ACTIVATION SITES INVOLVED IN HUMAN LYMPHOCYTE MEDIATED TUMOR CELL LYSIS

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ACTIVATION SITES INVOLVED IN HUMAN LYMPHOCYTE MEDIATED TUMOR CELL LYSIS

Aktivatiemoleculen betrokken bij humane lymfocyt-gemedieerde tumorcellysis

Proefschrift

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Aan mijn ouders,

Elly en Ewoud

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CHAPTER 1

GENERAL INTRODUCTION

The human defense system against foreign agents or aberrant cells is comprised of both a humoral and a cellular response. The humoral response consists mainly of the production of antibodies (Ab), that specifically recognize foreign antigens. Binding of Ab to antigens facilitates clearance of the antigen from the body by other cells of the immune system. These Ab are produced by B lymphocytes upon antigenic stimulation (humoral adaptive immunity). The cellular immune response can be divided into a natural and an adaptive component. Natural immunity is provided by natural killer (NK) cells, granulocytes, and monocytes/macrophages, which display their function without prior antigenic stimulation. NK cells are thought to provide a first line of defense against malignant or virus infected cells. Adaptive immunity is provided by T lymphocytes, which are activated by antigen-specific stimulation. Both NK cells and T lymphocytes can discriminate between self and non-self (foreign) cells. Cell mediated cytolysis of non-self cells is a major function of NK cells as well as cytotoxic T lymphocytes (CTL). The interaction between NK or CTL cells and non-self cells (target cells) resulting in target cell lysis, is a multistep process involving lymphocyte-target cell adhesion and, subsequently, lymphocyte activation.

In this chapter the characteristics of distinct types of cytotoxic lymphocytes and the various steps of the cytolytic process will be discussed.

Classification of cytotoxic lymphocytes

At present three major subsets of cytotoxic lymphocytes can be distinguished by phenotypic features as their surface receptor(s) for

target cell recognition, the expression of other cell surface proteins, so-called cluster designation (CD) determinants, and the type(s) of cytolytic activity which they mediate (see below). These three subsets are: 1) T cell receptor (TCR) $\alpha\beta^+$ lymphocytes, 2) TCR$\gamma\delta^+$ lymphocytes, and 3) TCR$^-$ NK cells. The relevant distinguishing characteristics are summarized in Table 1.1.

Table 1.1 Characteristics of cytotoxic lymphocyte subsets.

Cloned lymphocyte subsets	Immunophenotype				Cytolytic activity		
	CD3	CD4	CD8	CD16	MHC-restricted	MHC-unrestricted	ADCC
TCR$\alpha\beta^+$	+	+	-	-	+	-(+[a])	-
	+	-	+	-	+	-(+[a])	-
TCR$\gamma\delta^+$	+	-	-(+[b])	-/+	+	+	-/+[c]
TCR$^-$	-	-	-(+[d])	+	-	+	+

[a] When cultured with a high dose of interleukin 2 (IL2).

[b] About 0-40%.

[c] When CD16 is expressed.

[d] About 30% of TCR$^-$ NK cells.

In this paragraph, the three types of cytotoxic lymphocytes will be discussed. TCR$\alpha\beta^+$ cells, which represent approximately 60-75% of peripheral blood lymphocytes (PBL), express a disulphide linked heterodimeric protein receptor for target cell antigen recognition. This receptor is composed of an α and a β chain of molecular weight (Mw) 40-45 kDa each (1-3). Like immunoglobulins (Ig) (4), TCRα and β chains are encoded by genes constructed via rearrangement of multiple variable (V), joining (J), constant (C) and, in case of the β chain, also diversity (D) region gene segments (5-8). The specificity of the TCR$\alpha\beta$ complex for antigen is determined by the rearrangement patterns of TCRα and β genes (9). The TCR$\alpha\beta$ is noncovalently associated in a 1:1 ratio with the CD3 complex (see below).

TCR$\gamma\delta^+$ cells represent approximately 0-10% of PBL (10-13). In mice, but not in humans, TCR$\gamma\delta^+$ lymphocytes are predominantly found in epithelium of the skin (about 50% of the lymphocytes) (14,15) or intestine (16,17). The TCR$\gamma\delta$ is composed of a γ and δ chain and similar to TCR$\alpha\beta^+$

cells noncovalently associated with the CD3 complex (8,11,12,18-22).

Like TCRα and β chains, TCRγ and δ chains are encoded by V, J, C and D region gene segments. The human TCRγ gene locus (21) contains two constant region genes Cγ, of which Cγ1, but not Cγ2, encodes a highly conserved cysteine residue involved in the disulphide bridge between the γ and δ chain (23,24). This subdivides the $TCR\gamma\delta^+$ T lymphocytes into two subsets, based on the expression of a disulphide or a non-disulphide linked heterodimer.

The third subset of cytotoxic lymphocytes are the NK cells, which represent about 10% of PBL. NK cells do not express a TCR/CD3 complex (25,26). Their surface receptor for target cell recognition has not yet been identified (see below).

The differential expression of other cell surface determinants (CD antigens) provides a second means to distinguish among the three subsets of cytotoxic lymphocytes. $TCR\alpha\beta^+/CD3^+$ lymphocytes either express CD4 or CD8 and lack cell surface expression of the Fc receptor (R)III for IgG (CD16). $TCR\gamma\delta^+/CD3^+$ lymphocytes do not express CD4 and a small subset expresses CD8 and/or CD16 (10,11,13,17,28). $TCR^-/CD3^-$ NK cells lack cell surface expression of CD4. They do express CD16 and about 30% of NK cells expresses CD8 (29-32).

Finally, the three cytotoxic lymphocyte subsets differ in types of cytotoxic activities displayed. The $TCR\alpha\beta^+$ CTL specifically recognize foreign antigen in the context of proteins of the major histocompatibility complex (MHC) through the TCRαβ complex (33,34). $TCR\alpha\beta^+$ CTL can also recognize non-self MHC molecules. The specificity of $TCR\alpha\beta^+$ CTL is reflected by their target cell spectrum; only those target cells that express the relevant combination of MHC determinant and antigen are recognized and subsequently lysed, a phenomenon referred to as MHC-restricted lysis. Coexpression of either cell surface CD4 or CD8 correlates with restriction of lysis by MHC Class II or Class I molecules, respectively (reviewed in 35 and 36). Upon exposure to a high dose of interleukin 2 (IL2), some cloned $TCR\alpha\beta^+$ CTL may, in addition to MHC-restricted lysis, also exert MHC-unrestricted lysis (37-39).

Freshly isolated $TCR\gamma\delta^+$ CTL do not exert cytolytic activity. Their function in man is still unknown. In mice, the local accumulation of $TCR\gamma\delta^+$ lymphocytes in epithelial tissue suggests an important barrier function against microorganisms (40). In humans, however, similar accumulations have not been found.

Cloned TCR$\gamma\delta^+$ CTL display MHC-unrestricted lysis (10,11,41,42). Cloned TCR$\gamma\delta^+$ CTL from both mice and humans may also display antigen specific and/or MHC-restricted recognition (42–46). It is assumed that antigen recognition by TCR$\gamma\delta^+$ cells occurs via the TCR$\gamma\delta$ complex. The homology with the rearrangement patterns of TCRα and β chain genes and Ig gene segments, suggests similar specificity for the TCR$\gamma\delta$ receptor. Thus, cloned TCR$\gamma\delta^+$ CTL may display both antigen specific/MHC-restricted and MHC-unrestricted cytotoxic activity. This might be explained by assuming that there are two receptors: the TCR$\gamma\delta$ receptor involved in antigen-specific/MHC-restricted lysis and an unidentified receptor involved in MHC-unrestricted lysis. When expressed, CD16 (IgG receptor) enables cloned TCR$\gamma\delta^+$ CTL to lyse Ab-coated target cells, a phenomenon known as antibody dependent cellular cytotoxicity (ADCC) (10,11,13).

NK cells do not express a TCR/CD3 complex and their lytic activity is MHC-unrestricted. In the past years several cell surface structures have proven to be critical in MHC-unrestricted lysis (38,47,48), but thus far there is no consensus on a common receptor. Instead, it is hypothesized that there is not just one receptor, but that multiple receptors on NK cells are involved in target cell recognition (49,50). The target cell structure(s) recognized by NK cells have not been identified (47). However, the expression of so-called tumor associated antigens (TAA) on a number of tumors suggests that tumor cell recognition occurs via these antigens. NK cells are also characterized by their ability to exert ADCC through expression of CD16 (29–32).

NK cell or CTL mediated lysis of target cells

NK cell or CTL mediated lysis is a multistep event. This process is initiated by antigen nonspecific formation of lymphocyte-target cell conjugates (51–54). Conjugates are stabilized by subsequent lymphocyte receptor-antigen interaction. Thereafter, the lytic mechanisms become activated, followed by delivery of the lethal hit and subsequent lysis of the target cell. Once the lethal hit has been delivered, the lymphocyte dissociates from the target cell and may bind to a new one (recycling) (55).

There is still controversy about the mechanism of target cell lysis. Two major models have been proposed (56,57), one of which supposes a direct lysis of target cells through exocytosis of pore-forming molecules (perforin or cytolysin) from granules (Figure 1.1) (58,59). The

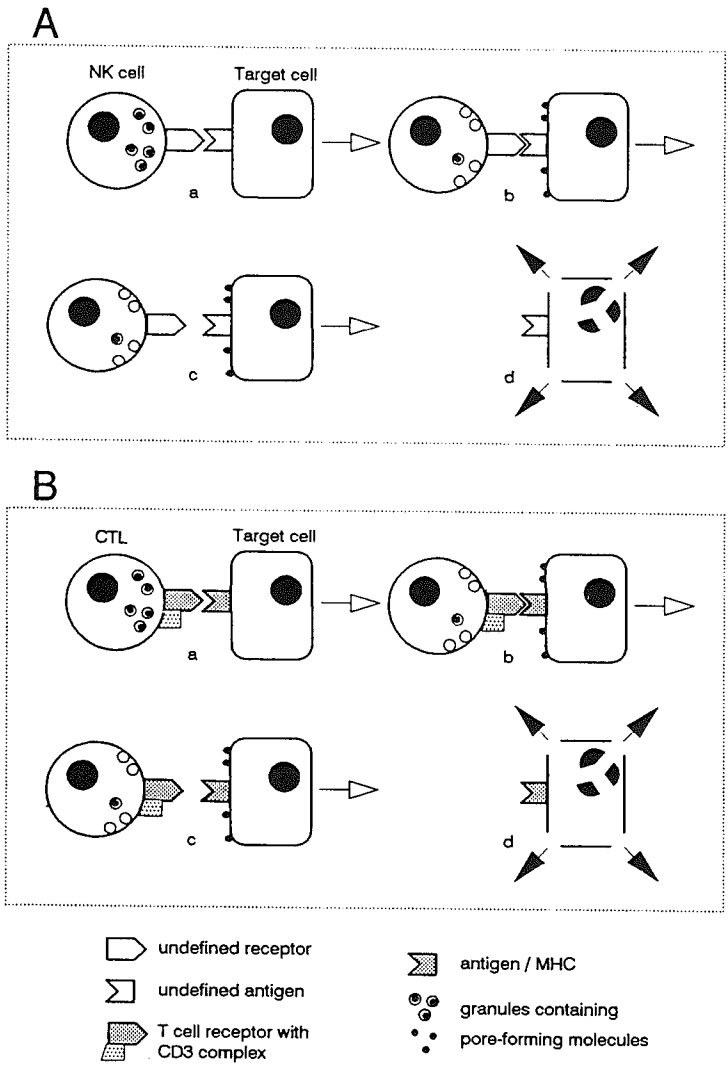

Figure 1.1 Target cell lysis by pore-forming molecules from NK cells (A) or CTL (B).

For reasons of simplicity, only the lymphocyte receptors involved in target cell recognition are depicted here. Lymphocyte receptor-antigen interaction (a) induces the release of pore-forming molecules at the interface (b). Thereafter lymphocyte and target cell dissociate (c) and the target cell is lysed through binding of pore-forming molecules (d).

observations that in some cases degradation of the nuclear membrane (60) and DNA fragmentation (61) preceed destruction of the cytoplasmic membrane indicate a second lytic mechanism. Compatible with this notion is that CTL, and possibly NK cells, may kill target cells without release of perforin (62,63). It was therefore proposed that the effector cell induces metabolic changes within the target cell which in turn lead to its lysis (60,61,64) (Figure 1.2). It is likely that a cytotoxic cell can activate multiple lytic mechanisms which may collectively lead to target cell destruction.

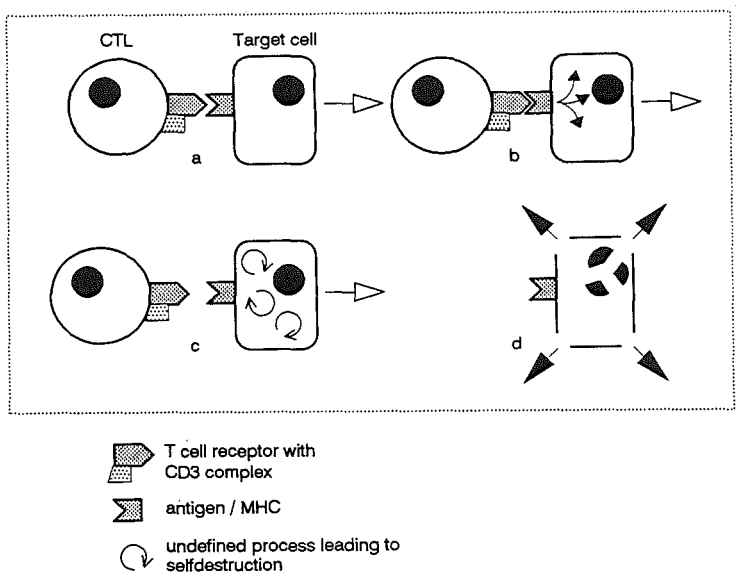

Figure 1.2 CTL induce metabolic changes within the target cell leading to its lysis.

TCR-antigen interaction (a) induces metabolic changes within the target cells (b). Thereafter, the CTL dissociates from the target cell (c), while the target cell is lysed (d).

Adhesion and/or signal transducing molecules

Inhibition of conjugate formation with monoclonal antibodies (mAb) directed against cell surface molecules on CTL and/or target cells have demonstrated the existence of various accessory molecules (65). Accessory molecules are operationally defined here as cell surface structures which mediate adhesion, transduce regulatory signals or both (listed in Table 1.2). Cell surface molecules that transduce signals

Table 1.2 Accessory molecules on lymphocytes involved in cytotoxicity.

	Lymphocyte subset		
Accessory molecules	$TCR\alpha\beta^+$ CTL	$TCR\gamma\delta^+$ CTL	TCR^- NK
CD2	+	+	+
CD3	+	+	-
CD4	+	-	-
CD8	+	unknown	unknown
CD11a/CD18	+	+	+
CD16	-	+[a)]	+
CD28	+	+	-
CD45	+	unknown	+
Tp103	+	+	-

[a)] when expressed.

across the membrane are defined as activation molecules. Multivalent Ab-accessory molecule interactions generally activate lymphocytes, whereas mono- or bivalent interactions usually inhibit lymphocyte functions. The valency of Ab-antigen interactions is increased by cross-linking mAb by their IgG-Fc to $Fc\gamma R$ expressed on target cells (Figure 1.3A). This interaction may activate the lytic mechanisms of the lymphocyte, resulting in target cell lysis (66-70).

The functional involvement of the major accessory molecules in lymphocyte mediated lysis is detailed below. Successively, the bio-

chemical features of the accessory molecules, their role in lymphocyte adhesion and finally their function in signal transduction, are described.

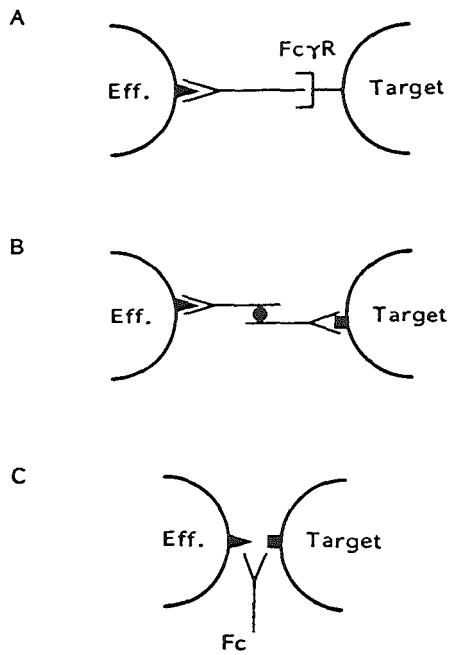

Figure 1.3 Induction of cytotoxic activity with antibodies.

Cytolysis of target cells by cytotoxic effector cells (Eff.) can be induced by A) single mAb directed against an activation site, crosslinked via its Fc to FcγR expressed on the target cell, B) chemically coupled bispecific Ab-heteroconjugate, comprising a mAb directed against an activation site and a mAb against a target cell determinant, and C) bispecific quadroma derived mAb, obtained by somatic fusion of two hybridomas producing a mAb against an activation site and a target cell determinant, respectively.

CD3 complex

The CD3 complex consists of five nonpolymorphic proteins γ, δ, ε, and a homodimer ζ (71–73). The CD3 complex is noncovalently associated in a 1:1 ratio with either the TCR$\alpha\beta$ or the TCR$\gamma\delta$. TCR–antigen interactions induce local accumulation of TCR molecules, and hence CD3, at the interaction site between lymphocyte and antigen presenting cell (74). It is thought that this accumulation induces signal transduction by CD3 (75). Crosslinking of anti-CD3 mAb mimics TCR–antigen interactions by inducing accumulation of TCR/CD3 complexes, leading to T cell activation. In contrast, soluble (noncrosslinked) anti-TCR or anti-CD3 mAb restrict this accumulation which decreases the TCR–antigen interaction, and subsequently MHC-restricted lysis. However, soluble anti-CD3 mAb also block lysis induced via other activation sites such as CD2 (see below). Thus, soluble anti-CD3 mAb may deliver a negative signal, which nonspecifically blocks transmembrane signaling via a number of other activation sites.

CD2

CD2 is a transmembrane protein of 50 kDa. It is expressed on virtually all thymocytes and mature TCR^+ T and TCR^- NK cells (35,76,77). Three distinct epitopes, T11.1, T11.2 and T11.3, have been identified on the CD2 molecule (78). The T11.1 epitope is associated with the sheep red blood cell binding site, whereas the T11.2 and T11.3 epitopes are not.

CD2 mediates cell–cell adhesion through interaction with its natural ligand leucocyte function associated antigen-3 (LFA-3) (CD58) (79–82), which is expressed on all leucocytes and various other cells (77). The CD2/LFA-3 interaction participates in adhesion of cytotoxic lymphocytes and target cells, thymocytes to thymic epithelium (83) and rosetting of human T lymphocytes with human or sheep erythrocytes (80,84–87). In addition to its adhesion function, CD2 acts as an activation site through which cytotoxicity can be induced by NK cells or CTL using anti-CD2 mAb (78,88–90).

Although CD2 is expressed on all cytotoxic lymphocytes, the requirements for activation via CD2 are different for the three distinct lymphocyte subsets. A combination of two stimuli, provided by mAb against T11.1 + T11.3, or T11.2 + T11.3 (78,89,90), or by anti-T11.3

mAb plus phorbol myristate acetate (PMA) (91) induces proliferation or cytotoxicity in all three subsets. In addition, cloned TCR$\gamma\delta^+$, but not TCR$\alpha\beta^+$, lymphocytes can be activated via a single anti-CD2 (anti-T11.1) mAb (88,92). In some, but not all, lymphocyte-target cell combinations, cytotoxicity by TCR$^-$ NK cell clones can also be induced by a single anti-CD2 mAb (88,92,93).

Recent studies have shown that target cell LFA-3 costimulates TCR$\alpha\beta^+$ lymphocytes activated with antigen, phytohemagglutinin (PHA), or anti-CD2 mAb (85,94-97). Activation of TCR$\alpha\beta^+$ lymphocytes via CD2 is functionally linked to CD3, since removal of the TCR/CD3 complex from the surface by anti-CD3 mAb (a process referred to as modulation) renders these lymphocytes refractory to activation via CD2 (78,98), and CD2 activation of TCR$\gamma\delta^+$ clones is blocked by anti-CD3 mAb (92). Moreover, stimulation of TCR$\alpha\beta^+$ lymphocytes with anti-T11.2 + anti-T11.3 mAb causes phosphorylation of CD3 chains (99). Recently, it has been found that CD2 is not only functionally, but also physically, associated with CD3 (100). In CD3$^-$ NK cells, the CD2 activation pathway is functionally linked to that of CD16 (93, A. Moretta, personal communication).

LFA-1 (CD11a/CD18)

LFA-1 is a member of a subgroup of the leucocyte integrin family, which includes several proteins with adhesion-like functions (101,102). This subgroup contains three members which share a common β chain (CD18) noncovalently associated with one of the three α chains, CD11a (LFA-1), CD11b (CR3 or Mac-1) or CD11c (p150/95) (Table 1.3). LFA-1 is expressed on all leucocytes.

Table 1.3 Members of the leucocyte integrin LFA-1 (β2) subgroup.

	Subunit α	Mw (kDa) β	CD	Known ligands
LFA-1	180	95	CD11a/CD18	ICAM-1,ICAM-2
CR3 or Mac1	165	95	CD11b/CD18	C3bi receptor
p150/95	150	95	CD11c/CD18	C3bi receptor

Natural ligands for LFA-1 are the intercellular adhesion molecules ICAM-1 (CD54) and ICAM-2 (103-106). ICAM-1 is a 90 kDa, monomeric, membrane glycoprotein with homology to neural cell adhesion molecule (NCAM) and myelin-associated glycoprotein (MAG) (105,107,108). ICAM-1 is also the receptor for the major Rhinovirus (109,110).

The LFA-1/ICAM-1 interaction is involved in homotypic adhesion of B cells, T cells and myeloid cells (111), and adhesion of $TCR\alpha\beta^+$ lymphocytes to a variety of cell types, including endothelial cells (112-114). The latter interaction is thought to play a role in the normal process of lymphocyte homing (115,116) and in T cell infiltration into areas of inflammation (117). Inflammation results in the producton of cytokines such as tumor necrosis factor (TNF), interleukin-1 (IL-1) and interferon-γ (IFNγ) (reviewed by Beutler (118)). Although ICAM-1 is normally present at only low levels on endothelial cells (112,119), these factors induce its expression and subsequently increase adhesion of lymphocytes to the endothelial tissue (112,119-121).

Recent evidence suggests that the LFA-1/ICAM-1 interaction is also involved in regulation of T cell function. Anti-CD11a mAb enhances, whereas anti-CD18 mAb decreases, adhesion-independent T cell proliferation (induced by anti-CD3 mAb) (122,123). In mice, anti-CD11a mAb enhance proliferation of preactivated T or B lymphocytes (124,125). Moreover, the LFA-1/ICAM-1 interaction coactivates: a) lysis triggered via the TCR/CD3 complex of both $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ CTL (126,127,Chapter 5), b) MHC-unrestricted lysis by TCR^- NK cells or $TCR\gamma\delta^+$ CTL (126,128,Chapter 6), c) lysis triggered via the CD16 activation antigen expressed on TCR^- NK cells (128,Chapter 6).

CD16

At present, three types of receptors for immunoglobulin IgG (FcγR) have been identified: FcγRI, FcγRII, and FcγRIII (129-131) (Table 1.4). CD16 (FcγRIII) is expressed on TCR^- NK cells, a small proportion of $TCR\gamma\delta^+$ lymphocytes, neutrophils, eosinophils, tissue macrophages, and cultured monocytes (29,31,32,132,133). CD16 is a monomeric transmembrane protein of 50-70 kDa (129). NK cells and $TCR\gamma\delta^+/CD16^+$ CTL lyse IgG-coated target cells through interaction with CD16 (ADCC) (29,31). Alternatively, anti-CD16 mAb can induce lysis of $Fc\gamma R^+$ target cells (68). CD16 on NK cells is functionally linked to LFA-1 and CD2

(88,93,128,134). CD16 on TCR$\gamma\delta^+$ cells is functionally linked to LFA-1 and CD3, but not to CD2 (126,128). Moreover, crosslinked anti-CD16 mAb induce lymphokine production (TNF,IFN-γ,etc.) by NK cells (135,136).

