lines YAC-1, P815, G1, and MADB106, as well as Con-A activated MHC-compatible and incompatible cells were used as targets. Pretreatment of the effector cells with either PT or CT reversed their killing of all tumor and allogeneic target cells. However, such treatment did not reverse their inability to kill syngeneic target cells. These results are the first to show that recognition of tumor cells as well as non-self molecules by **rat** IL-2-activated NK cells is coupled to G proteins. However, recognition of self molecules is not coupled to bacterial toxin-sensitive G proteins, suggesting that NK recognition of non-self molecules utilizes different signaling pathway than the putative recognition of self MHC molecules. Supported by the Norwegian Cancer Society.

Role of the MHC class I subunits in NK cell development

Margarita Salcedo[1], Petter Höglund[1], Luc Van Kaer[2], and Hans-Gustaf Ljunggren[1]

[1]Microbiology and Tumor Biology Center, Karolinska Institute, S-104 01 Stockholm, Sweden and [2]Howard Hughes Medical Institute, Department of Microbiology and Immunology, Vanderbilt University School of Medicine, Nashville, TN 37232, USA

We have addressed the role of individual MHC class I subunits as well as MHC class I bound peptides in the development of NK cell specificities. Targeted disruption of genes affecting MHC class I ($H\text{-}2^b$) cell surface expression influences development of NK cells. Such mutations include TAP1, b2m and combined TAP1/b2m deficiencies. NK cells in such mice are normal in numbers, readily kill YAC-1 cells, but are tolerant towards endogenous Con A blast targets and bone marrow grafts.

Although TAP1, b2m, and TAP1/b2m mutant mice superficially appear similar, expression of class I molecules differs between the three types of mice: Mice devoid of b2m express low numbers of class I heavy chains. Mice devoid of TAP1 express low numbers of class I heavy chains complexed with b2m, either totally devoid of peptide, or occupied by peptides that are delivered to the class I molecules independent of the TAP complex. Mice devoid of both TAP1 and b2m lack serologically detectable levels of class I molecules.

Initial comparative analysis of NK cell specificities in such mice revealed that NK cells that develop in b2m mutant mice kill target cells from TAP1/b2m mutant mice. These results indicate that NK cells can be selected and acquire a specificity when selected on free class I heavy chains devoid of b2m, and that free class I heavy chains can mediate protection from NK cell mediated lysis. Furthermore, the data indicate that even very low levels of MHC class I expression may be able to shape the NK cell repertoire.

To address the role of MHC class I bound peptides, we have studied the specificity of NK cells that develop in mice harboring bm mutant class I alleles. Such bm mutant class I alleles are presumed to be very similar to the corresponding wild type alleles with regard to 3D-structure while they harbor a different peptide repertoire in the antigen binding cleft. Preliminary data did not reveal any differences with respect to development of NK cell specificity imposed by a bm mutant peptide repertoire compared to that of the wild type.

Based on these and other results we will present a model encompassing the role of individual MHC class I subunits in shaping the NK cell repertoire in vivo.

NKR-P1 CROSSLINKING ON NATURAL KILLER CELLS INDUCES CYTOSOLIC AND PHOSPHOLIPASE A_2 ACTIVITIES

M.G. Cifone, C. Festuccia, L. Cironi, P. Roncaioli, A. Meccia, @D. Botti and #A. Santoni. Departments of Experimental Medicine and @Cellular Biology and Physiology, University of L'Aquila; Department of Experimental Medicine and Pathology, University "La Sapienza", and #Laboratory of Pathophysiology, Regina Elena Cancer Institute, Rome