Table 1.4 Characteristics of human FcγR.

Type of FcγR	CD	Mw (kDa)	Cell distribution
FcγRI	-	70	monocytes, macrophages, neutrophils, myeloid cells
FcγRII	CDw 32	40	B cells, monocytes, macrophages, eosinophils, neutrophils, myeloid cells
FcγRIII	CD16	55-70	monocytes, macrophages, neutrophils, eosinophils, NK cells, TCR$\gamma\delta^+$ CTL

CD4/CD8

Both CD4 and CD8, which are generally expressed on mutually exclusive TCR$\alpha\beta^+$ blood lymphocyte subsets, are involved in antigen non-specific conjugate formation, because anti-CD4 or anti-CD8 mAb block lymphocyte-target cell adhesion (36). The target cell ligands for CD4 or CD8 molecules on the CTL are monomorphic determinants of MHC Class II or Class I molecules, respectively (137,138). These interactions increase the overall avidity between lymphocyte and target cells, thereby compensating a low affinity TCR-antigen interaction or lowering the requirement for high antigen density on target cells.

In addition to their adhesion function, CD4 and CD8 are also involved in CTL activation after the formation of lymphocyte-target cell conjugates (139,140). Interaction of the TCR with its relevant MHC/antigen induces the accumulation of both TCR/CD3 and CD4 (or CD8) at the interaction site (74,141). This physical association might alter the conformation of the TCR/CD3 complex. The formation of a quarternary complex consisting of MHC and antigen on the target cell, and TCR/CD3 and CD4 (or CD8) on the lymphocyte is required for optimal lymphocyte activation (142,143). Whether CD4 (or CD8) initially associates with the TCR or the CD3 is presently unknown. The precise conformational changes in the TCR that lead to its association with CD4 (or CD8) is

also unknown. Soluble anti-CD4 or anti-CD8 mAb inhibit T lymphocyte activation possibly because they block coclustering of CD4 (or CD8) with the CD3/TCR (141,144,145).

Other accessory molecules

Physiologic lymphocyte receptor-ligand interactions which lead to proliferation and/or lymphokine production or induction of target cell lysis can be mimicked by mAb. Thus the role of particular cell surface structures in these processes can be studied without knowing their natural ligand. This has revealed the existence of additional accessory molecules involved in the lytic cycle, such as CD28, Tp103 and CD45.

CD28 (Tp44) is a disulphide-linked homodimer. It is expressed on the majority of $TCR\alpha\beta^+$ lymphocytes as well as $TCR\gamma\delta^+$ lymphocytes and thymocytes (146-148). Anti-CD28 mAb have been shown to augment cytolytic activity or proliferation triggered via either TCR/CD3 or CD2, indicating a functional linkage (147,149,150). However, modulation experiments using anti-CD3 or anti-CD2 mAb demonstrated that CD28 is not physically linked to either the TCR/CD3 complex or CD2 (151).

Tp103 is expressed on in vitro cultured NK cells and CTL (152,153). Anti-Tp103 mAb induced lysis by CTL clones, but not by NK cell clones (153, our unpublished observations). Modulation of the TCR/CD3 complex rendered cloned CTL refractory to activation by anti-Tp103 mAb. These data demonstrate that a functional TCR/CD3 complex is required for activation via Tp103 (153), probably because the Tp103 and CD3 signaling pathways intracellularly converge to a common pathway.

The CD45 antigen family consists of a series of high Mw proteins ranging in weight from 160-220 kDa expressed on all hemopoietic cells. Anti-CD45 mAb block lysis by NK or CTL cells (154,155).

New mAb functionally reactive with structures on the lymphocyte membrane will undoubtedly reveal other accessory molecules involved in lymphocyte activation. However, the physiologic role of these activation signals largely remains to be defined.

Induction of cytolysis by bispecific mAb

Cytotoxic lymphocytes can be triggered to lyse $IgG\text{-}FcR^+$ target cells, which are normally resistant to lysis, by using mAb which cross-

link activation sites on the effector cell to FcγR on the target cell (Figure 1.3A). The use of bispecific mAb which can recognize two distinct antigens circumvents the requirement of FcγR on the target cell. Two types of bispecific Ab are currently used to crosslink lymphocytes and target cells. First, chemically coupled Ab-heteroconjugates, which comprise a mAb directed against an activation site on the effector cell crosslinked to a mAb directed against a target cell determinant (75,92,156,157) (Figure 1.3B). Crosslinked Fab or Fab_2 fragments of mAb also produce functionally active Ab-heteroconjugates (126,128,156,158). Secondly, the so-called quadroma derived bispecific mAb, which are obtained by somatic fusion of two hybridomas producing mAb against lymphocyte activation sites and target cell structures, respectively (159-162). (Figure 1.3C). Bispecific mAb, comprising tumor cellspecific or virus-specific mAb may provide useful tools to enhance the elimination of tumor cells or virus infected cells in vivo (35,158,163,164).

Clinical relevance and future prospects

The ultimate objective of immunotherapy of cancer is eradication of tumor. This requires detailed knowledge of the immune network mechanisms. Despite the extensive information available on accessory molecules expressed on distinct lymphocyte subsets, as outlined in this general introduction, much is there to be learned. New accessory molecules on effector lymphocytes and ligands on target cells will be identified in the near future. Of particular interest will be to unravel the complexity and nature of interactions between these accessory molecules on the one hand and their ligands on the other. Also, the effect of biological response modifiers such as interleukins, interferons etc. on the expression and function of accessory molecules needs to be further explored. The various signal transducing pathways and their relations to accessory molecules will further clarify their physiological role at the molecular level.

Modulation of cytolytic activity by lymphocytes, in vitro as well as in vivo, will enable us to control and improve lymphocyte activation and eradicate tumor cells or viral infected cells.

Thus, immunologic studies as reported in this thesis will contribute to the understanding of diseases caused by immunological defects as well as for the development of new immunotherapy strategies for the treatment of these diseases.

Objective of this study

NK cells and subsets of CTL normally lyse target cells after signal transduction via particular surface receptor(s) for target cell recognition. The cytotoxic activity of NK or CTL cells can be manipulated using mAb (either mono- or bispecific) directed against such lymphocyte receptors or against lymphocyte accessory molecules.

The present study was initiated to determine 1) the requirements for selective in vitro generation and expansion of distinct cytotoxic lymphocyte subsets; 2) their proportions and cytotoxic capacities in tumor patients; 3) the requirements for activation of NK cells and CTL subsets via CD2, CD3 and CD16; and 4) the role of LFA-1/ICAM-1 interactions in the cytolytic process. These in vitro studies provide information relevant to the application of such cytotoxic lymphocytes and bispecific mAb in immunotherapy of cancer.

In Chapter 2 culture conditions are described for selective in vitro generation and expansion of TCR$\alpha\beta^+$, TCR$\gamma\delta^+$ or TCR$^-$ NK cells. This allowed to determine the proportions and cytolytic capacities of NK cells and CTL subsets derived from PBL and tumor infiltrating lymphocytes (TIL) (Chapter 3). It was found that the proportions of NK cells and CTL subsets with proliferative capacity in TIL was low as compared to PBL from either the same patients or from healthy donors. Those TIL with proliferative capacity in vitro, displayed cytotoxic activity comparable to that of PBL.

The requirements for activation of NK cells or CTL via the actition sites CD2, 3 or 16 are described in Chapters 4, 5 and 6. In Chapter 4 it is shown that in TCR$\gamma\delta^+$ CTL the requirements for activation via CD2 qualitatively differ from those in TCR$\alpha\beta^+$ CTL and TCR$^-$ NK cells.

The role of accessory molecules and their ligands, in particular LFA-1/ICAM-1 interactions, was also studied (Chapters 5 and 6). The experiments described in Chapter 5 demonstrate that the LFA-1/ICAM-1 interaction promotes CD3/TCR mediated lysis by CTL through: a) an enhanced CTL-target cell binding, and b) the delivery of postconjugate formation costimulatory signals.

The functional importance of LFA-1/ICAM-1 interactions in lymphocyte activation, in particular their role in MHC-unrestricted lysis, are described in Chapter 6. It was found that LFA-1/ICAM-1 interactions also enhance MHC-unrestricted lysis by TCR$^-$ NK cells as well as by TCR$\gamma\delta^+$ CTL.

Finally, the relevance of these in vitro studies for immunotherapy protocols is discussed (Chapter 7).

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CHAPTER 2

LEUCOAGGLUTININ INDUCES DIFFERENTIAL PROLIFERATION OF LYMPHOCYTE SUBSETS

Introduction

Functional, phenotypical and especially biochemical studies on lymphocyte subsets require large numbers of relatively pure cells. Lectin, antigen, monoclonal antibodies (mAb) and interleukin 2 (IL2) or combinations thereof, have proven to be efficient inducers of in vitro proliferation of lymphocytes (1-8). Study on the heterogeneity of lymphocytes requires nonselective expansion of distinct lymphocyte subsets. Nonselective culture systems for T cells using anti-CD3 mAb (2,4) or phytohemagglutinin (PHA) (5) have been described. In addition culture conditions have been described for the cloning of natural killer (NK) cells, using PHA plus IL2 (6,9,10), or irradiated Epstein Barr virus (EBV) transformed lymphoblastoid B cell lines (B-LCL) plus IL2 (6,7,10).

We previously described a culture system that allows large scale and long-term expansion of major histocompatibility complex (MHC)-restricted T cell receptor (TCR) $\alpha\beta^+/CD3^+16^-$ clones (8); MHC-unrestricted $TCR^-/CD3^-16^+$ NK clones (7) and the recently described $TCR\gamma\delta^+/CD3^+16^+$ clones (11,12). This culture system is based on the use of a combination of two types of allogeneic irradiated feeder cells, i.e. peripheral blood lymphocytes (PBL) and EBV transformed B-LCL, plus leucoagglutinin. Here, we describe modifications of this culture system, i.e. at various concentrations of leucoagglutinin (range 0-1.0 μg/ml) selective expansion of $TCR^-/CD3^-$ NK cells, (subsets of) T cells or unselective expansion occurs. Selective expansion of particular subsets of lymphocytes may be important for laboratory controlled cellular immunotherapy. Moreover, the availability of such culture systems allows

quantitative and qualitative analysis of lymphoid cell subsets derived from different (organ) sites.

Materials and Methods

Isolation of PBL

PBL were isolated from healthy donors by centrifugation on Ficoll-Isopaque (d=1.077 g/cm^3) (Pharmacia Fine Chemicals, Uppsala, Sweden). The number of lymphocytes was determined by May-Grünwald-Giemsa staining of cytospin preparations. PBL were either directly used, or cryopreserved into liquid nitrogen.

Culture conditions

PBL were cultured in round-bottomed 96 well microtiter plates (Greiner Labor Technik, Phidelsheim, FRG) in the presence of irradiated feeder cells (20 Gy). Two x 10^4 irradiated allogeneic PBL and 10^4 irradiated EBV transformed lymphoblastoid B cell lines (B-LCL) were added to each well (8,13). RPMI 1640 (Flow Laboratories, UK) culture medium was buffered with 20 mM Hepes, supplemented with 10% pooled human serum, 4 mM glutamine, 1 μg/ml indomethacin, 100 IU/ml penicillin/streptomycin and 5% v/v of IL2 containing supernatant from concanavalin-A activated PBL. The lectin leucoagglutinin (purified phytohemagglutinin (PHA), HA-15 Pharmacia, Uppsala, Sweden) was added to the cultures at various concentrations (range 0-1.0 μg/ml). Plates were incubated at 37°C in 5% atmosphere of CO_2. Lymphocytes were harvested and replated with new feeder cells in fresh medium at weekly intervals.

Cloning and expansion of lymphocyte subsets

Lymphocyte clones were generated by limiting dilution: PBL were plated at 1, 3, 10, 30, 100, 300, 1000 and 3000 cells per well to determine the fraction of negative wells. The frequency of proliferating lymphocytes (plating eficiency) was calculated by the minimum χ^2 method from a Poisson distribution relationship between the logarithm of the percentage of negative wells and the cell number (14). Wells were

microscopically screened for the presence of proliferating lymphocytes. Between day 12 and 14 proliferating lymphocytes were transferred into 8–24 wells. Fresh medium plus feeder cells were added as described above. Lymphocytes were replated weekly. Proliferation of cloned and bulk cultured lymphocytes was analysed by cell counting and calculation of the multiplication factor. The multiplication per week of PBL cultures was calculated by dividing the 7-day cell yield by the cell number at day 0.

Calculation of the doubling time and cumulative cell yield of cultured $TCR^+/CD3^+4^+$ and $TCR^+/CD3^+8^+$ lymphocytes

The plating efficiency of a subpopulation of lymphocytes within bulkcultured PBL (here CD4 or CD8), was calculated using the Poisson formula and the plating eficiency of PBL from limiting dilution experiments. The plating efficiency of $CD4^+$ or $CD8^+$ lymphocytes was used to calculate the doubling time of these lymphocyte subsets in bulk cultures, using the formulas:

$$M_d = \frac{\%\, d \times C}{PE_d \times N_d}$$

$$M_d = 2^b,\ G = \frac{T}{b}$$

in which M_d = Multiplication factor of a single lymphocyte in bulk culture.
C = Cumulative cell yield.
%d = Percentage of a lymphocyte subset d (= $TCR^+/CD3^+4^+$ or $TCR^+/CD3^+8^+$).
N = Total number of lymphocytes plated.
PE_d = Plating efficiency of subset d.
b = Number of cell divisions.
T = Number of hours to obtain C.
G = Doubling time in hours.

Cell surface marker analysis

Fresh, bulk cultured and cloned PBL were immunophenotyped using monoclonal antibodies (mAb): anti-CD3, WT32 (Dr. W. Tax, Nijmegen, The Netherlands), OKT4 and OKT8 (Ortho Pharmaceutical, Raritan, NJ, USA), anti-CD25, Tac (Dr. T. Uchiyama, Kyoto, Japan), and anti-CD16 VD2

(CLB-FcR granl) (Dr. P.A.T. Tetteroo, Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands) and B73.1 (15) (Dr. G. Trinchieri, Wistar Institute, Philadelphia, USA). The fluorescein isothiocyanate-conjugated goat anti-mouse IgG Ab was obtained from Nordic, Tilburg, The Netherlands. Lymphocytes were labeled with mAb as described elsewhere (8) and analysed with a fluorescence activated cell sorter (FACS II) (Becton Dickinson, Sunnyvale, California, USA).

Results

Leucoagglutinin increases the multiplication factor of PBL cultured in bulk

PBL obtained from healthy donors were cultured for 3-4 weeks with 1.0 μg/ml and without leucoagglutinin. High concentrations of leucoagglutinin (> 2 μg/ml) impaired optimal lymphocyte proliferation (data not shown). Without leucoagglutinin, the multiplication factor varied from 6 to 13 in the first week of culture among 4 donors tested (Table 2.1).

However, in the presence of 1 μg/ml leucoagglutinin the number of PBL multiplied > 53 times. In both culture conditions the multiplication factor obtained in the second week was significantly higher than in the first week of culture since 1) the number of responder lymphocytes was increased at the start of the second week and 2) there was a lag period preceding proliferation at the start of the first week of culture. Nevertheless, the cell yield obtained in leucoagglutinin stimulated cultures always exceeded that of non-leucoagglutinin stimulated cultures by a factor 1.5-11.

Leucoagglutinin in bulk cultures promotes the expansion of $TCR^+/CD3^+16^-$ T cells over $TCR^-/CD3^-16^+$ NK cells

The proliferation enhancing effects of different doses of leucoagglutinin (range 0.1-1.0 μg/ml) on the lymphocyte subset composition of PBL was studied longitudinally. When PBL were cultured without leucoagglutinin, selective expansion of $TCR^-/CD3^-16^+$ NK cells occurred in

Table 2.1 Leucoagglutinin increases the multiplication factor per week of bulk cultured PBL.

Experiment[a)]	Donor	Multiplication factor			
		Day 6		Day 12	
		Leucoagglutinin (μg/ml)			
		0	1	0	1
1	HD1646	13	63	29	104
	HD1647	8	53	27	101
	HD1648	6	66	79	107
2	HD1649	9	75	149	324

[a)] Lymphocytes were directly cultured after isolation ($3x10^3$ PBL per well in experiment 1) or frozen and thawed ($1x10^3$ PBL per well in experiment 2).

the first 2 weeks. Among 10 healthy donors, the initial percentage of $CD16^+$ lymphocytes mean 16% (range 8–21%) increased to on average 49% (range 30–65%) at day 14. Consequently, the percentage $CD3^+$ lymphocytes decreased from 65% (range 53–79%) to 45% (range 35–70%). A representative example is shown in Figure 2.1. The increase of the percentage $TCR^-/CD3^-16^+$ NK cells during the second week resulted in a significant increase of the $CD16^+/CD3^+$ lymphocyte ratio from 0.30 to 2.0. Thereafter the proportion of $TCR^-/CD3^-16^+$ NK cells gradually decreased to less than 10% of the total lymphocyte population.

In contrast, when 1 μg/ml leucoagglutinin was added to the cultures there was no percentual increase in $TCR^-/CD3^-16^+$ NK cells. Instead, the proportion of $TCR^+/CD3^+16^-$ T cells rapidly increased to 95–100%, indicating an almost complete overgrowth by $TCR^+/CD3^+16^-$ lymphocytes. Intermediate concentrations of leucoagglutinin induced proliferation of both subsets, until day 14. Thereafter, the proportion of $CD3^+$ lymphocytes rapidly increased.

When absolute numbers of $TCR^-/CD3^-16^+$ lymphocytes were determined in cultures with and without leucoagglutinin higher numbers of $TCR^-/CD3^-16^+$ NK cells were found in cultures with leucoagglutinin. Without leucoagglutinin on average a 1000 ± 200-fold increase in

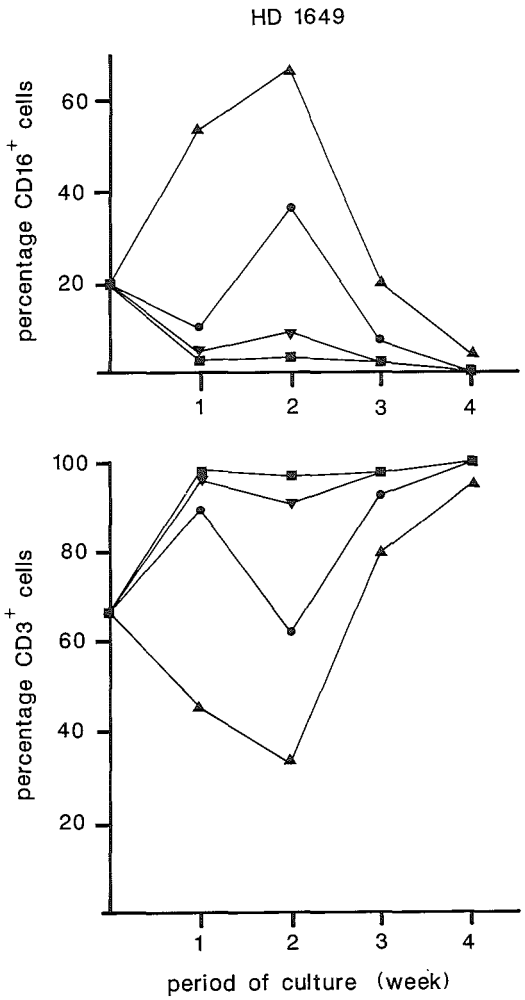

Figure 2.1 Rapid expansion of $TCR^+/CD3^+16^-$ T lymphocytes in PBL bulk cultures in the presence of leucoagglutinin.

PBL were cultured for 3 to 4 weeks with feeder cells and various concentrations leucoagglutinin as described in Materials and Methods. Lymphocytes were harvested at weekly intervals, and the percentage of $CD3^+$ and $CD16^+$ lymphocytes was determined by indirect immunofluorescence (flow cytometry). ▲ = 0 μg/ml; ● = 0.1 μg/ml; ▼ = 0.3 μg/ml; ■ = 1.0 μg/ml leucoagglutinin.

$TCR^-/CD3^-16^+$ NK cells was obtained, resulting in an approximately 70% pure NK cell population at day 14. In contrast, with 1 μg/ml leucoagglutinin, a > 2000±500-fold increase in $TCR^-/CD3^-16^+$ NK cells was obtained at day 14, but the percentage NK cells was only ≤ 6%.

The preferential outgrowth of $TCR^+/CD3^+8^+$ over $TCR^+/CD3^+4^+$ lymphocytes in bulk cultures is leucoagglutinin independent

To assess the effect of leucoagglutinin on $TCR^+/CD3^+4^+$ and $TCR^+/CD3^+8^+$ T cell subsets in bulk cultures, we have calculated at weekly intervals the multiplication factor for both lymphocyte subpopulations, and the $CD4^+/CD8^+$ lymphocyte ratio in cultures with and without leucoagglutinin. The multiplication of $TCR^+/CD3^+8^+$ lymphocytes under both culture conditions was about equal or exceeded that of $TCR^+/CD3^+4^+$ lymphocytes (Table 2.2) as reflected by the virtually linear decrease in the $CD4^+/CD8^+$ lymphocyte ratio (Figure 2.2). The iiial $CD4^+/CD8^+$ ratio (2.0–3.3) decreased to below 0.5 after 3 weeks of culture.

Table 2.2 Leucoagglutinin independent preferential proliferation of $TCR^+/CD3^+8^+$ lymphocytes.

Culture period in weeks	Multiplication factor[a] of $CD4^+$ cells				Multiplication factor[a] of $CD8^+$ cells			
	0[b]	0.1	0.3	1.0	0	0.1	0.3	1.0
1[c]	18	58	228	281	35	92	246	288
2	84	106	176	315	119	127	141	299
3	81	74	126	421	100	134	491	761
4	12[d]	21[d]	66	102	53[d]	88	153	125

[a] See Materials and Methods for calculation of the multiplication factor.

[b] Concentration of leucoagglutinin (μg/ml).

[c] PBL of HD1649 were cultured at $1x10^3$/well.

[d] The number of responder cells plated was $2x10^3$/well.

In order to investigate whether the selective proliferation of $TCR^+/CD3^+16^-$ or $TCR^-/CD3^-16^+$ lymphocyte (sub)populations in bulk cultures with or without leucoagglutinin was influenced by the original PBL subset composition, lymphocytes were also cultured under limiting dilution conditions.

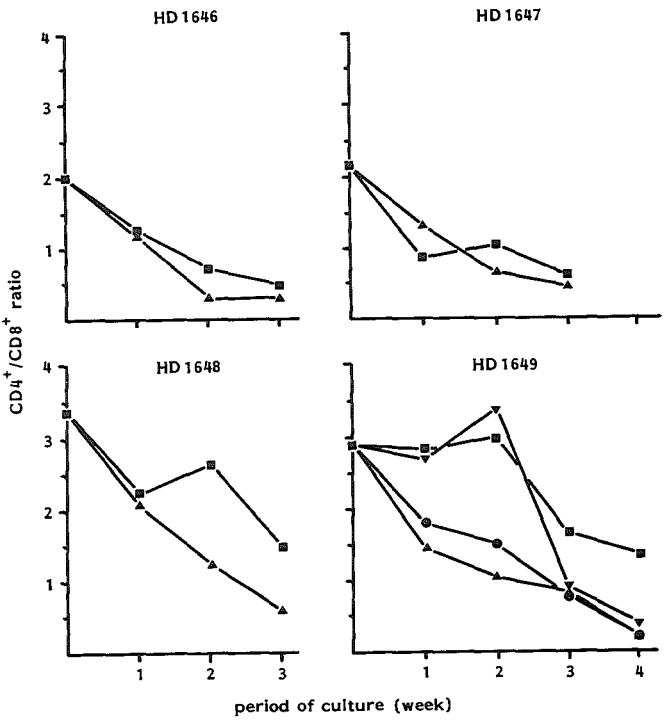

Figure 2.2 Leucoagglutinin independent outgrowth of $TCR^+/CD3^+8^+$ lymphocytes in bulk cultures.

PBL were cultured for 3 to 4 weeks with feeder cells and various concentrations leucoagglutinin as described in Materials and Methods. Lymphocytes were harvested at weekly intervals and the percentage of $CD4^+$ and $CD8^+$ lymphocytes was determined by indirect immunofluorescence (flow cytometry) and the $CD4^+/CD8^+$ ratio was calculated. ▲ = 0 μg/ml; ● = 0.1 μg/ml; ▼ = 0.3 μg/ml; ■ = 1.0 μg/ml leucoagglutinin.

Under limiting dilution leucoagglutinin increases the plating efficiency and life span of $TCR^+/CD3^+4^+$ and $TCR^+/CD3^+8^+$ clones

Lymphocyte clones were generated with and without 1 μg/ml leucoagglutinin. After 13 days microtiter wells were microscopically screened for proliferation of lymphocytes to determine the plating efficiency. The fraction of "negative wells" (no proliferating lymphocytes) showed a logarithmic correlation with the number of cells seeded (Figure 2.3), indicating a "single hit" phenomenon, i.e. no influence of the cell culture composition on the plating efficiency of individual lymphocytes. The plating efficiency of resting PBL was less than 12% when cultured without leucoagglutinin, but in contrast more than 44% PBL proliferated in the presence of 1 μg/ml leucoagglutinin (Table 2.3). Randomly picked clones were further expanded for another (third) week to obtain sufficient cell numbers to allow immunophenotypic analysis. At the end of the third week only 50% of the clones still proliferated when no or 0.1 μg/ml leucoagglutinin was present in the medium. However, up to 90% still proliferated provided 1 μg/ml leucoagglutinin was present (Table 2.4).

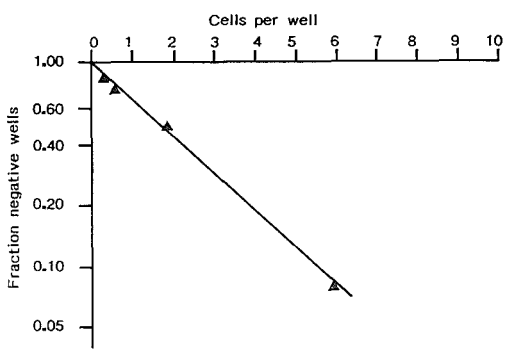

Figure 2.3 Frequency of proliferating PBL of a healthy donor (HD 1780).

PBL were cultured under limiting dilution conditions. The number of lymphocytes plated was plotted against the log scale of the fraction of negative wells. The plating efficiency was calculated as indicated in Materials and Methods.

Table 2.3 Leucoagglutinin increases the plating efficiency of PBL in limiting dilution experiments.

Experiment[a)]	Donor	Leucoagglutinin (μg/ml)	Plating efficiency (%)
1	HD1649	0	11
2	HD1385	0	7
3	HD1714	1.0	53
4	HD1733	1.0	45
5	HD1780	1.0	48

a) Lymphocytes were frozen and thawed (experiment 1, 2 and 3) or directly cultured after isolation (experiment 4 and 5).

Table 2.4 Preferential outgrowth of $TCR^+/CD3^+4^+$ clones under limiting dilution conditions.

Experiment[a)]	Donor	Leucoagglutinin (μg/ml)	No. of clones expanded week 2	Percentage of proliferating clones week 3[b)]	Number and phenotype
1	HD1649	0	55	44	8 ($CD3^+4^+$)[c)] 1 ($CD3^+8^+$) 1 ($CD3^-4^-8^-$)
1	HD1649	0.1	70	51	18 ($CD3^+4^+$) 2 ($CD3^+8^+$) 2 ($CD3^+4^-8^-$)
2	HD1714	1.0	40[d)]	100	11 ($CD3^+4^+$) 4 ($CD3^+8^+$) 2 ($CD3^+4^-8^-$)
3	HD1733	1.0	40	98	11 ($CD3^+4^+$) 3 ($CD3^+8^+$) 1 ($CD3^+4^-8^-$)
4	HD1780	1.0	40	88	23 ($CD3^+4^+$) 6 ($CD3^+8^+$)

a) Lymphocytes were thawed (experiment 1 and 2) or directly cultured after isolation (experiment 3 and 4).

b) Data expressed as percentage of the number of clones expanded at week 2.

c) The phenotype of the clones is given in parenthesis.

d) In experiment 2, 3 and 4, 40 clones were taken for expansion using a randomization table.

Immunophenotypic analysis of cloned lymphocytes generated with and without leucoagglutinin

In limiting dilution either with or without leucoagglutinin about 75% of the clones obtained was $TCR^+/CD3^+4^+$, 10-20% was $TCR^+/CD3^+8^+$; about 10% was $TCR^+/CD3^+4^-8^-$ and only one $TCR^-/CD3^-4^-8^-$ NK clone was obtained (Table 2.4)

This contrasts the above described preferential outgrowth of $TCR^+/CD3^+8^+$ lymphocytes in bulk cultures. We therefore calculated the doubling time of cloned versus bulk cultured $TCR^+/CD3^+4^+$ and $TCR^+/CD3^+8^+$ lymphocytes.

Calculation of the doubling time of $TCR^+/CD3^+4^+$ and $TCR^+/CD3^+8^+$ lymphocytes in bulk cultures and after cloning

In order to calculate the doubling time of $TCR^+/CD3^+4^+$ and $TCR^+/CD3^+8^+$ lymphocytes in PBL bulk cultures the frequency of actually

Table 2.5 Reduced doubling time of bulk cultured $CD8^+$ lymphocytes compared to cloned $CD8^+$ lymphocytes.

Donor	Leucoagglutinin µg/ml	Phenotype	Cell yield x 10^6 Average per clone (day 20)		Cell yield x 10^6 Bulk (day 19)	Doubling time (h) Clones	Doubling time (h) Bulk
HD1649	0	CD4	1.9	(8)[a]	1.0[b]	23.0	22.9
	0	CD8	0.8	(1)	9.4	24.6	19.7
HD1649	0.1	CD4	2.2	(18)	2.0	22.8	21.8
	0.1	CD8	2.1	(2)	37.5	22.8	18.1
HD1714	1.0	CD4	2.1	(11)	nd[c]	22.8	nd
	1.0	CD8	3.6	(4)	nd	22.0	nd
HD1780	1.0	CD4	1.3[d]	(23)	1.8	24.8	22.0
	1.0	CD8	1.2[d]	(6)	40.1	24.9	18.1

[a] The number of clones is given in parenthesis.

[b] See Materials and Methods for calculation.

[c] nd = not done.

[d] Cell yield at day 21.

triggered lymphocytes was determined. Since the plating efficiency determined in limiting dilution experiments was not influenced by cell-cell interactions, we used this plating efficiency also for bulk cultures (see Materials and Methods). The doubling times for cloned $TCR^+/CD3^+4^+$ and $TCR^+/CD3^+8^+$ lymphocytes varied from 22 to 25 h (Table 2.5). In bulk cultures also the doubling time of $TCR^+/CD4^+$ lymphocytes varied from 22 to 24 h, but was much shorter for $TCR^+/CD8^+$ lymphocytes, i.e. between 18 to 20 h. The shorter doubling time for $TCR^+/CD3^+8^+$ lymphocytes in bulk cultures as compared to cloned $TCR^+/CD3^+8^+$ lymphocytes was leucoagglutinin independent. These data indicate that $TCR^+/CD3^+4^+$ lymphocytes promote proliferation of preactivated $TCR^+/CD3^+8^+$ lymphocytes, e.g. by production of lymphokines.

Discussion

Large scale in vitro expansion of lymphocyte subsets serves two important purposes. Firstly, analysis of immune cell interactions and biochemical as well as functional characterization of the cell surface molecules involved, require large numbers of cells. Secondly, it is of particular importance for these studies as well as for the design of immunotherapy protocols to be able to selectively expand distinct subsets of immune cells. These two factors of the culture system, i.e. multiplication capacity and selective proliferation capacity are determined by the various components of the culture system, such as feeder cells, endogenous as well as exogenous cytokines, lectins and/or mAb. We previously determined the number and type of irradiated EBV transformed B-LCL and irradiated allogeneic PBL, and exogenous IL2 concentrations, optimal for the expansion of both NK cell derived clones and T cell derived clones (7,8,13).

This report describes how the multiplication capacity and selective proliferation capacity of the culture system can be modified by changing the concentration of leucoagglutinin, allowing selective expansion of $TCR^+/CD3^+16^-$ T cells or $TCR^-/CD3^-16^+$ NK cells or the nonselective expansion of both subsets of lymphocytes.

The multiplication factor of bulk cultured PBL gradually increased with the amount of leucoagglutinin added, reaching a plateau at 1 μg/ml. This gradual increase of the multiplication factor is probably due to an increased fraction of lymphocytes triggered to proliferate,

as determined in limiting dilution experiments (Table 2.3). Similarly, Moretta et al. (5) observed a 4-5-fold increase in plating efficiency for PBL when PHA was added.

Not only the multiplication factor but also the subset composition of bulk cultured PBL harvested at weekly intervals was markedly influenced by leucoagglutinin. In the presence of leucoagglutinin, the initial percentage $TCR^-/CD3^-16^+$ NK cells (5-20%) declined within 2 weeks to undetectable low levels, whereas the percentage $TCR^+/CD3^+16^-$ T lymphocytes increased to approximately 95.

In contrast, cultures without leucoagglutinin contained about 70% pure $TCR^-/CD3^-16^+$ NK cells after 2 weeks culture (Figure 2.1). This result is comparable with the observation of Perussia et al. (16), who obtained 50% $TCR^-/CD3^-16^+$ NK cells among PBL cultured with irradiated Daudi cells at day 10.

Although leucoagglutinin counteracts the selective proliferation of $TCR^-/CD3^-16^+$ NK cells among PBL, higher absolute numbers of NK cells were obtained in the presence of 1 μg/ml leucoagglutinin: without leucoagglutinin an average 1000-fold increase of $TCR^-/CD3^-16^+$ NK cells was obtained at day 13, but $\geq$ 2000-fold increase was obtained in the presence of 1 μg/ml leucoagglutinin. These data suggest that in addition to $TCR^+/CD3^+16^-$ T lymphocytes leucoagglutinin also stimulates $TCR^-/CD3^-16^+$ NK cells. This was also suggested by others (17,18), who observed that the number of IL2 receptors is increased on NK cell clones and LGL respectively upon stimulation with PHA. However, the proliferation stimulating effect of leucoagglutinin is much more pronounced on $TCR^+/CD3^+16^-$ T lymphocytes as illustrated by the undetectable percentage of $TCR^-/CD3^-16^+$ NK cells after 2 weeks of culture.

The high multiplication factors reported here for $TCR^-/CD3^-16^+$ NK cells exceeds those reported by others. For comparison: Perussia et al. (16) observed a 25-fold increase of $TCR^-/CD3^-16^+$ NK cells in PBL at day 13 cultured with irradiated Daudi cells as feeders; Talmadge et al. (17) obtained a 6-fold increase of Percoll enriched LGL at day 13 in response to recombinant (rec) IL2 (500 U/ml); Phillips et al. (19) observed a preferential stimulation of sorted Leu $11a^+$ (anti-CD16 mAb) cells upon stimulation with K562 cells, optimal at day 5 of culture as determined by [^{3}H] thymidine incorporation; Ochoa et al. (20) observed a 30-100-fold increase of lymphocytes displaying LAK activity at day 21, in response to recIL2 (1000 U/ml).

It should be noted that after a culture period exceeding 2 weeks the percentage $TCR^-/CD3^-16^+$ NK cells declined. This is probably due to the relatively short life span of $TCR^-/CD3^-16^+$ lymphocytes in vitro, and this would explain the extreme difficulty to clone and expand $TCR^-/CD3^-16^+$ NK cells. Although many investigators have tried to clone NK cells directly from PBL fractions (6,10), or from $B73.1^+$ sorted cells (21,22,23) or from LGL fractions obtained after Percoll isolation and E-rosette 29°C depletion (9,24), until now no generally applicable culture system has been described for the generation of stable NK cell clones. With a few exceptions (10,22) NK cell clones have a life span of maximally 4 weeks.

Interestingly, the preferential outgrowth of $TCR^+/CD3^+8^+$ lymphocytes over $TCR^+/CD3^+4^+$ lymphocytes observed in PBL bulk cultures was leucoagglutinin independent. Calculation of doubling times by extrapolation of data obtained under limiting dilution, showed a 18 to 20-h doubling time for $TCR^+/CD3^+8^+$ lymphocytes compared to 22-24 h for $TCR^+/CD3^+4^+$ lymphocytes. However, cloned $TCR^+/CD3^+4^+$ and $TCR^+/CD3^+8^+$ lymphocytes had equal doubling times of 22-25 h. Since there was no influence of the initial PBL subset composition on the plating efficiency in limiting dilution experiments, we conclude that $TCR^+/CD3^+4^+$ lymphocytes promote the proliferation of preactivated $TCR^+/CD3^+8^+$ lymphocytes, probably by production of lymphokines.

In conclusion, optimal expansion of lymphocytes, or lymphocyte subsets, can be accomplished by changing the concentration of leucoagglutinin in the culture system.

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CHAPTER 3

CYTOTOXIC LYMPHOCYTES IN PERIPHERAL BLOOD AND LUNG TUMOR TISSUE OF PATIENTS

Introduction

Human solid lung tumors often contain tumor infiltrating lymphocytes (TIL) (1), which may represent a favorable prognostic value (2,3) because they may exert an antitumor response (3,4). TIL isolated from most lung tumors comprise an enhanced percentage of the cytotoxic/suppressor $TCR\alpha\beta^+/CD3^+8^+$ lymphocyte subset as compared to PBL (5-7). $TCR^-/CD3^-16^+$ natural killer (NK) cells are virtually absent among lung TIL (7-9). Moreover, TIL, but not PBL, from lung tumor patients can show an increased expression of the activation markers CD25 (interleukin 2 [IL2] receptor) and/or HLA-DR (5-7).

Functional analysis has shown that fresh TIL and PBL exert little or no antitumor activity against autologous or allogeneic lung tumor cells (10-12). However, autologous lung tumor cells are efficiently lysed by $CD3^+$ enriched T lymphocytes (11,13). Others reported lysis of autologous lung tumor cells by large granular lymphocytes (LGL), isolated from PBL or pleural effusions (14,15). LGL reportedly represent $CD3^-$ NK cells (16). TIL from solid lung tumor tissue virtually display no NK lytic activity against K562 (7,10,17,18).

The in vivo escape of tumor cells may be due to a reduced number of cytotoxic lymphocytes in the tumor (9), or to an overall suppression of their activity as demonstrated for the response of lung TIL to PHA, which is suppressed by either autologous tumor cells (19), a subset of TIL (20), and possibly both.

Nevertheless, in vitro cloning and expansion of TIL and PBL from lung tumor patients, thereby excluding putative suppressive effects by particular subsets of lymphocytes, showed a poor mitogenic response

(8,21), especially the $TCR\alpha\beta^+/CD3^+8^+$ lymphocyte subset of TIL (22). This suppressed response of cloned lymphocytes suggests that this deficient proliferative capacity is intrinsic.

The small proportion of lymphocytes, however, that proliferated were $TCR^+/CD3^+$ and showed cytolytic activity to autologous and/or allogeneic tumor cells (23-25). No $TCR^-/CD3^-16^+$ NK cell clones were established.

Here we investigated the cytolytic activities and phenotypes of $TCR^-/CD3^-$ NK cells and $TCR^+/CD3^+$ T lymphocytes expanded from TIL and PBL obtained from healthy donors and lung tumor patients. Cytotoxicity against the NK-sensitive K562 (NK activity) and NK-resistant Daudi tumor cells (AK activity) were determined, as well as lectin dependent or antibody dependent cellular cytotoxic activity (LDCC and ADCC activity, respectively).

Because cloning and expansion of $TCR^-/CD3^-16^+$ NK cells is notoriously difficult, these cells were studied after culture in bulk under conditions which favor the expansion of TCR^- NK cells over other lymphocyte subsets (26,27). $TCR^+/CD3^+$ T lymphocytes were analysed after cloning and expansion in limiting dilution.

Results show that fresh and cultured PBL from 12 patients and 11 healthy donors have identical immunophenotypes. Also the lymphocyte subset composition of cytotoxic lymphocytes and their levels as well as types of cytotoxic activities were the same.

Fresh TIL displayed low NK or ADCC activities, whereas $TCR^-/CD3^-$ NK cell enriched bulk cultures displayed high levels of NK, AK or ADCC activities. Despite a significantly reduced plating efficiency of TIL as determined by limiting dilution, these lymphocyte clones showed comparable levels of cytotoxic activities as PBL clones either from patients or healthy donors.

We therefore conclude that TIL, but not PBL, derived from lung tumor patients are poor cytotoxic effector lymphocytes, because of the low proportion of $TCR^-/CD3^-$ NK cells as well as the low proportion of TCR^+ precursor CTL with proliferative capacity.

Materials and Methods

Patients and healthy donors

Twelve patients with a primary lung tumor, but no metastasis and no therapy before operation, were selected. Ten patients had moderately differentiated squamous lung cell carcinoma and 2 large cell carcinoma. Seven cases were classified as $T_2N_0M_0$ according to the TNM system (28), 1 case as $T_2N_1M_0$, 3 as $T_3N_0M_0$, and 1 as $T_3N_1M_0$. PBL and TIL were isolated from 12 patients, 11 male and 1 female. Eleven patients ranged in age from 60 to 76 and 1 patient was 27. PBL from 11 male healthy donors were used, ranging in age from 50 to 64.

Isolation of TIL

Single cell suspensions were made of mechanically and enzymatically dispersed fresh lung tumor tissue. Sections of 1–1.5 cm^3 were extensively washed with sterile RPMI 1640 (Flow Laboratories, UK), buffered with bicarbonate (2 g/l) and Hepes (4.8 g/l) and supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 IU/ml penicillin/streptomycin and 4 mM glutamine. Blood clots were removed with a scalpel and the tissue was further minced with scalpels and then forced through a nylon filter. The flow through was centrifuged, washed twice and resuspended in medium. In 2 cases the tissue remaining on the filter was washed in RPMI/Hepes plus antibiotics without serum and incubated for 10–20 min at 37°C with a mixture of collagenase (2 mg/ml), trypsine (2 mg/ml), and DNase (0.2 mg/ml) in RPMI/Hepes plus antibiotics. All chemicals were purchased from Sigma Chemical Co., St. Louis, USA. The suspension was again forced through a nylon filter and the flow through was washed twice in medium plus 10% FCS. Lymphoid and nonlymphoid cells were separated by centrifugation on a two-step Ficoll gradient (100% and 75% Ficoll-Isopaque, Pharmacia Fine Chemicals, Uppsala, Sweden) (29). The low density cell fraction was enriched for tumor cells and the high density cell fraction for lymphocytes. The viability of TIL exceeded 90% as determined by trypan blue exclusion. In only 6 cases the yield of viable TIL allowed phenotypical and/or functional analysis. Contamination with tumor cells ranged from 0–30% in these TIL fractions. After the isolation procedure, TIL were incubated for 16 h in RPMI/10% FCS at 37°C before use in experiments.

Isolation of PBL

PBL were isolated from healthy donors or lung tumor patients prior to surgery by centrifugation on Ficoll-Isopaque (d=1.077 g/cm³). The percentage of lymphocytes was determined by May-Grünwald Giemsa staining of cytospin preparations or by immunophenotyping using an anti-CD2 mAb. PBL were directly used or cryopreserved in liquid nitrogen.

In vitro lymphocyte cultures

TIL and PBL were cultured in bulk (3000/well) in 96 well round bottomed microtiter plates (Greiner Labor Technik, Phidelsheim, FRG) with γ-irradiated (25 Gy) feeder cells: allogeneic PBL ($2x10^4$ cells/well) and Epstein Barr virus (EBV) transformed lymphoblastoid B cell lines (10^4 cells/well) (21). RPMI 1640/Hepes culture medium, supplemented with 10% pooled human serum, 4 mM glutamine, 1 μg/ml indomethacin and 100 IU penicillin/streptomycin was added in a total volume of 200 μl per well. Two sources of IL2 were used; either 20% of the MLA144 cell line culture supernatant, which contained 100 U/ml IL2 as determined with the IL2 dependent murine cell line CTLL-2 (22), or 25 U/ml recombinant (rec) human IL2 (EuroCetus, Amsterdam, The Netherlands). To obtain selective proliferation of $TCR^-/CD3^-16^+$ NK cells, no lectin was added to the culture medium (27).

Plates were incubated at 37°C in 5% atmosphere of CO_2. Lymphocytes were harvested, counted and replated with new feeder cells in fresh medium at weekly intervals.

Cloning and expansion of lymphocytes

Lymphocyte clones were generated by limiting dilution as described previously (21). In short, PBL and TIL were plated at 0.5, 1, 3, 10, 30, 100 and 300 lymphocytes per well with culture medium plus 1 μg/ml leucoagglutinin (HA15, Pharmacia, Uppsala, Sweden) and irradiated feeder cells (as described above). Microtiter wells were microscopically screened for the presence of proliferating lymphocytes. The plating efficiency (PE) was calculated by the minimum χ^2 method from a Poisson distribution relationship between the logarithm of the percentage of negative wells and cell number (31). Between day 12 and 15, proliferating lymphocytes were transferred into 8-24 wells containing fresh

medium and new feeder cells. Thereafter, cloned lymphocytes were replated weekly.

Immunophenotype

Fresh; bulk cultured and cloned PBL and TIL were immunophenotyped using a panel of mAb: anti-CD2 mAb CLB T11.1 (CLB, Amsterdam, The Netherlands); anti-CD3, anti-CD4 and anti-CD8 mAb OKT3, OKT4 and OKT8 (Ortho Pharmaceutical, Raritan, NJ, USA); anti-CD16 mAb VD2 (CLB FcR gran 1, kindly provided by Dr. T. Huizinga, CLB, Amsterdam, The Netherlands); anti-CD25 mAb Tac (kindly provided by Dr. T. Uchiyama, Kyoto, Japan) and anti-HLA Class II mAb 7.5.10.1 (kindly provided by Dr. M. Jonker, TNO Rijswijk, The Netherlands). The fluorescein isothiocyanate conjugated goat anti-mouse IgG Ab was purchased from Nordic, Tilburg, The Netherlands. Lymphocytes were labeled with mAb and analysed with a fluorescence activated cell sorter (FACS II) (Becton Dickinson, Sunnyvale, California, USA) as described (32).

Determination of cytotoxicity

Cytotoxic activity was measured in triplicate in a 3-h ^{51}Cr release assay (32). A panel of histologically distinct tumor cell lines was used as target cells: K562, an erythromyeloid cell line; Daudi, a Burkitt's lymphoma derived cell line; and P815, a mouse mastocytoma derived cell line. Fresh tumor cells could not be used as target cells due to the low number of viable cells obtained. Lectin dependent cellular cytotoxic (LDCC) activity was determined by 15 min preincubation of ^{51}Cr labeled P815 cells with 100 $\mu g/ml$ leucoagglutinin followed by washing. Antibody dependent cellular cytotoxic (ADCC) activity was determined by preincubation of ^{51}Cr labeled P815 cells with rabbit anti-P815 Ab (1/40 final dilution of ascites), followed by washing. The standard error of the mean percentage lysis in triplicate determinations never exceeded 8% and was omitted for the clarity of presentation.

Results

Cytolysis by PBL and TIL

Twelve PBL fractions and 5 TIL fractions from lung tumor patients and 11 PBL fractions from healthy donors were tested for cytolytic activity. In order to compare the magnitude and type of cytolytic activities displayed by these lymphocyte fractions, LDCC, ADCC, NK and AK activity were determined. NK activity was tested against K562 cells, and NK-resistant Daudi cells were used to determine AK activity (for review, 33). PBL from both patients and healthy donors exerted similar ranges (5-60%) and average levels of NK, LDCC or ADCC activity, (Figure 3.1A). Moreover, all PBL fractions displayed low AK activity

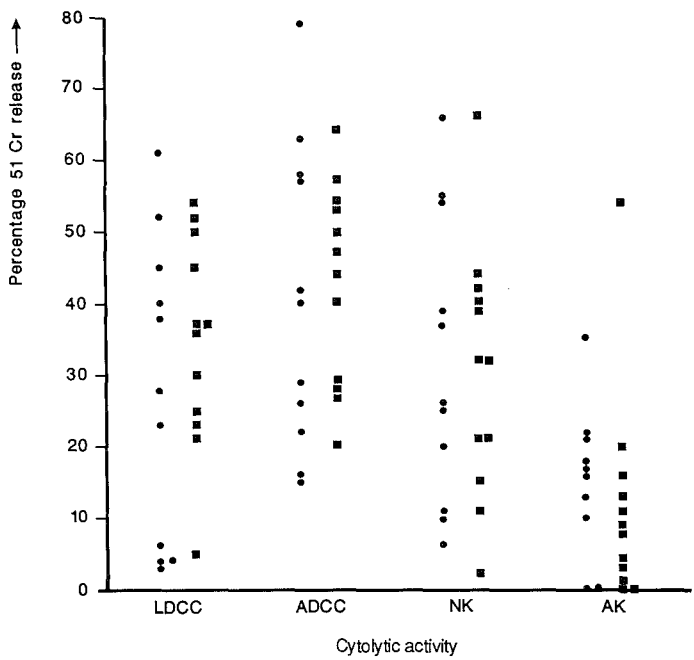

Figure 3.1A Cytolytic activity of PBL from 12 lung tumor patients (■) and 11 healthy donors (●). PBL from 1 healthy donor were not tested for AK activity. Effector to target cell (E/T) ratio was 27.