Several evidence clearly implicates NKR-P1 protein in the events leading to the NK-mediated tumor cell killing process and suggests that it may contribute to target cell recognition by NK cells. Perturbation of NKR-P1 has been shown in fact to stimulate PI turnover and intracellular Ca^{2+} mobilization, to induce granule exocytosis and to mediate redirect lysis by NK cells against FcγR+ target cells. We have previously reported that PLA_2, the enzyme which catalyzes the hydrolysis of phospholipids at the sn-2 position to yield fatty acid and lysophospholipid, is involved in NK cell activation and cytolytic functions. In this work we characterize the PLA_2 isoform demonstrating that NKR-P1 triggering induces the activation of both a type II secretory and a type IV cytosolic PLA_2 in freshly isolated rat NK cells. Our results also show that $sPLA_2$ is released from NK cells upon NKR-P1 crosslinking and mobilizes arachidonic acid which is then further metabolized through both cycloxygenase and lipoxygenase pathways. The involvement of PLA_2 on NKR-P1-induced cytotoxic functions was further investigated. Treatment of effector cells with the $sPLA_2$ inhibitor, para-bromophenacylbromide, or with the $cPLA_2$ inhibitor, AACOCF3 inhibited the redirected lysis against P815 target cells, and the granule content release induced by NKR-P1 crosslinking suggesting a crucial role for these enzymes in the induction of lytic activity of NK cells. Our results strongly suggest that cytosolic and secretory PLA_2 activation which takes place after NKR-P1 triggering, represents a crucial signalling pathway in the NK cell activation.

IDENTIFICATION OF "GROUP 0" NK CLONES WHICH RECOGNIZE ALL HLA-C ALLELES VIA TWO INDEPENDENT p58 RECEPTORS

M. VITALE, S. SIVORI, L. SANSEVERINO, D. PENDE, L. MORETTA and A. MORETTA.

I.S.T. and C.B.A. Genova, Istituto Istologia ed Embriol. Univ. Genova, Istituto Patologia Gen. Univ. Genova, Dipartimento di Scienze Biomediche e Biotecnologiche Univ. Brescia - ITALIA

A new group of NK clones (group 0) has been defined which fails to lyse all of the normal allogeneic target cells analyzed. Their specificity for HLA-class I molecules was suggested by the ability to lyse class I-negative target cells and by the fact that they could lyse resistant target cells in the presence of selected anti-class I mAbs. The use of appropriate target cells represented by either HLA-homozygous cell lines or cell transfectants revealed that these clones recognized all the HLA-C alleles. Indirect immunofluorescence and FACS analysis revealed that the presently defined group 0 NK clones express the GL183+EB6+ phenotype. MAbs directed to either GL183 or EB6 molecules were able to reconstitute lysis of these clones against Cw3+ or Cw4+ target cells respectively, thus demonstrating that EB6 molecules could recognize Cw4 and related alleles, while the GL183 molecules recognized Cw3 (and related C alleles).

These data suggest that the GL183 and the EB6 molecules can function, in individual NK clones, as independent receptors for two different groups of HLA-C alleles, (which include all known alleles of locus C), thus resulting in their inability to lyse all normal HLA-C^+ target cells. It is of note that, as group 0 clones, also group 2 clones (in spite of their inability to recognize Cw4) express the GL183+EB6+ phenotype. However FACS analysis revealed that, while group 0 and 1 clones brightly express EB6 molecules ($EB6^{bright}$), group 2 clones express an $EB6^{dull}$ phenotype.

A likely explanation for these findings could be that the density of EB6 receptors may be chritical for the generation of an optimal negative signal upon interaction with appropriate HLA-C alleles.

An alternative explanation for the low EB6 fluorescence intensity detected in group 2 NK clones could be that in these clones anti-EB6 mAbs may bind (with low affinity) to epitopes expressed by GL183 molecules. This would also provide an explanation for the fact that also anti-EB6 mAbs are able to reconstitute $Cw3^+$ target cell lysis by group 2 NK clones.