(range 0-20%). The 5 TIL preparations showed equal AK and LDCC activities as their autologous PBL (Figure 3.1B). In contrast, all 5 TIL preparations exhibited markedly lower levels of NK or ADCC activities than their autologous PBL or PBL derived from healthy donors.

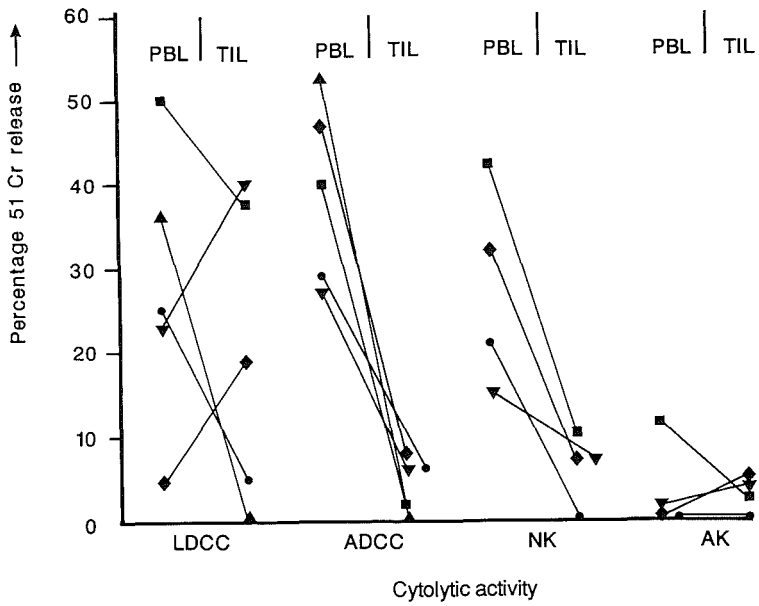

Figure 3.1B Cytolytic activity of fresh TIL from 5 lung tumor patients and their autologous PBL (■,▲,●,▼,◆). TIL from 1 patient (▲) were not tested for NK and AK activity. E/T ratio was 27.

Immunophenotype of PBL and TIL

PBL

One explanation for the variability in cytolytic activities between individuals is a difference in lymphocyte subset composition of PBL or TIL. Therefore, the percentages of CD3, CD4, CD8 and CD16 positive lymphocytes were determined. To identify activated lymphocytes, HLA-DR

and CD25 cell surface expression was determined. The range and mean percentages of CD3,4,8 and 16 positive lymphocytes in PBL from patients and healthy donors were about equal (Table 3.1). Also about equal percentages of lymphocytes expressed CD25 and HLA-DR ($\leq$ 7% and $\leq$ 12%), respectively (Table 3.1), indicating the presence of a low percentage of in vivo activated lymphocytes.

Table 3.1 PBL from lung tumor patients and healthy donors have similar phenotypes.

Surface antigen	PBL	
	Patients (n=8)[a]	Healthy donors (n=8)[a]
CD3	66 ± 7 (55-75)	68 ± 10 (54-78)
CD4	41 ± 5 (35-50)	47 ± 10 (28-60)
CD8	29 ± 9 (17-42)	27 ± 13 (18-53)
CD16	20 ± 9 (9-33)	19 ± 8 (6-29)
CD25	7 ± 3 (0-12)	5 ± 3 (0-10)
HLA-DR	10 ± 5 (0-18)	12 ± 3 (7-15)

[a] Data are expressed as mean percentage positive lymphocytes plus standard deviation. The range is given in parenthesis.

TIL

NK and ADCC activities are predominantly displayed by $TCR^-/CD3^-16^+$ lymphocytes, albeit that activated $TCR\gamma\delta^+/CD3^+$ T cells may also exert NK, AK or ADCC activities (34-36). The low NK or ADCC activity of TIL may therefore be due to a decreased proportion of $TCR^-/CD3^-16^+$ NK cells in TIL. From only 2 tumor specimens sufficient numbers of TIL were isolated to allow immunophenotypic analysis. $CD16^+$ NK cell containing lymphocytes could not be detected in these TIL fractions. In the 2 corresponding autologous PBL fractions, however, 11% and 23% $CD16^+$ lymphocytes were found, respectively. The $CD4^+/CD8^+$ lymphocyte ratio in TIL was considerably lower than that in autologous PBL (patient 1: 0.43 in TIL versus 1.19 in PBL; patient 2: 0.94 in TIL versus 1.68 in PBL). HLA-DR and CD25 expressing lymphocytes were not detected.

Functional and phenotypic analysis of *in vitro* activated and expanded PBL and TIL

Bulk cultured PBL

To investigate the phenotype and type of cytolytic activity of lymphocytes, PBL and TIL were cultured *in vitro*. Since it is difficult to establish and maintain $TCR^-/CD3^-16^+$ NK cell clones, PBL were cultured in bulk in IL2 containing medium plus irradiated allogeneic feeder cells but without leucoagglutinin, in order to selectively expand $TCR^-/CD3^-16^+$ NK cells (27).

After 2 weeks culture, in all except 1 case (Table 3.2A, patient 1), the initial percentage of $CD16^+$ lymphocytes (on average 20%) had increased (healthy donors: mean 60±11%, range, 40-75%; patients: mean 53±24%, range, 8-92%). In contrast, the initial percentage $TCR^+/CD3^+16^-$ lymphocytes (67%) had decreased to on average 40% and 29% in PBL from patients and healthy donors, respectively. All but one $CD16^+$ lymphocyte enriched fractions displayed high levels of NK, AK, LDCC or ADCC activities (more than 50% lysis at an E/T ratio of 27) (Table 3.2A).

Bulk cultured TIL

Because only 2 TIL fractions contained enough lymphocytes for immunophenotypic analysis, TIL from 6 patients were cultured in IL2 containing medium plus irradiated feeder cells but without leucoagglutinin, to determine whether the low NK or ADCC activity of TIL were due to a relatively low proportion of $TCR^-/CD3^-16^+$ lymphocytes.

Multiplication factors for TIL were 1.5-5-fold lower than for autologous PBL at 1 week of culture (data not shown). Interestingly, at 2 weeks of culture, the mean percentage $CD16^+$ lymphocytes in TIL was about 20% less than in autologous PBL (Table 3.2B), possibly due to a lower initial percentage of $CD16^+$ lymphocytes in TIL, as was found for 2 patients. These 2 TIL fractions virtually displayed no NK, ADCC or AK activities when cultured without leucoagglutinin. In contrast, the $CD16^+$ enriched lymphocyte fractions displayed high levels of NK, AK, LDCC or ADCC activities (Table 3.2B). Although the major subset of lymphocytes lysing K562 or Daudi target cells are $TCR^-/CD3^-16^+$ NK lymphocytes, various $TCR^+/CD3^+16^-$ T lymphocyte subsets may also lyse these target cells (33-35). Therefore, PBL and TIL were cloned to determine the percentages and subsets of precursor CTL as well as their type and level of cytotoxic activity.

Table 3.2A Cytolysis by $CD16^+$ enriched PBL from lung tumor patients is comparable to that of healthy donors.

	Phenotype[a]		Cytolytic activity[b]				
Patient	CD3	CD16	LDCC	P815	ADCC	NK	AK
1	80	8	73	3	51	9	1
2	30	62	52	10	50	56	6
3	53	50	45	0	38	52	23
4	3	72	46	66	70	56	75
5	35	50	59	62	76	67	72
6[c]	32	46	73	50	51	80	79
7	43	46	46	0	56	51	57
8	42	92	64	10	71	65	66
Mean ± SD	40±22	53±24	57±12	25±29	58±13	55±21	47±32
Healthy donors (n=7)	29±20	60±11	57± 8	24±25	58±11	61±14	48±34
Range	5–64	40–75	43–65	0–58	41–70	41–86	9–85

Table 3.2B Cytolysis by $CD16^+$ enriched TIL from lung tumor patients is comparable to that of $CD16^+$ enriched PBL from healthy donors.

	Phenotype[a]		Cytolytic activity[b]				
Patient	CD3	CD16	LDCC	P815	ADCC	NK	AK
1	72	0	77	5	33	17	1
6[c]	60	27[d]	59	45	44	85	79
7	82	0[d]	54	0	0	0	19
8	31	70	55	25	70	70	58
9	65	40	69	24	76	60	76
10	22	55	72	59	68	57	80
Mean ± SD	55±24	32±29	64±10	26±23	49±29	48±33	52±34

Lymphocytes were cultured for 2 weeks in IL2 containing medium plus allogeneic irradiated feeder cells without leucoagglutinin.

[a] Percentage positive cells.

[b] Percentage ^{51}Cr release at an E/T ratio of 27.

[c] Analysed after 1 week of culture.

[d] No initial $CD16^+$ lymphocytes were detected.

Immunophenotypic, proliferative and lytic properties of cloned PBL and TIL

PBL and TIL were cloned in limiting dilution in complete medium plus leucoagglutinin. Plating efficiencies were calculated using the Poisson formula and ranged from 9-59% and from 14-53% for PBL derived from patients and from healthy donors, respectively. The 2 TIL fractions had a lower plating efficiency (PE) than the corresponding PBL (patient 1: PE_{TIL} = 2%, PE_{PBL} = 9%; patient 2: PE_{TIL} = 17%, PE_{PBL} = 45%), which corresponded to the lower multiplication factors for bulk cultured TIL as compared to the corresponding autologous PBL bulk cultures.

About 20 clones of each donor were randomly picked for functional and phenotypic analysis. The majority of these clones from all healthy donors was $TCR\alpha\beta^+/CD3^+4^+$ (mean 70%±12%) (Table 3.3), of which only 20% exerted LDCC activity; about 25% was $TCR\alpha\beta^+/CD3^+8^+$ and virtually all

Table 3.3 Immunophenotypic and functional analysis of cloned PBL and TIL from lung tumor patients and healthy donors.

Experiment		Total number	Immunophenotype			
			% $TCR\alpha\beta^+/CD4^+$	% $TCR\alpha\beta^+/CD8^+$	% $TCR\gamma\delta^+/CD4^-8^-$	% lytic[a]
PBL (HD)	1	16	62	25	12	44
	2	20	70	25	5	35
	3	9	78	22	0	nt
	4	18	50	39[b]	11	61
	5	29	79	21	0	35
	10	23	78	22	0	39
PBL (P)	1	17	29	71	0	47
	2	20	85	10	5	25
	3	6	83	17	0	nt
	4	9	22	67	11	78
	5	28	18	54	28	82
	8	22	64	36	0	37
	10	26	85	8	8	24
TIL (P)	8	25	56	44	0	64
	10	29	34	62	0	73

HD = healthy donor; P = patient; nt = not tested.

[a] Lytic is defined as 10% or more ^{51}Cr release above spontaneous release at an E/T ratio of 10 in the LDCC assay.

[b] One clone is $TCR^-/CD3^-4^-8^\pm$.

displayed LDCC activity, but only occasionally NK activity. No AK activity was observed. In 3 out of 6 healthy donors also $TCR\gamma\delta^+/CD3^+4^-8^-$ clones were obtained and these all showed NK, AK, LDCC or ADCC activities (data not shown).

In contrast to healthy donors, the majority of PBL clones from 3 lung tumor patients were $TCR\alpha\beta^+/CD3^+8^+$. Again, only 20% of the $TCR\alpha\beta^+/CD3^+4^+$ clones showed LDCC activity and, occasionally, NK activity. The levels of cytolytic activities of all the $TCR\alpha\beta^+/CD3^+8^+$ clones were comparable to that of healthy donor derived $TCR\alpha\beta^+/CD3^+8^+$ clones. In PBL from 1 patient a high percentage of $TCR\gamma\delta^+/CD3^+4^-8^-$ clones (28%) was found (Table 3.3, experiment 5), which exerted NK, AK, LDCC and occasionally ADCC activities.

In 1 of the 2 TIL fractions studied, the number of $TCR\alpha\beta^+/CD3^+8^+$ clones was significantly increased as compared to the number derived from its autologous PBL.

Discussion

One of the mechanisms underlying the in vivo escape of tumor cells from the immunosurveillance may be an insufficient number of cytolytic lymphocytes that can be recruited. Alternatively, precursors of cytotoxic lymphocytes are present, but their cytotoxic potential becomes blocked due to suppressor mechanisms.

To address these questions fresh and cultured PBL as well as TIL from lung tumor patients were immunophenotypically and functionally analysed using mAb and by determination of NK, AK, LDCC and ADCC activities, respectively. Fresh PBL from lung tumor patients and healthy donors displayed similar phenotypic features. In contrast, fresh TIL contained no $CD16^+$ lymphocytes and the $CD4^+/CD8^+$ lymphocyte ratio was decreased as compared to their autologous PBL, in agreement with others (5-9). No lymphocytes were found in PBL or TIL expressing elevated levels of the activation markers CD25 and HLA-DR as compared to PBL from healthy donors, confirming the findings of Whiteside et al. (8). However, this contrasts with data obtained by others, who found more than 30% $CD25^+$ lymphoid cells in lung tumors, respectively (6,7). Differences in stage and histology of the lung tumors may explain these differences. However, also the different methods of detection, i.e. immunohistologic evaluations of lung tumor sections (6) versus iso-

lation of TIL by enzymatic processing of lung tumor tissue may account for the different results (this manuscript, 7). The lack of detectable levels of $CD25^+$, $HLA\text{-}DR^+$ or $CD16^+$ lymphocytes was not due to enzymatic digestion of the tumor tissue, because we obtained similar results with TIL derived from mechanically dispersed tumors. Moreover, although enzyme treatment decreased lymphocyte surface expression of CD16 and HLA-DR, it was fully recovered after incubation for 18 h in medium without enzymes (unpublished observations, 25).

In contrast to PBL, TIL exerted virtually no NK or ADCC activities. Firstly, NK and ADCC activities may be suppressed by tumor cells or subsets of TIL. Secondly, only a few $TCR^-/CD3^-16^+$ NK cells may be present in TIL (7,9, Table 3.2B), and those NK cells may be unable to lyse more than one target cell (18).

To exclude putative suppressive effects by tumor cells or subsets of TIL, and to phenotypically analyse the various types of cytotoxic lymphocytes and their precursors, PBL and TIL were cultured in bulk and cloned in limiting dilution and expanded (25). Since it is notoriously difficult to clone and expand $CD3^-16^+$ NK cells for prolonged periods of time, $CD16^+$ enriched lymphocyte bulk cultures were used (27). At 2 weeks of culture PBL were enriched for $CD16^+$ lymphocytes from initially 20% to 53% and from 20% to 60% for patients and healthy donors, respectively. However, TIL cultured in bulk for 2 weeks contained on average 32% $CD16^+$ NK cells, indicating that the proportion of $TCR^-/CD3^-16^+$ NK cells is relatively small in TIL, as indeed shown for 2 patients. In spite of the lower overall multiplication factor for bulk cultured TIL, there was no initial lag time of proliferation of $CD16^+$ lymphocytes from lung cancer, as was reported for $CD16^+$ TIL isolated from untreated primary squamous cell carcinomas of the head and neck (37).

$CD16^+$ enriched PBL from patients or healthy donors exerted similar, high levels of NK (55% and 61% lysis, respectively), AK (47% and 48% lysis, respectively) and ADCC activities (58% and 58% lysis, respectively). Unexpectedly, in spite of the lower percentage of $CD16^+$ TIL these lymphocytes exerted comparable levels of NK, AK and ADCC activities as control PBL, suggesting an enhanced recycling capacity, i.e. individual $CD16^+$ TIL can lyse more target cells per unit of time.

It should be noted that the percentage of lysis, also of K562 or Daudi cells, is the net effect of lytic activities of all subsets of lymphocytes and not only of $TCR^-/CD3^-16^+$ NK cells. Indeed, $TCR^+/CD3^+$

T lymphocyte subsets display MHC-unrestricted lysis upon activation in vitro (33,34,37). Limiting dilution analysis was therefore performed to determine the fraction size of these $TCR^+/CD3^+$ lymphocyte subsets and their cytotoxic activities.

The plating efficiency for PBL from healthy donors and patients ranged from 14-53% and 9-59%, respectively. TIL, however, showed a 3-4-fold lower plating efficiency than their autologous PBL. Although only 2 TIL fractions could be tested, our data are in agreement with those of others, who also found a 2-50-fold lower plating efficiency for lung TIL as compared to PBL (8,21). The majority of PBL clones derived from healthy donors were $TCR^+/CD3^+4^+$, but PBL clones of 3 out of 7 patients were $TCR^+/CD3^+8^+$. These contrasting data might be due to different subsets of lymphocytes within the $CD4^+$ or $CD8^+$ lymphocyte fractions, which differ in proliferative capacities.

Subset differences within the $CD4^+$ or $CD8^+$ lymphocyte fractions might also account for the higher proportions of $TCR^+/CD3^+8^+$ clones generated from TIL. In addition, this might be due to the higher initial percentage $CD8^+$ lymphocytes in TIL.

Despite the reduced plating efficiency of TIL, more than 60% of the established clones displayed LDCC activity and, occasionally, NK activity. On average 48% and 43% LDCC clones could be generated from autologous PBL and healthy donors respectively. Phenotypic analysis showed that virtually 100% $CD8^+$ clones and about 20% of the $CD4^+$ clones from TIL and PBL exerted LDCC activity, confirming data by Miescher et al. (22).

In conclusion, fresh TIL, but not PBL, from lung tumor patients, contain a low proportion of $TCR^-/CD3^-16^+$ NK cells. Moreover, $TCR\alpha\beta^+/CD3^+$ lymphocytes in TIL, but not in PBL, showed a decreased proliferative capacity in vitro, indicating that the proportion of in vivo TCR^+ precursor CTL that can be recruited may be reduced.

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CHAPTER 4

INDUCTION OF LYSIS BY TCR$\gamma\delta^+$/CD3$^+$ T LYMPHOCYTES VIA CD2 REQUIRES TRIGGERING VIA THE T11.1 EPITOPE ONLY

Introduction

The 50 kDa transmembrane glycoprotein CD2, originally identified as the sheep red blood cell (SRBC) receptor is also known as T11 or lymphocyte function associated antigen 2 (LFA-2). CD2 is involved in both cell-cell interactions and lymphocyte activation (1-5). CD2 is the earliest differentiation antigen to appear in human T cell ontogeny and is expressed on virtually all thymocytes, mature TCR$^+$/CD3$^+$ and TCR$^-$/CD3$^-$ NK cells (5-7). Three distinct epitopes, T11.1, T11.2 and T11.3 have been identified on the CD2 molecule (1,2). The T11.1 epitope is associated with the SRBC binding site whereas the T11.2 and T11.3 epitopes are not. Activation of TCR$\alpha\beta^+$/CD3$^+$ T cells through the "alternative" CD2 pathway requires two stimuli, i.e. a combination of two anti-CD2 mAb recognizing different CD2 epitopes (1,8-10) or a single anti-CD2 mAb plus either PMA (11) or submitogenic concentrations of PHA (3). The activation of T cells through CD2 may induce IL2 receptor (R) expression (1,12), IL2 production (10), IL2 dependent proliferation (2), and may trigger the cytolytic mechanism (3,13,14).

In contrast, activation of the cytolytic mechanism of TCR$^-$/CD3$^-$ NK cells via CD2 can be obtained by both a mitogenic combination of two anti-CD2 mAb (T11.2 and T11.3) (15) and by a single anti-CD2 mAb (3,16). Induction of cytotoxicity by a single anti-CD2 mAb however requires crosslinking of CD2 and CD16 (16,17). Thus activation triggered via CD2 may employ different pathways in TCR$\alpha\beta^+$/CD3$^+$ T cells and NK cells (2,3).

T cells expressing the TCR$\gamma\delta^+$/CD3$^+$ complex on their cell surface share characteristics with both TCR$^-$/CD3$^-$ NK cells and "classic"

$TCR\alpha\beta^+/CD3^+$ lymphocytes (18–20). All three lymphocyte subsets express CD2, can be induced to express IL2R, and subsequently show IL2 dependent proliferation. $TCR\gamma\delta^+/CD3^+$ cells resemble $TCR^-/CD3^-$ NK cells in the expression of FcγRIII (CD16), in the ability to exert FcγRIII mediated ADCC and, upon activation, in the ability to display MHC-unrestricted cytotoxic activity (18–20). $TCR\alpha\beta^+/CD3^+$ and $TCR\gamma\delta^+/CD3^+$ T cells both express polymorphic TCR that is noncovalently associated with CD3 proteins. These TCR are involved in antigen specific recognition (21,22) which may be MHC-restricted (21, Danuta Kozbor, personal communication).

In the present study we examined the requirements to trigger lysis via the CD2 activation site in human $TCR\gamma\delta^+/CD3^+$ clones. To this end we used bispecific Ab-heteroconjugates, containing a mAb directed against an activation site on the effector cell which is covalently coupled to an antibody directed against a target cell bound determinant. Bispecific mAb efficiently bridge target cells and effector cells and subsequently trigger the lytic machinery of the effector cell (23,24, 25,26).

In the experiments described herein we used an Ab-heteroconjugate containing anti-CD2 mAb crosslinked to anti-DNP Ab (anti-CD2 x anti-DNP) to target $TCR\gamma\delta^+/CD3^+$ clones against TNP coated target cells. It was demonstrated that heteroconjugates containing a single anti-CD2 mAb (OKT11) can induce cytotoxic activity in human $TCR\gamma\delta^+/CD3^+$ T cell clones, but not in $TCR\alpha\beta^+/CD3^+$ T cell clones, and that CD2 is functionally linked to the TCRγδ/CD3 pathway.

Materials and Methods

Cytotoxic clones

The following clones were used as effector cells in the cytotoxicity assay: the $TCR^-/CD2^+,3^-$ NK cell clones NK76 and NK77, obtained by limiting dilution of PBL from a patient with Tγ lymphocytosis (27); clone WIK ($TCR\gamma\delta^+/CD2^+3^+$), kindly provided by Dr. D.A. Hafler (Harvard Medical School, Boston, USA) (28); the $TCR\gamma\delta^+/CD2^+3^+$ clones AK1005 and AK4 and the $TCR\alpha\beta^+/CD2^+3^+$ clones D11 (Cw3 specific), G9 (Cw3), N+20 (Drw6), 609, 546 and 643, derived from PBL of healthy donors (29).

Clones were cultured in 96 well round bottomed microtiter plates (Greiner Labor Technik, Phidelsheim, FRG) as described (29). In short, 1-3x10^3 cloned cells/well were stimulated with 2x10^4 irradiated (25 Gy) allogeneic PBL plus 1x10^4 irradiated (25 Gy) EBV transformed lymphoblastoid B cell lines (BSM and APD) in a volume of 200 μl. The culture medium was RPMI 1640 (Flow Laboratories, Rockville, M.D.) buffered with bicarbonate (2 g/l) and HEPES (4.8 g/l) and supplemented with 10% non-heat-inactivated (pooled) human serum, 25 U/ml recombinant IL2 (EuroCetus, Amsterdam, The Netherlands), 4 mM glutamine, 100 IU/ml penicillin and streptomycin, 1 μg/ml indomethacin and 1 μg/ml leucoagglutinin (HA15, Pharmacia, Uppsala, Sweden). Clones were harvested and replated with new feeder cells and fresh medium at weekly intervals.

Target cells

A panel of histologically different tumor cell lines was used as target: APD, an EBV transformed B cell line; Licr-Lon, a leukemic plasma cell line; Raji, a B cell line derived from a patient with Burkitt's lymphoma, Molt-4, a T cell line; K562, an erythromyeloid cell line; GLC-2, a lung tumor derived cell line; IgR39, a melanoma derived cell line and Bonnet, a fibroblast cell line.

Antibodies

The following purified IgG antibodies and Fab fragments thereof were used to prepare heteroconjugates: OKT11 (IgG1), a mAb against the CD2 T11.1 epitope, obtained from Ortho Pharmaceutical Corp. (Raritan, NJ); OKT3 (IgG2a), a mAb against CD3 (30); w6/32 (IgG2a), a mAb against a common epitope on HLA Class I molecules (31) and purified rabbit anti-DNP antibodies (32). The Fab fragments of mAb, OKT11, w6/32 and anti-DNP were prepared by papain digestion and did not contain any intact antibody as judged by SDS page (32). Bispecific heteroconjugates were prepared with the use of the crosslinking reagent N-succinimidyl-3-(2-pyridyldithiol propionate) (SPDP) as described in detail (32). Heteroconjugates used in this study were: anti-CD2 x anti-DNP; anti-CD2 (Fab) x anti-DNP (Fab); anti-CD3 x anti-DNP; anti-HLA Class I x anti-DNP and anti-HLA Class I (Fab) x anti-DNP (Fab). mAb used for inhibition of cytotoxicity were OKT11 (anti-CD2) and CLB FcR gran 1

(VD2), a mAb against CD16, which was kindly provided by Dr. T. Huizinga (Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam, The Netherlands).

Assay for cytotoxic activity

Cytotoxic activity was measured in a standard 3-h ^{51}Cr release assay (20). Briefly, varying numbers of effector cells were seeded in triplicate in 75 μl/well in 96 well round bottomed microtiter plates. The Ab-heteroconjugate (75 μl) was added to the microtiter well at various concentrations, 30 min before addition of 100 μl ^{51}Cr-labeled target cells. Target cells were TNP modified by incubation with 0.3 mM trinitrobenzene sulfonate for 15 min at 37°C followed by washing. Ab-heteroconjugate induced cytotoxicity was blocked with mAb by the simultaneous addition of 50 μl mAb and 50 μl heteroconjugate to 50 μl effector cells 30 min prior to the addition of 100 μl target cells. Data are expressed as the mean percentage specific lysis in triplicate determinations. The standard error of the mean percentage lysis never exceeded 5% and was omitted for reasons of clarity of presentation.

Results

A single anti-CD2 mAb containing heteroconjugate shows differential activation of $TCR\gamma\delta^+$; $TCR\alpha\beta^+$ and TCR^- cloned lymphocytes

To analyse the ability of the CD2 and CD3 antigens to act as activation sites for the induction of cytotoxic activity in distinct subsets of cloned lymphocytes, anti-CD2 x anti-DNP and anti-CD3 x anti-DNP heteroconjugates were tested for their capacity to induce lysis of various TNP modified tumor cell lines. Anti-CD2 x anti-DNP heteroconjugate comprising the anti-T11.1 mAb, induced lysis of all TNP modified tumor cells by both $TCR^-/CD3^-$ NK cell clones and by $TCR\gamma\delta^+/CD3^+$ T cell clones but not by $TCR\alpha\beta^+/CD3^+$ T cell clones (Table 4.1). All clones expressed about equal levels of CD2 (Figure 4.1). The inability of anti-CD2 x anti-DNP heteroconjugate to retarget $TCR\alpha\beta^+/CD3^+$ T cell clones appears to be characteristic for all $TCR\alpha\beta^+/CD3^+$ lymphocytes, irrespective whether these cells express CD4 or CD8 and recognize HLA

Class II or HLA Class I determinants, respectively (Table 4.2). Even a fifty times higher concentration of anti-CD2 x anti-DNP heteroconjugate (5 μg/ml) did not trigger the lytic machinery in TCR$\alpha\beta^+$/CD3$^+$ cloned cells (data not shown): only lysis of the NK sensitive target K562 (Table 4.1) was moderately enhanced by anti-CD2 x anti-DNP heteroconjugate, but this was also observed using anti-HLA Class I x anti-DNP heteroconjugates probably resulting from increased conjugate formation of susceptible K562 targets to already lytic effector cells.

Table 4.1 Targeting of lysis mediated by cloned T and NK cells, by anti-CD2 and anti-CD3 containing Ab-heteroconjugates.

Effector clone	Addition	% lysis of TNP-target cells: Target cell K562[a]	Molt	APD	Raji	Licr-Lon	GLC-2	IgR39	Bonnet
TCR$\alpha\beta^+$/CD2$^+$3$^+$ (D11)	medium	23[b]	0	0	0	57[c]	0	0	1
	anti-CD3 x anti-DNP[d]	66	53	57	58	78	33	22	48
	anti-CD2 x anti-DNP	42	6	0	0	49	1	1	2
TCR$\gamma\delta^+$/CD2$^+$3$^+$ (AK4)	medium	11	9	0	19	1	15	6	13
	anti-CD3 x anti-DNP	60	52	10	66	47	44	16	36
	anti-CD2 x anti-DNP	61	60	49	80	56	60	65	51
TCR$^-$/CD2$^+$3$^-$ (NK76)	medium	69	51	3	73	3	38	1	13
	anti-CD2 x anti-DNP	71	71	54	85	76	72	58	65

[a] For histological origin of target cells see Materials and Methods.

[b] % specific lysis in a 3-h ^{51}Cr release assay is given at an E/T ratio of 9.

[c] Target Licr-Lon expresses the HLA Class I antigen (Cw3) specifically recognized by clone D11.

[d] Heteroconjugates (0.1 μg/ml, final concentration) were added to the effector cells 30 min before TNP-target cells.

Table 4.2 Anti-CD2 x anti-DNP Ab-heteroconjugate does not induce lysis by TCR$\alpha\beta^+$/CD3$^+$ T cell clones.

Effector clone	Addition	% lysis of TNP-tumor cells Licr-Lon	BSM	APD
TCR$\alpha\beta^+$/CD3$^+$ (609) CD8$^+$	-	1[a]	1	70[b]
	anti-CD3 x anti-DNP[c]	59	77	67
	anti-CD2 x anti-DNP	2	0	73
TCR$\alpha\beta^+$/CD3$^+$ (G9) CD8$^+$	-	24[b]	47[b]	2
	anti-CD3 x anti-DNP	56	80	78
	anti-CD2 x anti-DNP	30	60	5
TCR$\alpha\beta^+$/CD3$^+$ (546) CD8$^+$	-	7	13	1
	anti-CD3 x anti-DNP	77	70	71
	anti-CD2 x anti-DNP	8	15	3
TCR$\alpha\beta^+$/CD3$^+$ (N+20) CD4$^+$	-	1	2	52[b]
	anti-CD3 x anti-DNP	27	26	52
	anti-CD2 x anti-DNP	2	0	59
TCR$\alpha\beta^+$/CD3$^+$ (643) CD4$^+$	-	2	3	0
	anti-CD3 x anti-DNP	47	37	45
	anti-CD2 x anti-DNP	2	5	4

[a] Percent specific lysis in a 3-h ^{51}Cr release assay is given at an E/T ratio of 9.

[b] Target cells expressed the HLA antigens specifically recognized by that clone. Clone G9 is Cw3 specific; clone N+20 recognizes Drw6. The antigenic specificity of clones 609, 546 and 643 are unknown.

[c] Heteroconjugates (0.1 μg/ml, final concentration) were added to the effector cells 30 min before TNP coated target cells.

The anti-CD3 x anti-DNP heteroconjugate triggered the lytic machinery of both, TCR$\alpha\beta^+$/CD3$^+$ and TCR$\gamma\delta^+$/CD3$^+$ clones. As expected, TCR$^-$/CD3$^-$ NK cell clones could not be induced to lyse TNP modified target cells with anti-CD3 x anti-DNP heteroconjugate. Unmodified target cells were not lysed by cloned effector cells coated with heteroconjugates (data not shown). The level of lysis induced by heteroconjugate depended on the amount of heteroconjugate added (Figure 4.2). Concentrations as low as 4 ng/ml anti-CD2 x anti-DNP heteroconjugate were effective in triggering the lytic machinery, and half maximal lysis was achieved at about 15 ng/ml.

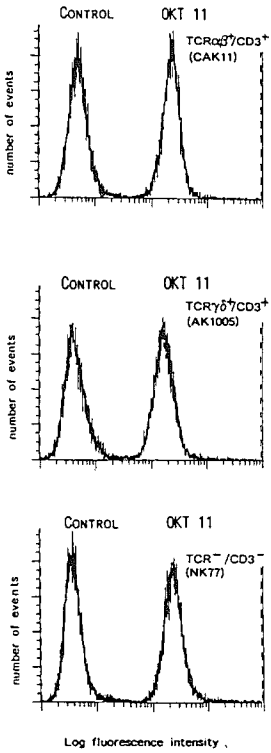
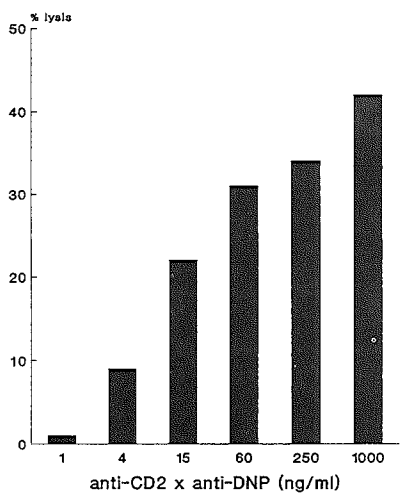


Figure 4.1 Cell surface expression of CD2 on cloned $TCR\alpha\beta^+/CD3^+$ (CAK11), $TCR\gamma\delta^+/CD3^+$ (AK1005), and $TCR^-/CD3^-$ (NK77) cells.

Clones were stained with anti-T11.1 mAb (OKT11, 1/100 dilution of ascites). FITC-conjugated goat anti-mouse Ig serum (dilution 1/40) (Nordic, Tilburg, The Netherlands) was used as second reagent. Cells were analysed with a FACScan (Becton Dickinson, Sunnyvale, California).

Figure 4.2 Induction of cytotoxic activity by anti-CD2 x anti-DNP Ab-heteroconjugate is dose dependent.

$TCR\gamma\delta^+CD3^+$ clone AK1005 was tested at E/T 30 for induction of lysis on TNP-coated melanoma cell line IgR39. Anti-CD2 x anti-DNP Ab-heteroconjugate was added to the effector cells 30 min before TNP-IgR39 target cells. The y-axis represents the percentage lysis above background lysis.



FcγRIII on TCRγδ$^+$/CD3$^+$ clones does not participate in lysis mediated by anti-CD2 x anti-DNP heteroconjugate

It has been reported that the activation of the lytic machinery of TCR$^-$/CD3$^-$ NK cells by a single anti-CD2 mAb required the participation of CD16 (FcγRIII), (17). Since some TCRγδ$^+$/CD3$^+$ clones express low levels of CD16 and mediate ADCC (19,20) it was possible that anti-CD2 x anti-DNP heteroconjugate induced cytotoxic activity by binding to FcγRIII on the TCRγδ$^+$/CD3$^+$ clones. Therefore heteroconjugates were prepared from Fab fragments. In TCRγδ$^+$/CD3$^+$ clones the anti-CD2 x anti-DNP and anti-CD2 (Fab) x anti-DNP (Fab) heteroconjugates induced comparable levels of lysis (Table 4.3). By contrast, anti-CD2 (Fab) x

Table 4.3 Anti-CD2 (T11.1) mAb induced lysis of TNP-targets by TCRγδ$^+$/ CD3$^+$ clones.

Phenotype	Effector clone	% lysis of TNP-IgR39 cells in the presence of		
		Medium	anti-CD2 x anti-DNP[a)]	anti-CD2 (Fab) x anti-DNP (Fab)
TCRγδ$^+$/CD3$^+$	(AK1005)	15[b)]	55	55
TCRγδ$^+$/CD3$^+$	(AK4)	13	44	41
TCRγδ$^+$/CD3$^+$	(WIK)	17	30	33
TCR$^-$/CD3$^-$	(NK76)	20	67	31

a) Heteroconjugates were added to the effector cells at a final concentration of 0.1 μg/ml 30 min before addition of TNP treated target cells.

b) % lysis at an E/T ratio of 24.

anti-DNP (Fab) heteroconjugate induced a small amount of lysis of TNP treated target cells by a TCR$^-$/CD3$^-$16$^+$ NK cell clone whereas the heteroconjugate prepared from intact antibody induced strong lysis. These results demonstrate that activation of TCRγδ$^+$/CD3$^+$ clones by anti-CD2 x anti-DNP heteroconjugate does not require interaction of the Fc portions of the heteroconjugate with FcγRIII on the effector cell. Further support for this notion comes from experiments in which we inhibited anti-CD2 x anti-DNP heteroconjugate induced cytolysis by

anti-CD2 or anti-CD16 mAb (Table 4.4). The anti-CD2 x anti-DNP heteroconjugate induced cytotoxic activity of $TCR^-/CD3^-$ NK cell clones was partly inhibited by both anti-CD2 and anti-CD16 mAb. In contrast, the heteroconjugate induced lytic activity of $TCR\gamma\delta^+/CD3^+$ T cell clones was only inhibited by anti-CD2 mAb but not by anti-CD16 mAb. Again, this shows that FcγRIII (CD16) is not involved in the anti-CD2 x anti-DNP heteroconjugate triggered cytotoxicity by the $TCR\gamma\delta^+/CD3^+$ T cell clones, but does participate in lysis mediated by the NK clone.

Table 4.4 Inhibition of anti-CD2 x anti-DNP heteroconjugate induced cytotoxicity in $TCR\gamma\delta^+/CD3^+$ clones by anti-CD2 but not by anti-CD16 mAb.

Addition of		% lysis of TNP-IgR39 by cloned effector cells							
Heteroconjugate	mAb	$TCR\gamma\delta^+/CD3^+$ (AK1005[a])		$TCR\gamma\delta^+/CD3^+$ (WIK)		$TCR\gamma\delta^+/CD3^+$ (AK4)		$TCR^-/CD3^-$ (NK77)	
		9[b]	27	12	24	15	30	12	24
none	none	7	16	7	16	17	33	10	23
none	anti-CD2[c]	3	7	5	12	16	22	6	12
none	anti-CD16	6	16	10	19	27	30	5	20
anti-CD2 x anti-DNP[d]	none	23	44	24	42	47	62	48	61
anti-CD2 x anti-DNP	anti-CD2	5	14	9	21	25	38	36	52
anti-CD2 x anti-DNP	anti-CD16	20	40	18	37	54	67	28	44

[a] Cloned effector cells, clones AK1005, AK4 and NK77 are $CD16^+$, clone WIK is $CD16^-$.

[b] E/T ratio.

[c] Anti-CD2 mAb (OKT11, 1:500 of ascites) and anti-CD16 mAb (VD2, 1:1000 of ascites) were added 30 min before addition of the target cells.

[d] Heteroconjugates were added to the effector cells at a final concentration of 0.1 μg/ml.

Only heteroconjugates directed against activation sites on the effector cell induce cytolysis

To establish whether the induction of lysis requires a mAb within the heteroconjugate directed against an activation site on the effector cell, we tested the cytotoxicity inducing capacities of heteroconju-

gates consisting of Fab fragments of either anti-CD2 or anti-HLA Class I crosslinked to anti-DNP (Fab). (Table 4.5). Anti-Class I (Fab) x anti-DNP (Fab) heteroconjugate did not induce cytotoxic activity in either $TCR\gamma\delta^+/CD3^+$ or $TCR^-/CD3^-$ clones. The lack of induction of cytotoxicity in both types of clones by anti-Class I (Fab) x anti-DNP (Fab) demonstrates that not all cell surface molecules on the effector cell are able to transduce activation signals. In contrast to $TCR\gamma\delta^+/CD3^+$ clones, in $TCR^-/CD3^-$ NK cell clones high levels of cytotoxicity could be induced by anti-Class I x anti-DNP but not by the heteroconjugated Fab fragments. This suggests that the anti-Class I x anti-DNP induced cytotoxicity in the $TCR^-/CD3^-$ NK cell clone is caused by interactions of Fc parts of the heteroconjugate with FcγRIII (CD16) on the surface of the $TCR^-/CD3^-$ NK cells. This activity was not detected in $TCR\gamma\delta^+/CD3^+$ clones, although low levels of CD16 were expressed by $TCR\gamma\delta^+/CD3^+$ clones AK4 and AK1005.

Table 4.5 Specificity of anti-CD2 x anti-DNP heteroconjugate induced cytolysis.

Addition of heteroconjugate	% lysis of TNP-IgR39 by cloned effector cells			
	$TCR\gamma\delta^+/CD3^+$ (AK1005)	$TCR\gamma\delta^+/CD3^+$ (WIK)	$TCR\gamma\delta^+/CD3^+$ (AK4)	$TCR^-/CD3^-$ (NK77)
none	26[a]	23	12	19
anti-CD2 x anti-DNP[b]	67	50	52	74
anti-Class I x anti-DNP	25	25	13	61
anti-Class I (Fab) x anti-DNP (Fab)	23	24	12	21

[a] % specific lysis at an E/T ratio of 15.

[b] Heteroconjugates were added to the effector cells at a final concentration of 0.1 μg/ml 30 min before addition of target cells.

CD2 and CD3 activation pathways in $TCR\gamma\delta^+/CD3^+$ cells are functionally linked

In $TCR\alpha\beta^+/CD3^+$ cells the activation through CD2 by a combination of two anti-CD2 mAb is interconnected with the CD3 activation pathway (3,14,33,34). Therefore we examined whether in $TCR\gamma\delta^+/CD3^+$ clones, the

CD2 and CD3 activation pathways are also functionally linked. For that purpose, we tested the effect of anti-CD3 mAb on the cytotoxicity induced by anti-CD2 x anti-DNP heteroconjugate. Addition of anti-CD3 mAb blocked the anti-CD2 x anti-DNP heteroconjugate induced cytotoxicity of $TCR\gamma\delta^+/CD3^+$ clones (Table 4.6). Of note, the baseline cytotoxic activity was also inhibited. This suggests that in $TCR\gamma\delta^+/CD3^+$ T cells the CD3 and CD2 activation pathways are functionally linked. Moreover, CD3 also appears to be involved, either directly or indirectly, in the regulation of the MHC-unrestricted cytotoxic activity of $TCR\gamma\delta^+/CD3^+$ T cell clones.

Table 4.6 Functional linkage between CD2 and CD3 activation pathways in $TCR\gamma\delta^+/CD3^+$ clones.

Addition of antibodies	% lysis of TNP-IgR39	
	$TCR\gamma\delta^+/CD3^+$ (AK4)	$TCR\gamma\delta^+/CD3^+$ (WIK)
none	32[a]	18
anti-CD2 (Fab) x anti-DNP (Fab)[b]	67	56
anti-CD2 (Fab) x anti-DNP (Fab) + anti-CD3[c]	15	14
anti-CD3	9	3

[a] % lysis at E/T ratio of 15.

[b] Heteroconjugate (0.1 μg/ml) was added to effectors 30 min before target cells.

[c] Anti-CD3 mAb (CLB T3/4. E, 1/5000 final concentration) was added simultaneously with the heteroconjugate.

Discussion

In the present study, the requirements for activation through CD2 in $TCR\gamma\delta^+/CD3^+$ clones was examined. The results demonstrate that in $TCR\gamma\delta^+/CD3^+$ cells the requirements for activation via CD2 qualitatively differ from those in $TCR\alpha\beta^+/CD3^+$ cells and $TCR^-/CD3^-$ NK cells. Contrary to the CD2 activation requirements in $TCR\alpha\beta^+/CD3^+$ cells, cytotoxic activity in $TCR\gamma\delta^+/CD3^+$ clones can be induced by heteroconjugates containing a single anti-CD2 mAb, namely anti-T11.1. Activation of

$TCR\gamma\delta^+/CD3^+$ clones via CD2 is independent of the FcγRIII (CD16) because the anti-CD2 (Fab) x anti-DNP (Fab) heteroconjugate was as active as the heteroconjugate prepared from intact antibodies. Moreover, anti-FcγRIII did not inhibit anti-CD2 x anti-DNP heteroconjugate induced cytotoxicity in $TCR\gamma\delta^+/CD3^+$ clones. In $TCR^-/CD3^-$ NK cell clones, however, the induction of cytotoxicity by anti-CD2 x anti-DNP heteroconjugate depended on FcγRIII (CD16) because the anti-CD2 (Fab) x anti-DNP (Fab) heteroconjugate induced considerably lower levels of lysis than the heteroconjugate prepared from intact antibodies. In addition, lysis induced by intact heteroconjugate was partially blocked by mAb against CD16 or CD2. Anti-CD2 mAb only competes with the binding of Fab parts of the heteroconjugate with CD2 on the effector cell surface but not with the interaction of IgG-Fc parts of the heteroconjugate with CD16. Therefore, anti-CD2 mAb only partially inhibits the heteroconjugate induced lysis by $TCR^-/CD3^-$ NK cells. Anti-HLA Class I x anti-DNP heteroconjugate was unable to promote lysis by the $TCR\gamma\delta^+/CD3^+$ clones (Table 4.5), indicating that not any cell surface structure on the $TCR\gamma\delta^+/CD3^+$ clones will serve as signal transducer for the induction of cytotoxicity.

How can the different activation requirements via CD2 in $TCR\alpha\beta^+/CD3^+$, $TCR\gamma\delta^+/CD3^+$ and $TCR^-/CD3^-$ lymphocytes be explained? The densities of the CD2 on the cell surfaces of the various distinct clones were similar as determined by flow cytometry (Figure 4.1) and hence provide no explanation. The differences in activation requirements may however be related with the interrelation of CD2 with other cell surface structures functionally or physically. We and others have shown that in $TCR\alpha\beta^+/CD3^+$ cells, the CD2 and CD3 activation pathways are functionally linked (3,14,33,34), and that in NK clones, the CD2 and CD16 activation pathways are interconnected (16). In the $TCR\gamma\delta^+/CD3^+$ clones the CD2 activation pathway is functionally linked to the CD3 pathway (Table 4.6), but not to the CD16 pathway (Table 4.4). The lack of detectable interplay between CD2 and CD16 may be due to the low level of CD16 expression on $TCR\gamma\delta^+/CD3^+$ clones as compared to $TCR^-/CD3^-$ NK cell clones (20). It remains to be established whether such functional linkage can occur when CD16 expression on $TCR\gamma\delta^+/CD3^+$ clones is enhanced, e.g. by cytokines (under investigation). Anti-CD3 mAb inhibited CD2 mediated activation of cytotoxicity. Such inhibition may result from blocking of a common intracellular pathway utilized in both CD2 and CD3 mediated activation. Alternatively, anti-CD3 mAb may

deliver a "negative" signal which interferes non-specifically with multiple forms of transmembrane signaling which do not normally use components of the CD3 activation pathway.

In conclusion, these results indicate that induction of cytotoxic activity via the CD2 antigen has different activation requirements, involving different epitopes and possibly different mechanisms in $TCR\alpha\beta^+/CD3^+$, $TCR\gamma\delta^+/CD3^+$ T cells and $TCR^-/CD3^-$ NK cells. The study of these various activation molecules and pathways with bifunctional heteroconjugates is not merely of academic interest. Tumor cells are heterogeneous and may therefore show differential susceptibilities to $TCR\alpha\beta^+/CD3^+$, $TCR\gamma\delta^+/CD3^+$ or $TCR^-/CD3^-$ lymphocytes, respectively. The availability of various bifunctional Ab preparations with distinct specificities may allow the selective activation, homing and lymphokine production of only (a) selected subset(s) of activated lymphocytes. Anti-CD3 containing heteroconjugate only activates $TCR\gamma\delta^+/CD3^+$ and $TCR\alpha\beta^+/CD3^+$ lymphocytes but not $TCR^-/CD3^-$ NK cells (25,35). Anti-CD16 containing heteroconjugate predominantly activates $TCR^-/CD3^-$ NK cells (24,32). Here it is shown that anti-T11.1 containing heteroconjugate triggers lysis by $TCR\gamma\delta^+/CD3^+$ and to a lesser extent by NK cells, whereas $TCR\alpha\beta^+/CD3^+$ cells remain unactivated. Finally, both subsets of T cells and NK cells can be activated by a combination of heteroconjugates containing both anti-T11.2 and anti-T11.3 specificities (35,36).