List of Invited Speakers

William H. Chambers	University of Pittsburgh Cancer Institute and Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, PA. 15213 (USA). Tel.: 1 412 624 77 00 Fax : 1 412 624 77 36
Marco Colonna	Basel Institute for Immunology, Grenzacherstrasse 487, CH-4005 Basel (Switzerland). Tel.: 41 61 605 12 58 Fax : 41 61 605 13 64
Ten Feizi	The MRC Glycosciences Laboratory, Northwick Park Hospital, Watford Road, Harrow, (U.K.). Tel.: 44 181 869 34 60 Fax : 44 181 869 34 55
Federico Garrido	Departamento Análisis Clínicos e Inmunología, Hospital Virgen de las Nieves, Universidad de Granada, 18014 Granada (Spain). Tel.: 34 58 24 11 10 Fax : 34 58 28 31 47
Erhard Hofer	Laboratory of Transplantation Immunology at VIRCC, Department of Dermatology, University of Vienna, Brunnerstrasse 59, A-1230 Vienna, (Austria). Tel.: 43 1 86 63 43 08 Fax : 43 1 86 63 46 23
Jeffrey P. Houchins	Department of Laboratory Medicine and Pathology, University of Minnesota, 420 Delaware Str., Minneapolis, MN. 55455 (USA). Tel.: 1 612 625 94 36 Fax : 1 612 626 26 96
Klas Kärre	Microbiology and Tumor Biology Center, Karolinska Institute, S-171 77 Stockholm (Sweden). Tel.: 46 8 728 62 82 Fax : 46 8 30 64 38
Vinay Kumar	Department of Pathology, University of Texas,Southwestern Medical School, 6000 Harry HGines Blvd.NB6.402, Dallas, TX.75235-9072 (USA). Tel.: 1 214 648 41 10 Fax : 1 214 648 40 33
Lewis L. Lanier	DNAX Research Institute of Molecular and Cellular Biology, 901 California Avenue, Palo Alto, CA. 94304 (USA). Tel.: 1 415 496 11 18 Fax : 1 415 496 12 00

Hans-Gustaf Ljunggren	Microbiology and Tumor Biology Center, Karolinska Institute, S-104 01 Stockholm (Sweden). Tel.: 46 8 728 63 00 Fax : 46 8 32 67 02
Eric O. Long	Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, NIH, 12441 Parklawn Drive, Rockville, MD 20852 (USA). Tel.: 1 301 496 82 66 Fax : 1 301 402 02 59
Miguel López-Botet	Servicio de Inmunología, Hospital de la Princesa, Diego de León 62, 28006 Madrid (Spain). Tel.: 34 1 402 33 47 Fax : 34 1 309 24 96
Alessandro Moretta	Department of Biomedical Sciences and Biotechnology, Univ. of Brescia and I.S.T. and C.B.A., 16132 Genova (Italy). Tel.: 39 10 353 78 62 Fax : 39 10 353 75 76
Lorenzo Moretta	Istituto Scientifico Tumori e Centro Biotecnologie Avanzate, Viale Benedetto XV, N.10, 16132 Genova (Italy). Tel.: 39 10 573 72 19 Fax : 39 10 35 41 23
Peter R. Parham	Department of Structural Biology, Stanford University, Stanford, CA. 94305 (USA). Tel.: 1 415 723 62 24 Fax : 1 415 723 84 64
José Peña	Dept. Immunology, Faculty of Medicine, Reina Sofía Hospital, University of Córdoba, s/nº Menéndez Pidal Avenue, 14004 Córdoba (Spain). Tel.: 34 57 21 82 66 Fax : 34 57 21 82 29
David H. Raulet	University of California, Berkeley, CA. 94720 (USA). Tel.: 1 510 642 95 21 Fax : 1 510 642 14 43
Bent Rolstad	Department of Anatomy, Institute of Basic Medical Sciences, University of Oslo, Postbox 1105, Blindern, N- 0317 Oslo 3 (Norway). Tel.: 47 22 85 11 50 Fax : 47 22 85 12 78

William E. Seaman — Department of Veterans Affairs Medical Center and University of California, San Francisco, 4150 Clement Street, San Francisco, CA. 94121 (USA).
Tel.: 1 415 750 20 37
Fax : 1 415 750 69 82