The selective activation of subsets of cytotoxic lymphocytes may induce different repertoires of lymphokines and cytolytic activities, and thereby may affect the host's local immune response at the tumor site. Therefore these basic studies could help direct the design of future generations of immunotherapy of cancer.

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CHAPTER 5

LFA-1/ICAM-1 INTERACTION COACTIVATES THE CD3/T CELL RECEPTOR COMPLEX IN TRIGGERING CELL MEDIATED LYSIS

Introduction

T cells can be activated when the antigen specific TCR interacts with non-self MHC proteins or with foreign antigen bound to self MHC proteins (1,2). T lymphocytes may also be activated via nonpolymorphic cell surface receptors such as CD2, CD3 or CD16 interacting with their ligands or mAb (3-6). Recent evidence suggests that these activation sites are functionally interrelated, for instance, activation via CD2 can be regulated via CD3 and vice versa (5,6).

The interaction between CTL and the relevant target cells results in target cell lysis by a multistep process (2,7). The initial adhesion step is the rapid formation of an intercellular bond (conjugate formation) which is antigen independent. Two major pathways of adhesion have been identified. First, LFA-1 on CTL interacts with intercellular adhesion molecule-1 (ICAM-1) on the target cell (8,9). Second, the effector cell CD2 interacts with LFA-3 on the target cell (10-13). Subsequently, antigen recognition via the TCR results in triggering of the CTL lytic machinery and target cell lysis (2).

LFA-1 is a transmembrane heterodimer composed of a 180 kDa α chain (CD11a) and a 95 kDa β chain (CD18) which is expressed by most leucocytes (14-16). In addition to its adhesion function, LFA-1 has also been suggested to be involved in T cell activation, possibly as a signal transduction molecule, as reported for CD2 (13). Binding of anti-LFA-1α or anti-LFA-1β mAb to T cells has been shown to up- or down-regulate, respectively, anti-CD3 mAb triggered T cell proliferation in the absence of intercellular adhesion (17). In mice, anti-LFA-1α mAb enhances proliferative responses of preactivated T and B lymphocytes

(18,19). Finally, a unique anti-LFA-1α mAb has been found to induce homotypic cell-cell interactions (20).

ICAM-1 is a monomeric 90-114 kDa glycoprotein which can be expressed on cells of many lineages, especially at specific differentiation stages or after exposure of cells to certain inflammatory mediators in vitro or in vivo (21). Biochemical and functional studies have conclusively demonstrated that ICAM-1 is one of the ligands for LFA-1 (9,22-25). Target cell ICAM-1 is involved in adhesion to and lysis of target cells; however, demonstration of this role is restricted to target cells which express ICAM-1 as the major LFA-1 ligand (24). Furthermore, ICAM-1 is a major contributor to a variety of other T cell responses dependent on cell-cell interaction (26-29). A previously suggested second ligand for LFA-1, i.e. ICAM-2, has recently been identified (30). However, at this point it is unclear whether binding of the LFA-1 to the ICAM-1 ligand molecule, not only facilitates the adhesion of the effector cell to the target cell, but also mediates signals that regulate CTL activation.

Recent reports have demonstrated that lysis resistant Burkitt's lymphoma cells express low levels of LFA-1, LFA-3 and ICAM-1 (31,32). It was shown that Epstein Barr virus (EBV) positive biopsy cells with a low expression of these molecules were relatively resistant to lysis by specific CTL. Prolonged in vitro culture of these cells induced an increased expression of the molecules which was associated with a more efficient interaction with specific CTL. These findings prompted speculation that the lack of expression of ICAM-1 and other adhesion molecules on tumor cells contributes to their escape of immunosurveillance (31,33,34).

The importance of the LFA-1/ICAM-1 interaction led us to further study its involvement in CTL/target cell conjugate formation and triggering of lysis. To this end, ICAM-1 positive or negative tumor cell lines were used as target cells and bispecific Ab-heteroconjugates, containing an anti-CD3 mAb which is covalently coupled to an anti-DNP Ab. Such bispecific reagents efficiently bridge effector cells and TNP modified target cells and simultaneously trigger the lytic machinery of the effector cell (6,35). The data demonstrate that induction of ICAM-1 expression on target cells enhances their susceptibility to CD3 triggered lysis. Associated findings suggest that this costimulatory effect results from enhanced conjugate formation as well as enhanced signal transduction by LFA-1/ICAM-1 interactions.

Materials and Methods

Cytotoxic clones

CTL clones were obtained by limiting dilution cloning under polyclonal activation conditions as we described earlier (5). In short, CTL clones were expanded in 96 well round bottomed microtiter plates (Greiner Labor Technik, Phidelsheim, FRG). One to $3x10^3$ cloned CTL per well were stimulated with a mixture of irradiated (25 Gy) allogeneic PBL ($2x10^4$) and $1x10^4$ Epstein Barr virus (EBV) transformed lymphoblastoid B cell lines (BSM and APD) in a final volume of 200 μl. The culture medium was RPMI 1640 (Flow Laboratories, Rockville, MD) buffered with bicarbonate (2 g/l) and HEPES (4.8 g/l) and supplemented with 10% non-heat-inactivated (pooled) human serum, 25 U/ml rec IL2 (EuroCetus, Amsterdam, The Netherlands), 4 mM glutamine, 100 IU/ml penicillin and streptomycin, 1 μg/ml indomethacin and 1 μg/ml leucoagglutinin (HA15, Pharmacia, Uppsala, Sweden). Clones were harvested and replated with new feeder cells and fresh medium at weekly intervals.

Antibodies

Bispecific hetero-crosslinked Ab were prepared with the use of the crosslinking reagent N-succinimidyl-3-(2-pyridyldithiol propionate) (SPDP) as described in detail elsewhere (35). Hetero-crosslinked Ab used in this study were: anti-CD3 (OKT3) x anti-DNP; anti-CD18 (MHM23) x anti-DNP and anti-HLA Class I (w6/32) x anti-DNP mAb. Anti-DNP Ab were purified polyclonal rabbit Ab. mAb used for the inhibition of lymphocyte functions and for immunofluorescence staining were: CLB54, (anti-CD18) (36), specific for the LFA-1β chain, a generous gift from Dr. R.A.W. van Lier (CLB, Amsterdam, The Netherlands); mAb 84H10, (anti-ICAM-1) (9) and w6/32 (anti-HLA Class I common epitope) (37).

Cytotoxicity assay

Cytotoxic activity was measured in a standard 3-h ^{51}Cr release assay (5,6). Briefly, varying numbers of effector cells were added in triplicate in 75 μl/well in 96 well round bottomed microtiter plates. The hetero-crosslinked Ab (75 μl) was added to the microtiter well at the

indicated concentrations 30 min before addition of ^{51}Cr-labeled target cells (100 μl). Target cells were incubated with 0.3 mM trinitrobenzene sulfonate (TNP) for 15 min at 37°C followed by washing. ICAM-1 expression on IgR39 tumor cells was enhanced by incubation with 1000 U/ml recombinant human TNF (recTNF) (Cetus Corp., Emmeryville, California, USA) for 24 h. Tumor cell lines used as target cells in this study include IgR39 and IgR37, melanoma derived cell lines; Licr-Lon, a leukemic plasma cell line; Molt-4, a T cell line, APD, an EBV transformed B cell line; K562, an erythromyeloid cell line and U937, a monocytic cell line (5).

Hetero-crosslinked Ab-induced cytotoxicity was blocked with mAb by the simultaneous addition of 50 μl blocking mAb and 50 μl heteroconjugated Ab to 50 μl effector cells 30 min prior to the addition of 100 μl target cells, unless indicated otherwise. The standard error of the mean percentage lysis never exceeded 5% and was omitted for clarity of presentation.

Conjugate formation assay

Antigen independent and hetero-crosslinked Ab-induced conjugate formation of CTL and target cells was assessed by FACS analysis (Becton Dickinson, Mountain View, CA, USA) (9,38,39). CTL ($5x10^6$) were labeled with 100 μg/ml hydroethidine in a volume of 2 ml at 37°C for 25 min. Target cells ($5x10^6$) were first modified with TNP (see above) and then labeled with 0.05 μg/ml carboxy fluorescein diacetate (CFDA) in a volume of 2 ml at 37°C for 25 min. CTL and target cells were washed before use. Twenty microliters of labeled effector cells (10^7/ml) were mixed with an equal amount of labeled target cells (10^7/ml) with and without Ab-heteroconjugates and/or blocking mAb. Ab-heteroconjugates and mAb were preincubated with the CTL for 15 min at 37°C before the addition of tumor target cells. The final volume was adjusted to 100 μl. Conjugate assay medium was RPMI 1640 supplemented with 10% FCS. CTL and target cell mixtures were centrifuged for 5 sec in a serofuge and conjugates were allowed to form at 21°C for 10 min. Conjugate formation was stopped by the addition of 1 ml ice-cold PBS + 1% BSA. Cells were immediately resuspended and analysed on a FACScan. Ten thousand total fluorescent events were collected and the number of events emitting simultaneously red and green fluorescence (conjugates) was divided by the total number of events emitting red fluorescence (total effector

cells) yielding the percentage of CTL that had formed conjugates. No conjugates were observed when CTL and target cells were mixed and analysed immediately.

Immunofluorescence assay

Five x 10^5 cells were pelleted and incubated with 10-20 μl mAb at 4°C for 30 min. After washing, the cells were incubated with 10 μl 1:40 diluted goat anti-mouse Ab, conjugated with FITC (Nordic, Tilburg, The Netherlands). The cells were washed twice with PBS + 1% BSA + 0.01% Sodium Azide and analysed on the FACScan.

Results

Melanoma IgR39 is relatively resistant to CD3 mediated lysis

A panel of histologically distinct tumor cell lines were compared for their susceptibility to lysis by TCR$\alpha\beta^+$/CD3$^+$ and TCR$\gamma\delta^+$/CD3$^+$ CTL clones triggered via CD3, using hetero-crosslinked anti-CD3 x anti-DNP. Most tumor cell lines were susceptible to CD3 mediated lysis (Figure 5.1). The melanoma derived cell line IgR39 however showed a relative resistance to CD3 mediated lysis. This was found for both TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$ CTL and hence is independent of the sublineage of the CTL.

The level of CD3 mediated lysis depended on the amount of anti-CD3 added. At higher anti-CD3 concentrations (15 ng/ml or more) the tumor cell line IgR39 was effectively lysed (Figure 5.1). Thus, the relative resistance to CD3 mediated lysis by cloned TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$ CTL can be overcome at higher effective densities of anti-CD3 mAb on the target cells.

Expression of cellular adhesion molecules on tumor cells

Because the relative resistance of IgR39 target cells to CD3 mediated lysis was independent of the CTL clone used, it was hypothesized that the composition of surface molecules on the target cells, particularly the known ligands for CTL/target cell interaction ICAM-1 and LFA-3, determined its relative resistance to CD3 mediated lysis by CTL.

The TNP modification of the IgR39 cells was not the cause of the relative resistance because IgR39 target cells reportedly showed comparable levels of sensitivity to CD2 mediated lysis as other tumor cell lines (6). The expression of LFA-3 and ICAM-1 on the tumor cell lines was determined. Contrary to the panel of other cultured tumor cells tested in Figure 5.1, the IgR39 cells lacked surface expression of ICAM-1 (Figure 5.2). All human tumor cell lines tested here expressed LFA-3. TNP modification of the tumor cell lines did not affect expression of ICAM-1 and LFA-3 (data not shown).

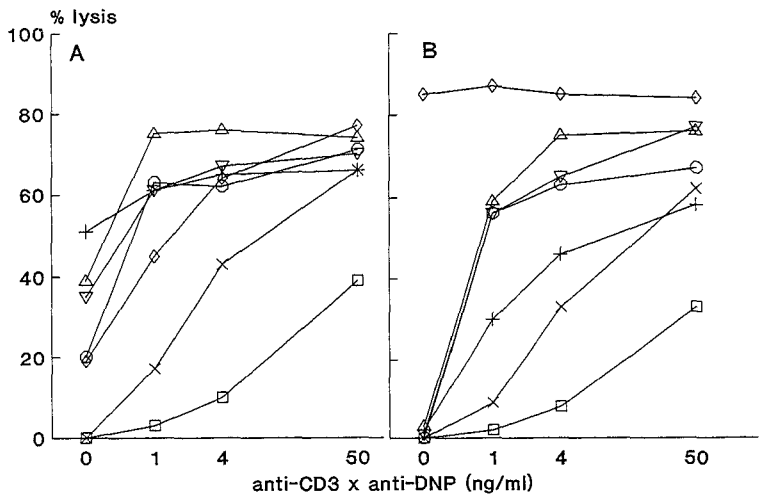

Figure 5.1 IgR39 melanoma cell line is relatively resistant to CD3 mediated lysis by $TCR\gamma\delta^+/CD3^+$ clone WiK (panel A) or $TCR\alpha\beta^+/CD3^+$ clone D11 (panel B).

TNP modified tumor cell lines used as target cells were: □, IgR39; +, IgR37; x, APD; △, Molt-4; ◇, Licr-Lon; ▽, K562 and O, U937. Target cell lysis was induced by addition of the indicated concentration hetero-crosslinked anti-CD3 mAb. Target cell line Licr-Lon expressed the HLA Class I antigen Cw3, specifically recognized by $TCR\alpha\beta^+/CD3^+$ clone D11. E/T ratio was 10.

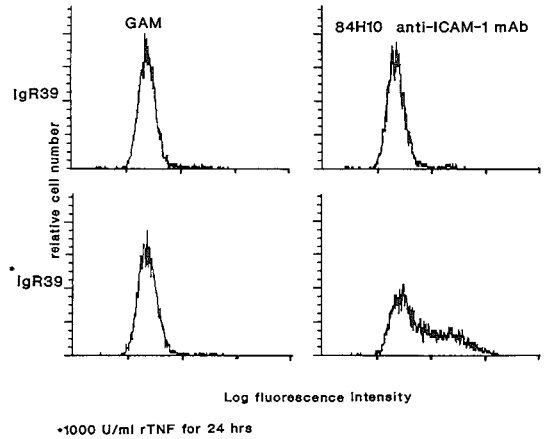

Figure 5.2 TNF induces ICAM-1 expression on the ICAM-1$^-$ melanoma cell line IgR39.

IgR39 was cultured in the presence of 1000 U/ml recTNF for 24 h.

The "resistance" of IgR39 target cells to CD3 mediated lysis is due to lack of ICAM-1 expression

To assess the functional correlation between lack of ICAM-1 expression and "resistance" to CD3 mediated lysis, TNF was used which is known to induce ICAM-1 expression on many cell types (21). Incubation of IgR39 cells with 1000 U/ml recTNF for 24 h induced ICAM-1 expression as determined by FACS-analysis (Figure 5.2). This expression of ICAM-1 was accompanied by a simultaneous increase in susceptibility to CD3 mediated lysis (Figure 5.3). Definitive proof that the lack of ICAM-1 is the critical factor in the "resistance" to CD3 mediated lysis of IgR39 target cells comes from anti-ICAM-1 mAb inhibition studies. ICAM-1 specific mAb abolished the, by TNF treatment obtained, increased levels of CD3 mediated lysis of IgR39 target cells (Figure 5.3). The level of lysis of untreated IgR39 cells was, as expected, not affected by anti-ICAM-1 mAb. Thus, TNF induced expression of ICAM-1 is sufficient to account for the TNF induced increase in susceptibility of IgR39 target cells to CD3 mediated CTL lysis.

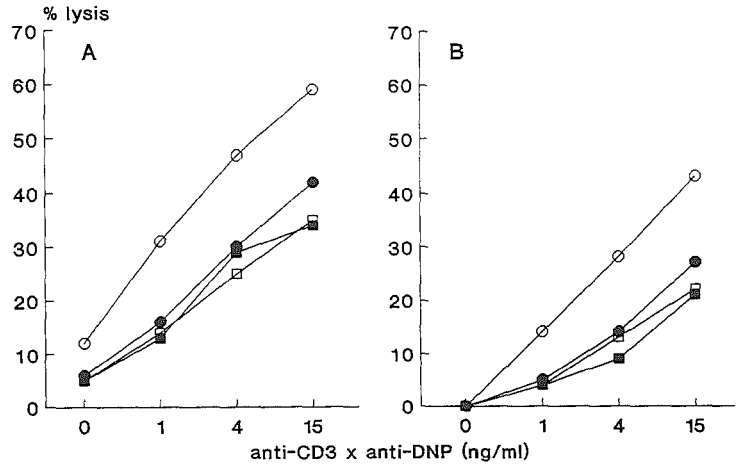

Figure 5.3 Anti-ICAM-1 mAb abolishes the increased susceptibility of TNF treated IgR39 cells to CD3 mediated lysis by $TCR\gamma\delta^+$/$CD3^+$ clone WiK (panel A) or $TCR\alpha\beta^+/CD3^+$ clone D11 (panel B).

IgR39 cells were cultured with or without 1000 U/ml recTNF for 24 h and TNP modified before use as target cells. Hetero-crosslinked anti-CD3 mAb and anti-ICAM-1 mAb (84 H10, dilution 1/1000 of ascites) were added simultaneously to the CTL effectors prior to the addition of target cells □, IgR39; ■, IgR39 + anti-ICAM-1 mAb; ○, TNF treated IgR39 and ●, TNF treated IgR39 + anti-ICAM-1 mAb. E/T ratio was 10.

Signaling properties of LFA-1 in CTL activation

To examine the signaling abilities of LFA-1 in the activation of the lytic machinery of CTL, the effect of anti-CD18 (LFA-1β) mAb on spontaneous and anti-CD3 mAb induced conjugate formation and cytolysis was tested. As shown in Table 5.1, anti-CD18 mAb inhibited spontaneous conjugate formation of CTL with $ICAM\text{-}1^+$ Licr-Lon cells but minimally with $ICAM\text{-}1^-$ IgR39 cells. The latter suggests that IgR39 cells also lack sufficient expression of alternative ligands for LFA-1 (e.g. ICAM-2) (30). Anti-CD3 mAb containing heteroconjugated Ab enhanced the percent conjugates formed between CTL and tumor cells. CD18 specific

Table 5.1 Inhibition of conjugate formation by anti-CD18 (LFA-1β) mAb.

Addition of heteroconjugate[b]	Anti-CD18 mAb[c]	% conjugates between TCRγδ+ CTL[a] and TNP-target cells	
		ICAM-1+ (Licr-Lon)	ICAM-1- (IgR39)
-	-	34	23
-	+	17	19
anti-CD3 x anti-DNP	-	52	35
anti-CD3 x anti-DNP	+	48	31

[a] TCRγδ+ effector cell: WiK.

[b] Heteroconjugate concentration 1 μg/ml.

[c] Anti-CD18 (CLB54 ascites, 1/1000).

[d] For calculation of % conjugates see Materials and Methods.

mAb had virtually no inhibitory effect on CD3 mediated conjugate formation of CTL to ICAM-1+ or ICAM-1- tumor cells (Table 5.1), but did inhibit CD3 mediated lysis of the ICAM-1+ or ICAM-1- target cells (Figure 5.4). This significant inhibitory effect of CD18 specific mAb on lysis contrasts with the marginal inhibitory effects on conjugate formation. Anti-CD18 mAb even inhibited CD3 mediated lysis when added 15 min after the addition of target cells, at which time conjugates were already formed (Figure 5.5). These findings are consistent with a regulatory role for LFA-1 in CD3 mediated CTL activation post CTL/target cell conjugate formation.

Anti-CD18 x anti-DNP hetero-crosslinked Ab coactivates CD3 mediated lysis of ICAM-1- target cells

Experiments were designed to mimic LFA-1/ICAM-1 interactions, using anti-CD18 x anti-DNP hetero-crosslinked Ab. Addition of anti-CD18 x anti-DNP Ab-heteroconjugate increased the susceptibility of ICAM-1- IgR39 target cells to CD3 mediated lysis by TCRγδ+ CTL (Table 5.2). Similar results were obtained using TCRαβ CTL (data not shown). The

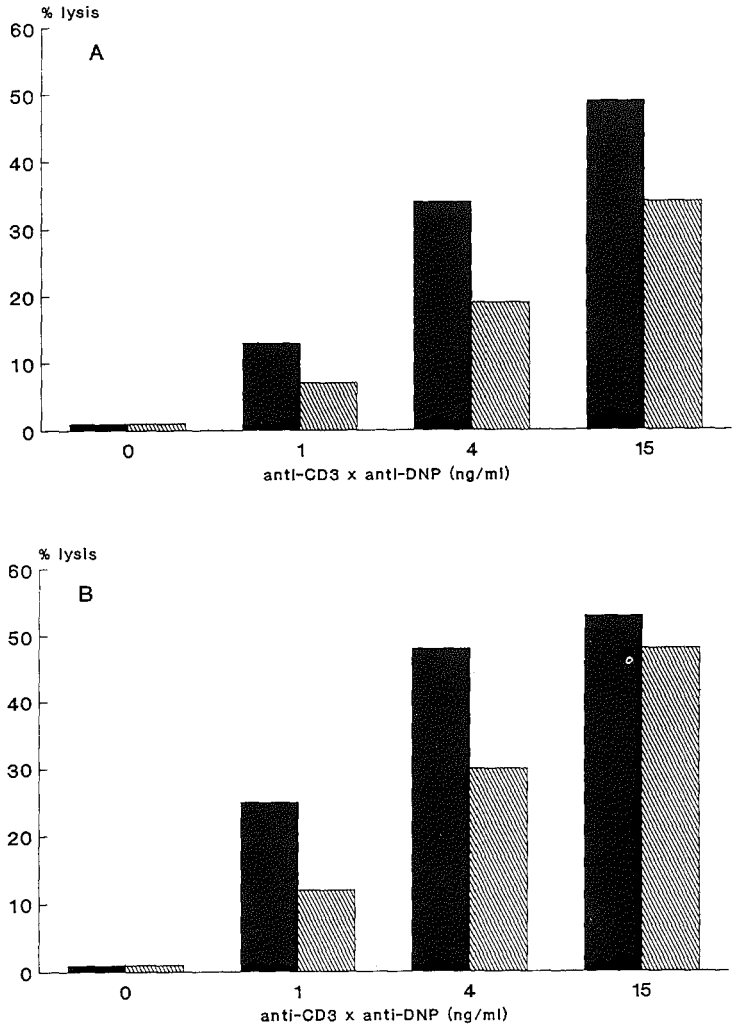

Figure 5.4 Anti-CD18 mAb inhibits CD3 mediated lysis of ICAM-1$^-$ IgR39 cells (panel A) or ICAM-1$^+$ Licr-Lon cells (panel B) by TCR$\gamma\delta^+$/CD3$^+$ clone WiK.

Target cell lysis is induced by the indicated concentration anti-CD3 mAb in the absence (solid bars) or presence (hatched bars) of anti-CD18 mAb (CLB54, 1/1000 of ascites). E/T ratio was 10.

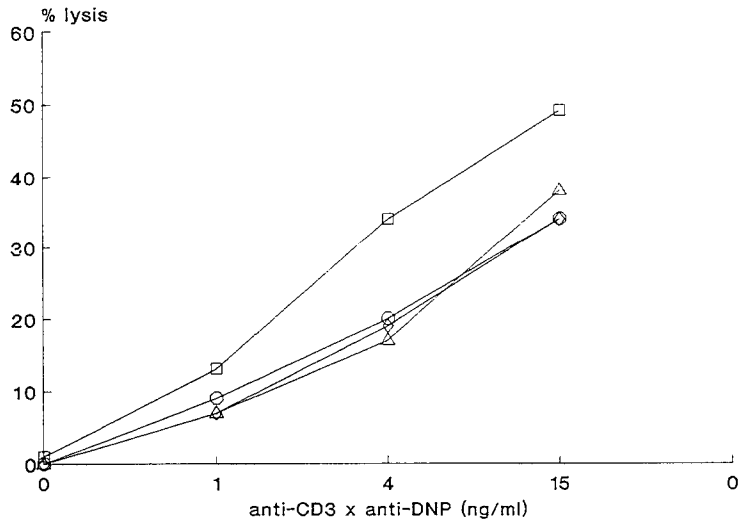

Figure 5.5 Anti-CD18 mAb inhibition of CD3 mediated lysis of IgR39 cells by TCR$\gamma\delta^+$/CD3$^+$ clone WiK.

Anti-CD18 mAb (CLB54, 1/1000 of ascites) is added 15 min before (◇); simultaneously with (△) or 15 min after (○) the addition of target cells to CTL and hetero-crosslinked anti-CD3. □ Control, without anti-CD18 mAb. E/T ratio was 10.

Table 5.2 Anti-CD18 x anti-DNP Ab coactivates lysis of the ICAM-1$^-$ target cell[a].

Ab heteroconjugates[b]	ICAM-1$^-$ (IgR39)				ICAM-1$^+$ (Licr-Lon)			
	Exp.1	Exp.2	Exp.3	Exp.4	Exp.1	Exp.2	Exp.3	Exp.4
– –	15[c]	2	13	3	3	3	4	11
– aCD18 x aDNP	40	6	37	9	7	3	5	13
– aClassI x aDNP	nt[d]	2	16	3	nt[d]	1	4	11
aCD3 x aDNP –	24	11	44	29	49	37	59	74
aCD3 x aDNP aCD18 x aDNP	42	31	70	56	49	46	69	76
aCD3 x aDNP aClassI x aDNP	nt[d]	12	40	23	nt[d]	33	63	68

[a] Effector TCR$\gamma\delta^+$/CD3$^+$ clone: WiK.
[b] Heteroconjugate concentration aCD3 x aDNP = 0.1 μg/ml
aCD18 x aDNP = 0.1 μg/ml
aClass I x aDNP = 0.1 μg/ml.
[c] % specific lysis at an E/T ratio of 24.
[d] nt = not tested.

susceptibility to lysis of ICAM-1$^+$ target cells was much less affected (Table 5.2). Remarkably, anti-CD18 x anti-DNP Ab alone also considerably enhanced MHC-unrestricted lysis of ICAM-1$^-$ but not of ICAM-1$^+$ target cells by TCR$\gamma\delta^+$ CTL. Control, anti-HLA Class I x anti-DNP Ab had no effect on MHC-unrestricted or CD3 mediated lysis.

Discussion

Initiation of CD3/TCR complex mediated T cell functions is believed to be interrelated with various accessory molecules. The transmembrane molecule LFA-1 is one of the most important accessory molecules. Here, the contribution of the LFA-1/ICAM-1 interaction to CD3/TCR complex mediated lysis by TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$ CTL at different stages of the lytic cycle was studied. The use of hetero-crosslinked anti-CD3 x anti-DNP Ab allows the comparison of susceptibilities to lysis of different tumor cell lines by an individual CTL clone. An ICAM-1$^-$ melanoma tumor cell line IgR39 shows a relative resistance to CD3/TCR mediated cytolysis by TCR$\alpha\beta^+$ as well as by TCR$\gamma\delta^+$ cloned lymphocytes. The low susceptibility of IgR39 tumor cells to CD3/TCR mediated cytolysis was directly related to the lack of ICAM-1 expression because TNF treatment of ICAM-1$^-$ IgR39 cells induced ICAM-1 expression and indeed simultaneously increased susceptibility to CD3/TCR mediated cytolysis. Moreover, anti-ICAM-1 mAb downregulated this TNF induced, increased susceptibility to lysis, demonstrating that it was functionally due to upregulation of ICAM-1 and not other unknown ligands.

The relative resistance of IgR39 cells to CD3 mediated cytolysis can be overcome by higher concentrations of anti-CD3 mAb. Thus an efficient LFA-1/ICAM-1 interaction may lower the requirement for either high antigen density on target cells or, alternatively, may compensate for a low affinity TCR on CTL by establishing a more effective CTL-target cell interaction.

The interaction between CTL and target cells, resulting in target cell lysis, is a multistep proces (2,7). Thus the relative resistance of the ICAM-1$^-$ IgR39 target cells to CD3/TCR mediated lysis may reflect either a less efficient conjugate formation, or a decreased level of activation of the lytic machinery. This study shows that CTL less efficiently formed conjugates with ICAM-1$^-$ than with ICAM-1$^+$ tumor cells. Hetero-crosslinked anti-CD3 mAb enhanced conjugate formation with both

the ICAM-1 negative and positive target cells. Addition of anti-CD18 x anti-DNP Ab increased spontaneous and CD3 mediated conjugate formation with the ICAM-1$^-$ cell line and consequently enhanced CD3/TCR mediated lysis of this target cell. Hetero-crosslinked anti-CD18 mAb failed to enhance CD3/TCR mediated lysis of ICAM-1$^+$ tumor cells, because it could not further enhance the relatively efficient conjugate formation with this target. Remarkably, hetero-crosslinked anti-HLA Class I did not enhance CD3 mediated conjugate formation with the ICAM-1$^-$ target cells (data not shown) and therefore did not affect CD3 mediated lysis.

The data suggest that the relative resistance to CD3 mediated lysis of the ICAM-1$^-$ tumor cells is not exclusively associated with a decreased conjugate formation. The LFA-1/ICAM-1 interaction also appears to synergize with CD3/TCR mediated lysis at a postconjugate stage. LFA-1 specific mAb reportedly block lysis by NK and CTL cells through inhibition of conjugate formation (24,39). Here we show that, although anti-CD18 mAb inhibited CD3 mediated cytolysis by CTL of an ICAM-1$^-$ tumor cells, it did not affect their conjugate formation. Anti-CD18 mAb blocked CD3 mediated lysis of both ICAM$^+$ or ICAM-1$^-$ target cells, even when added 15 min after mixing CTL and target cells at which time conjugates are already formed. These findings suggest that LFA-1 can transduce downregulatory signals postconjugate formation, although the possibility that the inhibitory effect of anti-CD18 mAb is solely due to decreased recycling of CTL is to be formally excluded. A further argument in favor of a signaling function for LFA-1 comes from the previously reported observation that ICAM-1$^-$ IgR39 tumor cells show comparable susceptibility to CD2 mediated lysis as ICAM-1$^+$ tumor cells (Chapter 4, 6). The relative resistance of ICAM-1$^-$ target cells to CD3, but not CD2, mediated lysis demonstrates that it is not merely due to a decreased conjugate formation ability but suggests a signaling function for the LFA-1- /ICAM-1 interaction. Hetero-crosslinked anti-CD18 mAb enhanced MHC-unrestricted lysis of the ICAM-1$^-$ target cell by TCR$\gamma\delta^+$ CTL but not by TCR$\alpha\beta^+$ CTL in two out of four experiments. Still, LFA-1 crosslinking to the target cell per se is not sufficient to trigger the cytolytic machinery of TCR$\alpha\beta^+$ and TCR$\gamma\delta^+$ CTL because anti-LFA-1 mAb expressing hybridomas, crosslinking LFA-1, were not lysed by the CTL (40, unpublished observation). Hence, LFA-1 transduces a coactivation signal rather than a primary signal. Whether the functional interrelation between LFA-1 and the CD3/TCR complex depends on coclustering of these molecules as has been demonstrated for CD3/TCR and the accessory

molecule CD4 (41), remains to be established.

In summary, these results demonstrate the central role of LFA-1 in T cell activation. The LFA-1/ICAM-1 interaction appears to be involved in CD3/TCR mediated lysis at two distinct stages of the lytic cycle. Firstly, it promotes binding of CTL to target cells and secondly, it delivers costimulatory signals to CD3/TCR in triggering cell mediated lysis. Consequently, tumor cells lacking ICAM-1 expression may escape lysis by CTL. The relative resistance of ICAM-1$^-$ tumor cells to lysis might be overcome by the induction of ICAM-1 on the tumor cell surface, e.g. with TNF or IFN-γ (21). Also the use of anti-CD2 x anti-target Ab which activate CTL independently of the additional LFA-1/ICAM-1 interaction or the combined use of bispecific anti-CD3 x anti-target and anti-LFA-1 x anti-target Ab may induce efficient lysis of ICAM-1$^-$ target cells.

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CHAPTER 6

LFA-1/ICAM-1 INTERACTION PROMOTES MHC-UNRESTRICTED AS WELL AS CD16 MEDIATED CYTOLYSIS

Introduction

Target cell lysis by cytotoxic lymphocytes involves adhesion between lymphocytes and target cells followed by triggering of the lytic machinery and delivery of the lethal hit (1). $TCR\alpha\beta^+/CD3^+16^-$ T lymphocytes are triggered via the TCR complex upon MHC-restricted antigen specific recognition (2,3). On the contrary $TCR\gamma\delta^+/CD3^+16^{+/-}$ T lymphocytes and $TCR^-/CD3^-16^+$ NK cells exert MHC-unrestricted lysis via a putative receptor (4,5). Target cell recognition is preceeded by nonspecific interactions between adhesion molecules with ligands (6). Two adhesion pathways are well documented: CD2 on T lymphocytes, interacting with LFA-3 (CD58) on target cells (7-9) and LFA-1 on T lymphocytes, interacting with intercellular adhesion molecule-1 (ICAM-1, CD54) on target cells (7,10,11). CD2 is also involved in signal transduction upon binding to LFA-3 (12,13).

The LFA-1 glycoprotein is a heterodimer consisting of a 180 kDa α chain (CD11a), which is noncovalently associated with a 95 kDa β chain (CD18). LFA-1 is expressed on most leucocytes (6,14) and involved in homotypic adhesion of leucocytes (15) and heterotypic adhesion of lymphocytes to a variety of lymphoid and nonlymphoid cells (16,17). Moreover, LFA-1 up- or downregulates adhesion independent lymphocyte proliferation (18,19). In addition, a unique anti-CD11a mAb has been described which induces homotypic T cell and B cell adhesion (20).

ICAM-1 is a monomeric glycoprotein of relative molecular mass 90 kDa and expressed on a variety of hemopoietic as well as nonhemopoietic cells (21). ICAM-1 expression can be increased by inflammatory lymphokines (22,23) which might enhance the binding of leucocytes at inflam-

matory sites. Functional studies have revealed that ICAM-1 is involved in LFA-1 dependent T cell functions such as B cell activation and T cell proliferation and cytolysis (24-27). The importance of the LFA-1-/ICAM-1 interaction was further indicated by reports which showed that Burkitt's lymphoma cells may escape cytolysis because of their low expression of LFA-1, ICAM-1 and LFA-3 (28,29). We recently demonstrated that LFA-1/ICAM-1 interactions coactivate TCR/CD3 complex mediated tumor cell lysis by $TCR\alpha\beta^+$ as well as $TCR\gamma\delta^+$ clones (Braakman et al., submitted for publication).

Here we studied the role of this LFA-1/ICAM-1 interaction in MHC-unrestricted and anti-CD16 mAb triggered lysis. Fresh and cloned $TCR^-/CD3^-16^+$ NK cells and cloned $TCR\gamma\delta^+/CD3^+16^{+/-}$ T lymphocytes were used as effector cells; $ICAM\text{-}1^+$ or $ICAM\text{-}1^-$ tumor cell lines were used as target cells. Ab-heteroconjugates, which comprised an anti-CD16 Fab_2 mAb chemically coupled to an anti-DNP Fab_2 Ab were used to trigger lysis of TNP modified target cells (30). Results show that the LFA-1/ICAM-1 interactions promote MHC-unrestricted lysis by both $TCR^-/CD3^-$ NK as well as $TCR\gamma\delta^+/CD3^+$ T cells. Moreover, LFA-1/ICAM-1 interactions also coactivate CD16 mediated lysis.

Materials and Methods

Human cytotoxic clones

The $TCR^-/CD3^-16^+$ NK cell clones NK76 and NK77 were derived from a patient with Tγ lymphocytosis (31) and clone NK472 from a patient with severe combined immunodeficiency. $TCR\gamma\delta^+/CD3^+4^-8^-16^+$ clone 1042 was derived from PBL from a lung tumor patient and $TCR\gamma\delta/CD3^+4^-8^-16^-$ clone WiK was a generous gift from Dr. D.A. Hafler (Harvard Medical School, Boston, USA) (32). All clones were generated by limiting dilution and maintained in culture as previously described (33).

$TCR^-/CD3^-16^+$ NK cell enriched PBL

Fresh PBL from a healthy donor were depleted for adherent cells by 1-h incubation on plastic at 37°C. The nonadherent lymphocyte fraction was incubated with SRBC at 29°C for 60 min to form rosettes. The E29°

non rosette forming lymphocyte fraction contained up to 25% $CD16^+$ lymphocytes as determined by immunophenotypic analysis using the anti-CD16 mAb B73.1 (34).

Cell lines

Tumor cell lines used in this study were: IgR37, IgR39 and Mel2A, three melanoma derived cell lines; T24, a bladder carcinoma cell line; U-937, a promonocytic cell line; K562, an erythromyeloid cell line; APD and BSM, two Epstein-Barr virus transformed B cell lines; Daudi, a Burkitt's lymphoma derived cell line; Licr-Lon, a leukemic plasma cell line, and Molt-4, a T cell leukemia. Cell lines were cultured in RPMI buffered with bicarbonate (2 g/l) and Hepes (4.8 g/l) and supplemented with 10% heat-inactivated fetal calf serum (FCS), 4 mM glutamine and 100 IU/ml penicillin and streptomycin. The melanoma and bladder derived cell lines growing in monolayer cultures were harvested by trypsinization. Alternatively a PBS/5 mM EDTA solution was used.

Reagents and mAb

Human recombinant (rec) TNF (Cetus Corporation Lot # NP200B, Emeryville, CA, USA) and human recIFN-γ (a gift from Dr. P.H. van der Meide, Primate Center TNO, Rijswijk, The Netherlands) were tested for their ability to increase the cell surface expression of ICAM-1 on various tumor cell lines as described (23). The tumor cells were cultured at $2x10^5$ cells/ml with medium, 25 U/ml recIFN-γ or 1000 U/ml recTNF for 24 h. mAb used for immunophenotypic analysis were: anti-CD18 (LFA-1β) mAb CLB54, kindly provided by Dr. R.A.W. van Lier (Central Laboratory for Blood Transfusion Service, Amsterdam, The Netherlands) (35); anti-CD11a (LFA-1α) mAb TS1/22 and anti-LFA-3 mAb TS2/9, generous gifts from Dr. A.M. Krensky (Stanford University Medical Center, Stanford, CA, USA) (14); anti-ICAM-1 mAb 84H10 (11) and anti-CD16 mAb 3G8 (36), which was unreactive with the $TCR\gamma\delta^+$ clones. Labeling procedures were performed as described elsewhere (31) and cells were analysed by flow cytometry on a FACScan (Becton Dickinson, Mountain View, CA, USA).

Chemical crosslinking of Ab

Fab_2 fragments were prepared from affinity chromatography purified rabbit anti-2,4 dinitrophenyl (DNP) Ab and anti-CD16 mAb (3G8) by papain digestion and did not contain any intact antibody as judged by SDS page (30). Bispecific Ab-heteroconjugates of 3G8 Fab_2 x anti-DNP Fab_2 were prepared using the crosslinking reagent dimethyl suberimidate (DMS) (30).

Lymphocyte-tumor cell conjugate formation assay

Tumor cells ($5x10^6$) and lymphocytes $5x10^6$ were intracellularly labeled with 0.05 μg/ml carboxy fluorescein diacetate (CFDA) or 100 μg/ml hydroethidine (HE) respectively, for 30 min at 37°C in a volume of 2 ml. Both tumor cells and lymphocytes were washed twice in RPMI 1640/Hepes plus 10% FCS and concentrated to 10^7 cells/ml. Blocking of conjugate formation was performed by preincubation (15 min) of lymphocytes with anti-CD18 mAb. Equal numbers of labeled tumor cells and lymphocytes were mixed, in a final volume of 100 μl, and centrifuged in a serofuge (5 sec). Subsequently, the mixture was incubated at 21°C for 10 min. Conjugate formation was stopped by the addition of 1 ml ice-cold PBS/1% BSA. Cells were gently resuspended and immediately analysed on a FACScan. The percentage of lymphocytes that formed conjugates with tumor cells was calculated by dividing the percentage of events emitting both red (HE) and green (CFDA) fluorescence by the total percentage of events emitting red fluorescence.

Cytotoxicity assay

Cytotoxic activity was measured in a standard 3-h ^{51}Cr release assay (31). In short, varying numbers of lymphocytes were seeded in triplicate in 96 well round bottomed microtiter plates (Greiner Labor Technik, Phidelsheim, FRG). To study anti-CD16 x anti-DNP Ab-heteroconjugate induced lysis, tumor cells were incubated with 0.3 mM trinitrobenzene sulfonate (TNP) for 15 min at 37°C, followed by washing. Ab-heteroconjugates were added to the lymphocytes 30 min before addition of ^{51}Cr-labeled TNP-tumor cells. Cytotoxicity was blocked by anti-CD18 mAb, added 30 min prior to addition of the TNP-tumor cells. The standard error of the mean percentage lysis never exceeded 6% and was omit-

ted for clarity of presentation.

Results

IgR37 and IgR39 melanoma cells are relatively resistant to MHC-unrestricted or anti-CD16 mAb triggered lysis

Histologically distinct tumor cell lines were tested for their susceptibility to MHC-unrestricted lysis by cloned $TCR^-/CD3^-16^+$ lymphocytes. Most tumor cell lines were susceptible to MHC-unrestricted lysis, but two melanoma derived cell lines IgR37 and IgR39 were relatively resistant to lysis by TCR^- NK cell clones (Figure 6.1). Tumor cell lines were also coated with TNP and tested for their susceptibility to anti-CD16 mAb triggered lysis using anti-CD16 x anti-DNP Ab heteroconjugate. IgR37 and IgR39 melanoma cells were also relatively

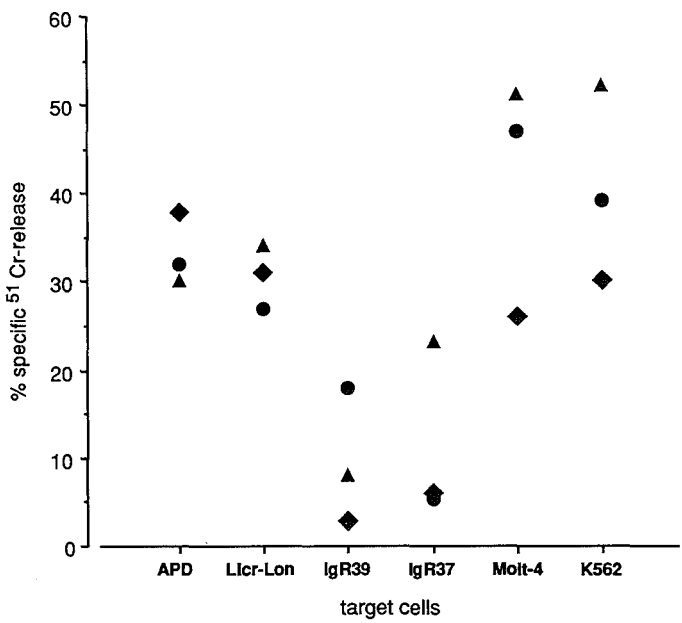

Figure 6.1 IgR37 and IgR39 melanoma are relatively resistant to MHC-unrestricted lysis by $TCR^-/CD3^-16$ NK cell clones: NK76 (▲), NK77 (●) and NK472 (◆). E/T ratio was 12.

resistant to CD16 mediated lysis (Figure 6.2). This was found for both NK cell enriched PBL and cloned $TCR^-/CD3^-16^+$ NK cells showing that the relative resistance to lysis was not donor dependent. The relative resistance of IgR37 and IgR39 to CD16 mediated lysis was not caused by the TNP-coating of the tumor cells, because both melanoma derived cell lines were efficiently lysed using an anti-CD2 x anti-DNP Ab-heteroconjugate (37).

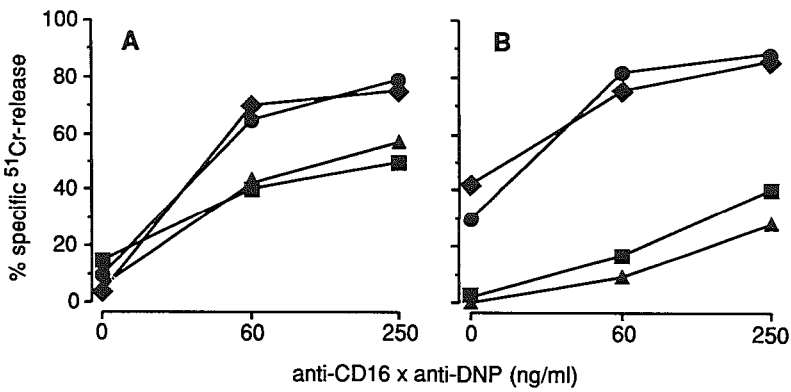

Figure 6.2 IgR37 and IgR39 melanoma show relative resistance to anti-CD16 mAb triggered lysis by $CD16^+$ NK cell enriched PBL (panel A) or $TCR^-/CD3^-16^+$ NK cell clone 472 (panel B).
TNP-APD (●), TNP-Licr-Lon (◆), TNP-IgR37 (■) and TNP-IgR39 (▲) were tested for their susceptibility to anti-CD16 x anti-DNP Ab-heteroconjugate induced lysis at E/T ratio 21 (panel A) or 12 (panel B).

IgR39 and IgR37 melanoma cells express little or no ICAM-1: upregulation of ICAM-1 expression by TNF or IFN-γ

The differential susceptibility to lysis of distinct tumor cell lines may be due to differential levels of expression of particular target cell surface ligands involved in effector/target cell interaction. Therefore, LFA-3 and ICAM-1 expression on the tumor cell surface were quantified by FACS analysis. Most histogenetically distinct tumor cell lines expressed high levels of ICAM-1 and LFA-3 (100% positive cells). However, the relatively lysis resistant melanoma cell

lines IgR39 and IgR37 expressed either little or no ICAM-1 ($\leq$ 5% or 25% ICAM-1$^+$ cells, respectively). A third melanoma cell line (Mel2A), normally expressed ICAM-1 and thus the lack or low expression of ICAM-1 is not melanoma related. TNP modification of tumor cell lines did neither affect LFA-3 nor ICAM-1 expression (data not shown).

It was tempting to speculate that lack of ICAM-1 expression was functionally related to the low susceptibility to MHC-unrestricted or anti-CD16 mAb triggered lysis. Thus, upregulation of ICAM-1 expression should increase the level of lysis. Induction or enhancement of cell surface expression of ICAM-1 by IgR37 and IgR39 was achieved by IFN-γ and TNF (Figure 6.3). Both lymphokines induced comparable levels of

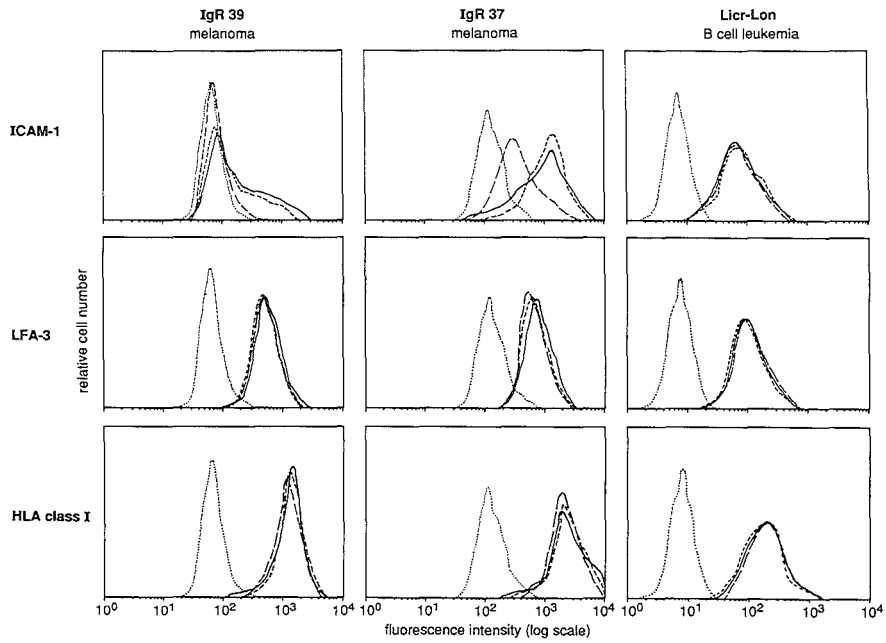

Figure 6.3 TNF and IFN-γ upregulate ICAM-1 expression on ICAM-1$^-$ tumor cells.