Jack Strominger — Department of Molecular and Cellular Biology, Harvard University, 7 Divinity Av., Cambridge, MA. 02138-2092 (USA).
Tel.: 1 617 495 27 33
Fax : 1 617 496 83 51

Wayne M. Yokoyama — NEW ADDRESS: Rheumatology Division, Washington University Medical Center, 660 South Euclid Avenue, Box 8045, CSRB 10027, St. Louis, MO. 63110 (USA).
Tel.: 1 314 362 74 80
Fax : 1 314 454 10 91

List of Participants

Balbino Alarcón	Centro de Biología Molecular "Severo Ochoa" CSIC, Facultad de Ciencias, Universidad Autónoma, Cantoblanco, 28049 Madrid (Spain). Tel.: 34 1 397 80 49 Fax : 34 1 397 47 99
Pedro Aparicio	Departamento de Bioquímica B e Inmunología, Facultad de Medicina, Universidad de Murcia, Campus de Espinardo, 30100 Murcia (Spain). Tel.: 34 68 36 39 60 Fax : 34 68 83 09 50
Antonio Arnaiz-Villena	Servicio de Inmunología, Hospital Universitario "12 de Octubre", 28041 Madrid (Spain). Tel.: 34 1 390 83 15 Fax : 34 1 390 83 99
Roberto Biassoni	IST, Istituto Nazionale per la Ricerca sul Cancro, Centro Biotecnologie Avanzate, Viale Benedetto XV nº 10, 16132 Genova (Italy). Tel.: 39 10 573 72 21 Fax : 39 10 35 41 23
Oscar de la Calle	Servicio de Inmunología, Hospital de Sant Pau, Sant Antoni Mª Claret, 167, 08025 Barcelona (Spain). Tel.: 34 3 291 90 00 Fax : 34 3 291 94 27
Angel Ezquerra	CISA-INIA, 28130 Valdeolmos, Madrid (Spain). Tel.: 34 1 620 23 00 Ext. 34 Fax : 34 1 620 22 47
Christine S. Falk	Institute of Immunology, University of Munich, Goethestr. 31, D-80336 Munich (Germany). Tel.: 49 89 59 96 684 Fax : 49 89 59 96 667
Elena Fernández	Servicio de Inmunología, Hospital de la Princesa, Diego de León 62, 28006 Madrid (Spain). Tel.: 34 1 402 80 00 Fax : 34 1 402 90 00

Paul Fisch	Freiburg University Medical Center, Department of Medicine I, Hugstetter Str. 55, D-79106 Freiburg (Germany). Tel.: 49 761 270 71 83 Fax : 49 761 270 71 77
María García-Barcina	Departamento de Biología Celular y Ciencias Morfológicas, Facultad de Medicina, Universidad del País Vasco, Bº Sarriena s/nº, 48945 Leioa, Vizcaya (Spain). Tel.: 34 4 464 88 00 Ext. 2733 Fax : 34 4 464 89 66
Katinka Giezeman-Smits	Department of Pathology, Faculty of Medicine, Leiden University, P.O. Box 9600, 2300 RC Leiden (The Netherlands). Tel.: 31 71 26 66 31 Fax : 31 71 24 81 58
Dolores Jaraquemada	Departament de Biologia Cel.lular i de Fisiologia, Unitat d'Immunologia, Hospital Universitari Germans Trias i Pujol, Ctra. del Canyet s/nº, 08916 Badalona (Spain). Tel. 34 3 465 12 00 Ext. 346 Fax : 34 3 395 42 06
Paul J. Leibson	Mayo Clinic, 200 First Street Southwest, Rochester, MN. 55905 (USA). Tel.: 1 507 284 81 76 Fax : 1 507 284 16 37
Manuel López Cabrera	Unidad de Biología Molecular, Hospital de la Princesa, Diego de León 62, 28006 Madrid (Spain). Tel.: 34 1 402 80 00 Ext. 334 Fax : 34 1 309 24 96
José Antonio López de Castro	Centro de Biología Molecular "Severo Ochoa", Facultad de Ciencias Biológicas, Universidad Autónoma, Cantoblanco, 28049 Madrid (Spain). Tel.: 34 1 397 80 50 Fax : 34 1 397 83 44
Azzam A. Maghazachi	Department of Anatomy, Institute of Basic Medical Sciences, University of Oslo, Postbox 1105, Blindern, N-0317 Oslo 3 (Norway). Tel.: 47 22 85 11 55 Fax : 47 22 85 12 78

Mauro S. Malnati	Laboratory of Human Virology, HSR San Raffaele, Via Olgettina 58, 20132 Milano (Italy). Tel.: 39 2 26 43 47 85 Fax : 39 2 26 43 47 86
Manuel Nieto-Sampedro	Department of Neural Plasticity, Instituto Cajal, CSIC, 37 Doctor Arce Ave, 28002 Madrid (Spain). Tel.: 34 1 585 47 20 Fax : 34 1 585 47 54
Sumati Rajagopalan	Lab. of Immunogenetics, NIAID - NIH, 12441 Parklawn Drive, Rockville, MD. 20852 (USA). Tel.: 1 301 496 95 89 Fax : 1 301 402 02 59
Antonio Rodríguez Márquez	Servicio de Inmunología, Hospital de la Princesa, Diego de León 62, 28006 Madrid (Spain). Tel.: 34 1 402 33 47 Fax : 34 1 309 24 96
Susana Rojo	National Institute of Allergy and Infectious Diseases, Dept. of Health & Human Services, Twinbrook II Facility, 12441 Parklawn Drive, Rockville, MD. 20852 (USA). Tel.: 1 301 496 29 51 Fax : 1 301 402 02 59
Margarita Salcedo	Microbiology and Tumor Biology Center, Karolinska Institute, S-104 01 Stockholm (Sweden). Tel.: 46 8 728 63 02 Fax : 46 8 32 67 02
Angela Santoni	Laboratory of Pathophysiology, Regina Elena Cancer Institute, Viale Regina Elena 324, 00161 Roma (Italy). Tel.: 39 6 446 84 48 Fax : 39 6 445 48 20
Rafael Solana	Servicio de Inmunología, Hospital Universitario Reina Sofia, Avda. Menéndez Pidal s/nº, 14004 Córdoba (Spain). Tel.: 34 57 21 75 32 Fax : 34 57 21 82 29

Fumio Takei	University of British Columbia and The Terry Fox Laboratory, B.C. Cancer Agency, 601 West 10th Avenue, Vancouver, British Columbia, Canada V5Z 1L3. Tel.: 604 877 60 70 Fax : 604 877 07 12
Mª del Mar Vales	Fairchild Biochemistry Building, Harvard University, 7 Divinity Ave, Cambridge, MA. 02138 (USA). Tel.: 1 617 495 56 13 Fax : 1 617 496 83 51
B. Carlos Vilches	Servicio de Inmunología, Hospital Puerta de Hierro, San Martín de Porres 4, 28035 Madrid (Spain). Tel.: 34 1 316 06 44 Fax : 34 1 316 06 44
Massimo Vitale	IST, and C.B.A. Università di Genova, 10 Viale Benedetto XV, Genova (Italy). Fax : 39 10 353 75 76
Eric Vivier	Centre d'Immunologie INSERM-CNRS de Marseille-Luminy, Case 906, 13288 Marseille Cedex 9 (France). Tel.: 33 91 26 94 00 Fax : 33 91 26 94 30
Steven Ziegler	Darwin Molecular, 1631 220th Street, SE, Bothell, WA. 98021 (USA). Tel.: 1 206 489 80 11 Fax : 1 206 489 80 17

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Instituto Juan March de Estudios e Investigaciones
Castelló, 77 • Teléfono 34 - 1 - 435 42 40 • Fax 576 34 20
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