Tumor cells were cultured with 1000 U/ml human recTNF (——), 25 U/ml recIFN-γ (——) or medium (— —) for 24 h and stained with mAb. Negative controls (....) were stained with FITC-conjugated goat anti-mouse Ab serum (dilution 1/40) (Nordic, Tilburg, The Netherlands) and gave comparable results after the various incubations.

ICAM-1 on the ICAM-1$^-$ cell lines. By contrast, no further enhancement of ICAM-1 expression was observed on the ICAM-1$^+$ cell lines. The expression of LFA-3 or HLA Class I was virtually not affected by TNF or IFN-γ. TNF was used in further experiments to increase ICAM-1 expression.

Upregulation of ICAM-1 expression on tumor cells reconstitutes susceptibility to MHC-unrestricted or anti-CD16 mAb triggered lysis

Subsequently, we addressed the question whether induction of ICAM-1 expression by target cells simultaneously enhanced their formation of conjugates with lymphocytes and their susceptibility to MHC-unrestricted or anti-CD16 mAb triggered lysis. TNF-induced ICAM-1 expression by tumor cells coincided with an increased percentage of conjugates between TCR$^-$/CD3$^-$16$^+$ NK cell clones and tumor cells (Table 6.1). Simultaneously, the level of MHC-unrestricted lysis by TCR$^-$ or TCR$\gamma\delta^+$ clones as well as the level of anti-CD16 mAb triggered lysis by TCR$^-$ clones was increased (Table 6.2). This increased cytotoxicity was inhibited by the anti-ICAM-1 mAb 84H10 (Figure 6.4), demonstrating that this increased susceptibility to lysis was proportionally related to the ICAM-1 expression on the tumor cells.

Table 6.1 Upregulation of ICAM-1 expression on ICAM-1$^-$ tumor cells enhances conjugate formation.

	% Effector cell-tumor cell conjugates					
	ICAM-1$^-$ melanoma (IgR39)		ICAM-1$^\pm$ melanoma (IgR37)		ICAM-1$^+$ B cell leukemia (Licr-Lon)	
Effector cells			TNF[a)]			
TCR$^-$ NK clones	–	+	–	+	–	+
NK472	7	16	–	–	22	17
NK 77	17	26	35	45	30	24
NK 76	9	15	20	47	14	18

[a)] Incubated with 1000 U/ml recTNF for 24 h.

Table 6.2 Upregulation of ICAM-1 expression on ICAM-1$^-$ tumor cells enhances MHC-unrestricted or anti-CD16 mAb triggered lysis.

Effector cells	Addition	% lysis of TNP-tumor cells			
		ICAM-1$^+$ B cell leukemia (Licr-Lon)		ICAM-1$^-$ melanoma (IgR39)	
		TNF[b]			
TCR$^-$/CD3$^-$16$^+$ clones[a]	Anti-CD16 x anti-DNP Ab-heteroconjugate ng/ml	−	+	−	+
NK472	0	34	31	0	6
	60	82	77	13	27
	250	93	86	39	53
NK76	0	27	28	2	9
	60	57	56	4	13
	250	69	69	7	22
NK77	0	30	35	9	19
	250	69	73	23	35

[a] NK472 and NK76 were tested at an E/T ratio of 12, NK77 at 24.

[b] Incubated with 1000 U/ml recTNF for 24 h.

LFA-1 has a postconjugate regulatory role in cytolysis

The LFA-1 molecule, in addition to its adhesion function, may also play a role in transducing regulatory signals. Therefore, we studied the effect of anti-CD18 (LFA-1β) mAb in conjugate formation and cytotoxicity experiments. In agreement with earlier reports (14,38,39), MHC-unrestricted lysis of ICAM-1$^+$ tumor cells was inhibited by anti-CD18 mAb (Figure 6.5), due to inhibition of conjugate formation (Table 6.3). Importantly, whereas anti-CD18 mAb inhibited MHC-unrestricted lysis of ICAM-1$^-$ tumor cells, it did not affect their formation of conjugates with lymphocytes (Table 6.3).

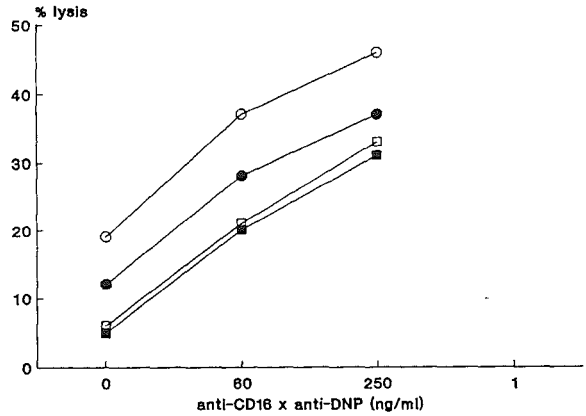

Figure 6.4 Anti-ICAM-1 mAb blocks the increased susceptibility of TNF treated IgR39 cells to MHC-unrestricted or anti-CD16 mAb triggered lysis.

ICAM-1$^-$ IgR39 cells were incubated with medium (□) or 1000 U/ml recTNF (○) for 24 h. Anti-CD16 mAb triggered lysis by the TCR$^-$/CD3$^-$16$^+$ clone NK76 was blocked with anti-ICAM-1 mAb, preincubated for 15 min with target cells. E/T ratio was 12. ■ = anti-ICAM-1 mAb (1/1000 dilution of ascites) + IgR39, ● = anti-ICAM-1 mAb + TNF-IgR39.

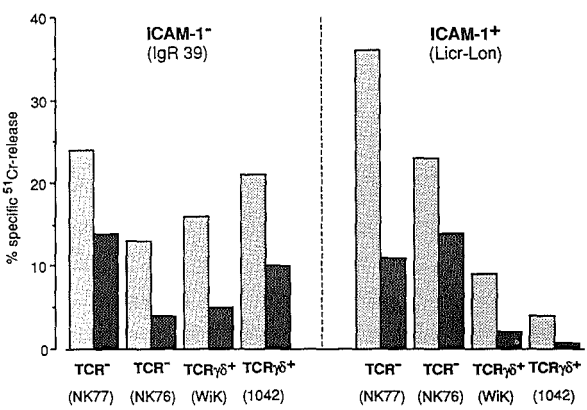

Figure 6.5 Anti-CD18 mAb inhibits MHC-unrestricted lysis of ICAM-1$^+$ or ICAM-1$^-$ tumor cells by TCR$^-$ NK or TCRγδ$^+$ T cell clones.

E/T ratio was 24. ▭ medium, ▬ anti-CD18 mAb (1/400 dilution of ascites).

Table 6.3 Anti-CD18 mAb blocks conjugate formation with ICAM-1$^+$, but not with ICAM-1$^-$ tumor cells.

Lymphocyte clones	Anti-CD18 mAb[a)]	% Conjugate formation: Tumor cells: ICAM-1$^+$ B cell leukemia (Licr-Lon)	% Conjugate formation: Tumor cells: ICAM-1$^-$ melanoma (IgR39)
TCR$\gamma\delta^+$/CD3$^+$16$^\pm$	-	18	8
(1042)	+	3	8
TCR$\gamma\delta^+$/CD3$^+$16$^-$	-	28	18
(WiK)	+	16	17
TCR$^-$/CD3$^-$16$^+$	-	22	13
(NK76)	+	18	14
TCR$^-$/CD3$^-$16$^+$	-	30	17
(NK77)	+	21	17

[a)] Anti-CD18 mAb was added at a final concentration of 1/1000 dilution of ascites.

Discussion

In this Chapter we show that the LFA-1/ICAM-1 interaction coactivates MHC-unrestricted lysis by TCR$^-$ as well as TCR$\gamma\delta^+$ cloned lymphocytes. Anti-CD16 mAb triggered lysis by TCR$^-$/CD3$^-$16$^+$ lymphocyte clones is also regulated by LFA-1/ICAM-1 interactions. The latter was demonstrated with anti-CD16 x anti-DNP Ab-heteroconjugate using TNP-coated tumor target cells. This approach allows to compare tumor cell susceptibilities to mAb triggered lysis by individual TCR$^-$ clones.

The tumor cell lines IgR37 and IgR39 appeared to be relatively resistant to MHC-unrestricted or CD16 mediated lysis by TCR$^-$ NK cells. IgR39 melanoma cells, but not IgR37, were also relatively resistant to MHC-unrestricted lysis by TCR$\gamma\delta^+$ T cell clones, as reported earlier (4). The relative resistance of the melanoma cells to lysis was not due

to trypsinization of the in monolayer cultures growing melanoma cell lines, because similar results were obtained when a PBS/5 mM EDTA solution was used to make a single cell suspension. Moreover, trypsinization of the ICAM-1^+ cell lines T24, Mel 2A, Licr-Lon or Molt-4 did not affect the expression of ICAM-1 nor their susceptibility to lysis. The relative resistance to MHC-unrestricted or anti-CD16 mAb triggered lysis correlated with the lack of ICAM-1 expression on IgR37 and IgR39, because induction of tumor cell surface ICAM-1 expression by TNF reconstituted susceptibility to MHC-unrestricted or CD16 mediated lysis.

TNF treatment of ICAM-1^+ cell lines did not affect ICAM-1 expression nor their susceptibility to MHC-unrestricted or anti-CD16 mAb triggered lysis. Definite proof that this increased susceptibility to lysis was due to upregulation of ICAM-1 on the tumor cells, comes from ICAM-1 specific mAb inhibition studies. Anti-ICAM-1 mAb significantly inhibited MHC-unrestricted and CD16 mediated lysis by TCR^- NK clones.

The up- or downregulatory role of LFA-1 in cytolysis depends on the structural nature of the stimulus to LFA-1. Crosslinking of LFA-1 through interaction with ICAM-1 on the target cells resulted in enhancement of MHC-unrestricted or anti-CD16 mAb triggered lysis. Mimicking of the LFA-1/ICAM-1 interaction by anti-CD18 x anti-DNP Ab-heteroconjugates promoted MHC-unrestricted or anti-CD3 mAb triggered lysis by $TCR\alpha\beta^+$ as well as $TCR\gamma\delta^+$ clones (Braakman et al., submitted for publication). However, crosslinking of LFA-1 per se did not activate the cytolytic machinery, because anti-CD11a or anti-CD18 mAb producing hybridomas were not lysed by fresh or clones TCR^- or $TCR\gamma\delta^+$ cells (41, unpublished observations). Thus crosslinking of LFA-1 via ICAM-1 or mAb delivers a coactivation signal for MHC-unrestricted or anti-CD16 mAb triggered lysis.

By contrast, soluble anti-CD18 or CD11a mAb inhibit both CTL and NK cell lysis (14,38,39). It was shown that this inhibition was due to inhibition of antigen-nonspecific conjugate formation (40). Here, we show that anti-CD18 mAb blocked MHC-unrestricted lysis of ICAM-1^+ or $ICAM^-$ tumor cells. However, anti-CD18 mAb only inhibited formation of conjugates using ICAM-1^+ target cells. These data demonstrate a post-conjugate regulatory role of LFA-1 in MHC-unrestricted as well as CD16 mediated lysis.

In conclusion, LFA-1/ICAM-1 interactions determine target cell susceptibility to lyis. ICAM-1$^-$ tumor cells or viral infected cells may therefore escape the antigen specific and MHC-unrestricted immune surveillance as suggested earlier (28,29) and shown here. Bifunctional Ab are powerful tools for the induction of target cell lysis (30,37,43) and are already applied in immunotherapy protocols (42). However, as we demonstrate here and elsewhere (Braakman et al., submitted for publication), LFA-1/ICAM-1 interactions may also regulate bifunctional Ab triggered cytolysis. This might be overcome by induction of ICAM-1 on target cells (as was shown here), or by using other bifunctional Ab, e.g. comprising anti-CD2 mAb (37).

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CHAPTER 7

CONCLUDING REMARKS

The host immune system-tumor relationship can be viewed as a delicate balance which is influenced by internal as well as external signals. The immune system is designed to recognize and destroy tumor cells in the earliest possible stage. However, a tumor may break through the defense provided by the immune system. This Chapter deals with the host immune system-tumor relationship, the mechanisms which may lead to escape of tumor cells from immune surveillance, and discusses several approaches to immunotherapy of cancer which are presently used or open for experimentation in the future.

Tumors can be induced by chemical substances, irradiation, viruses or arise spontaneously. It was first proposed by Ehrlich (1) and later substantiated by others (2,3), that cells which are antigenically sufficiently distinct from normal cells are recognized by components of the immune system and subsequently eliminated (immune surveillance theory). The effectiveness of a persons *in vivo* cellular immune response against tumor cells depends on the number of cytotoxic effector cells and antibodies that can be recruited, their ability to recognize and lyse tumor cells and the persons tumor load. Evidence has emerged from animal models that an intact immune response can be effective in the elimination of distinct histological types of tumors (4,5).

Tumor cell escape from immune surveillance

Several mechanisms can be envisaged which may cause escape of tumor cells from immune surveillance. First, tumors may be insufficiently immunogenic to elicit an immune response. Secondly, tumor cells may survive and proliferate because of a deficient immune response.

Tumor escape may occur because tumor cells lack antigens which discriminate them from normal cells. In some cases the transformation of normal cells to malignant cells is due to the uncontrolled expression of normal proteins. Therefore, these tumors do not express neo-antigens, but only differ from normal cells in their level of expression of these antigens. Alternatively, tumor cells may express neo-antigens, but their density is too low to be immunogenic in vivo. Escape from immune surveillance may also be caused by shedding of neo-antigens from the cell surface (6). Shedding of surface antigens from both normal and neoplastic cells was demonstrated by Cone et al (7).

Tumor cells expressing neo-antigens may still be resistant to lysis by cytotoxic lymphocytes because they do not express sufficient levels of ligands for accessory molecules on the immune cells. Accessory molecules on lymphocytes (activation sites and/or adhesion molecules) play an important role in lymphocyte activation (as shown in Chapter 4, 5 and 6). These accessory molecules not only increase the overall avidity of lymphocyte-tumor cell interactions, but also transduce signals that facilitate lymphocyte activation. The overall avidity is extremely important when tumor cell-associated antigens are expressed at low densities (see above). The crucial role of accessory molecule-ligand interactions in tumor cell resistance to lysis was demonstrated here and suggested by Gregory et al. (8). LFA-1, LFA-3 and ICAM-1 negative Burkitt's lymphoma derived cells were resistant to lysis by specific CTL. Expression of LFA-1, LFA-3 and ICAM-1 was induced on the Burkitt's lymphoma cells during culture, which coincided with an increased susceptibility to lysis. We obtained similar data with ICAM-1$^-$ melanoma derived cells that were relatively resistant to cytolysis by TCR$\alpha\beta^+$ CTL, TCR$\gamma\delta^+$ CTL as well as TCR$^-$ NK cells. Induction of ICAM-1 expression on the melanoma cells by TNF or IFN-γ, increased susceptibility to lysis by cytotoxic lymphocytes. Conclusive evidence that indeed LFA-1/ICAM-1 interactions were critical for susceptibility to lysis by NK cells and CTL came from inhibition studies on lysis using anti-ICAM-1 mAb (9,10, Chapter 5 and 6). Thus, the antigenic make-up of a tumor cell critically codetermines its susceptibility to lysis.

Another cause for the development of a tumor out of a limited number of transformed cells may be a deficiency in the immune system. A number of studies has clearly demonstrated an association between the potency of the immune system and tumor development. The tumor incidence in immunodeficient individuals, e.g. patients suffering from a variety of

hereditary immunodeficiencies, is increased (11). Another well known example is the occurence of Non-Hodgkin's lymphoma or Karposi sarcoma in acquired immunodeficiency syndrome (AIDS) (12,13). Kidney graft recipients who received prolonged immunosuppressive treatment are highly sensitive to viral infections and tumor development (14,15). It is generally accepted that the increased incidence of tumors in elderly people is related to their impaired immune responsiveness.

In addition, local immune disfunction may be induced by tumor cells, subset(s) of tumor infiltrating lymphocytes (TIL) or both, through immunosuppressive mechanisms. Tumor cells may either directly suppress antitumor activity via production of factors which act on cytotoxic lymphocytes, or activate lymphocytes with suppressor activity. Instead, the partial development of immune disfunction to e.g. viral associated tumor antigens in a long infected host may be caused by tolerance. The reduction of the *in vitro* proliferative capacity of precursor (p)-CTL in TIL as compared to pCTL from PBL of patients or healthy donors, is supposedly due to immunosuppressive mechanisms exerted by the tumor (Chapter 3).

Immunotherapy of tumors

Having summarized a number of parameters involved in tumor cell lysis, the question is: how can the present knowledge be applied to restore the distorted balance between the host immune system and the established tumor. Various treatment modalities are applied, i.e. surgery, chemotherapy, radiotherapy and combinations thereof. The rapid developments in the fields of immunology and recombinant DNA technology have provided new strategies for therapy, based on manipulation of the patients own immune system. Most initial approaches to tumor-immunotherapy attempted active specific immunisation. Active specific immunisation is a procedure in which tumor cells from the patient are combined with nonspecific immunostimulants, such as Bacillus Calmette-Guerin (BCG) or Corynebacterium parvum, to develop an antitumor vaccine. Such a vaccine may enhance the immunogenicity of the tumor cell antigens and/or create new epitopes. This may lead to an increased immune response against otherwise weakly immunogenic tumors (16,17).

Other strategies have been applied, based on the activation of the immune system *in vivo*. Administration of biological response modifiers (BRM) such as interleukins, or interferons, have been succesfully used

in the treatment of tumors (18-22). Because administration of high doses of interleukin 2 (IL2) causes significant toxicity, immune cells were activated ex vivo with IL2. Both PBL and TIL derived from patients were used. TIL were believed to be more "specific" for the tumor from which they were derived and thus more effective in the treatment of cancers. This form of adoptive immunotherapy (reviewed in 23) has yielded encouraging results, especially in patients with immunogenic tumors, such as melanoma and renal cell carcinoma (24,25). Melanomas may express a variety of TAA, as reflected by the generation of tumor specific oligo clonal $TCR\alpha\beta^+/CD3^+$ CTL which recognize distinct antigens on the melanoma cell (Lotze, Mitchell, personal communication). In patients, however, the fraction of immune cells responding to melanoma and other immunogenic tumors is apparently insufficient.

The use of IL2 activated lymphocytes, together with continuous IL2 infusion, involves lymphocytes of which only 2-5% are endowed with anti-tumor cytolytic activity. We and others therefore stated to use bispecific mAb targeted lymphocytes for the selective attack of tumor cells, because T cells (approximately 60% of the lymphocyte population) are activated by anti-CD3 x anti-tumor mAb (26-30). Consequently, a large proportion of the adoptively transferred activated lymphocytes can then be mobilized to attack tumor cells. The mechanisms of bispecific mAb induced lysis are described in detail in Chapter 1, 4, 5, and 6. The use of these mAb-retargeted lymphocytes will hopefully improve their homing into the tumor and elicit a localized immune network response at the tumor site. This will not only result in the induction of cytolytic activity but also in lymphokine production. Bispecific mAb targeted lymphocytes may also attack tumors which lack the expression of ligands for accessory molecules (Chapter 5 and 6).

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SUMMARY

The studies presented in this thesis were initiated to define a rationale for the immunotherapy of cancer using cytotoxic lymphocytes in combination with bispecific mAb.

The major subsets of cytotoxic lymphocytes, TCR^- NK cells, $TCR\alpha\beta^+$ CTL and $TCR\gamma\delta^+$ CTL are described in Chapter 1. These lymphocyte subsets differ in their surface receptor(s) for target cell recognition, expression of accessory molecules and type(s) of cytotoxic activities which they display. For instance lytic activity by $TCR\alpha\beta^+$ CTL is MHC-restricted, whereas NK cell activity is MHC-unrestricted. $TCR\gamma\delta^+$ CTL display both types of activities.

A short overview of the best known accessory molecules expressed on NK cells or CTL and their ligands expressed on target cells is also presented in Chapter 1. The role of accessory molecules in lymphocyte-target cell adhesion and in lymphocyte activation is discussed, as well as the mechanisms through which these processes may occur.

In vitro studies on distinct subsets of lymphocytes require large numbers of pure (cloned) cells. Therefore culture conditions were established that allow the preferential and rapid expansion of either TCR^- NK cells or TCR^+ lymphocytes, or the nonselective outgrowth of both subsets from PBL of healthy donors (Chapter 2). Optimal proliferation of TCR^- NK cells was obtained using a combination of irradiated allogeneic PBL and irradiated Epstein Barr virus (EBV) transformed lymphoblastoid B cell lines (B-LCL) plus interleukin 2 (IL2). Addition of 1 μg/ml of the lectin leucoagglutinin to the culture medium induced a preferential outgrowth of TCR^+ T lymphocytes. Although the proportion of TCR-NK cells was decreased to 5% or less, still a 2000-fold multiplication of TCR^- NK cells was obtained at day 13. Without leucoagglutinin a 1000-fold increase of about 70% pure TCR^- NK cells was obtained at day 13.

Under limiting dilution conditions the majority of the clones generated was $TCR\alpha\beta^+/CD4^+$. $TCR\gamma\delta^+$ clones were occasionally obtained, and

could be maintained in culture for several months. Although the life-span of most TCR^- NK cell clones is restricted to about 4 weeks, a panel of NK clones had a life-span of 2-3 months.

Chapter 3 deals with the subset composition and cytotoxic features of PBL and TIL derived from lung tumor patients. TIL, but not PBL, from lung tumor patients have lower proportions of TCR^- NK cells and exert little or no NK or ADCC activity as compared to PBL from healthy donors. Upon in vitro culture using the conditions for selective expansion described in Chapter 2, the TCR^- enriched bulk cultures displayed high levels of NK and ADCC activity. Cloning of PBL and TIL in limiting dilution demonstrated a reduced proliferative capacity of precursor CTL in TIL, but cytotoxic activity of the few established clones was comparable to that of PBL clones.

The requirements for activation of the lytic machinery of cytotoxic lymphocyte subsets through CD2 were examined, using bispecific Ab-heteroconjugates containing anti-CD2 mAb crosslinked to anti-DNP and TNP coated target cells (Chapter 4). Contrary to the CD2 activation requirements in $TCR\alpha\beta^+$ CTL clones, cytotoxic activity in $TCR\gamma\delta^+$ CTL clones and TCR^- NK cell clones can be induced by heteroconjugates containing a single anti-CD2 (anti-T11.1) mAb. Activation of $TCR\gamma\delta^+$ cells via CD2 is independent of Ab-heteroconjugate binding to CD16 ($Fc\gamma RIII$) since Ab-heteroconjugates prepared from Fab fragments induced equal levels of cytolysis. Moreover, anti-CD16 mAb did not inhibit triggering via CD2 in $TCR\gamma\delta^+$ CTL. In TCR^- NK cells, however, induction of cytotoxicity via CD2 is co-dependent on interplay with CD16. Anti-CD3 mAb blocked the anti-CD2 x anti-DNP Ab-heteroconjugate induced cytotoxicity of $TCR\gamma\delta^+$ cells, indicating a functional linkage between CD2 and CD3 on these cells. It was concluded that induction of lysis via CD2 shows qualitatively distinct activation requirements in TCR^- NK cells, $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ CTL.

The major activation pathway of T cells is via the TCR/CD3 complex, which is functionally interrelated with various accessory molecules. The contribution of the LFA-1/ICAM-1 interaction to CD3/TCR mediated lysis by $TCR\alpha\beta^+$ or $TCR\gamma\delta^+$ CTL is described in Chapter 5. $ICAM\text{-}1^+$ or $ICAM\text{-}1^-$ tumor cell lines were used as target cells. Anti-CD3 and anti-CD18 (LFA-1β) containing hetero-crosslinked mAb were used to bridge CTL and target cells and to activate CTL. The $ICAM\text{-}1^-$ melanoma derived cell line IgR39 appeared relatively resistant to CD3 mediated lysis by both $TCR\alpha\beta^+$ and $TCR\gamma\delta^+$ CTL, when compared with $ICAM\text{-}1^+$ cell

lines. Induction of ICAM-1 expression on the membrane of IgR39 cells by TNF rendered these cells more susceptible to CD3 mediated lysis. Anti-ICAM-1 mAb inhibited this enhanced susceptibility to lysis, demonstrating that the induction of ICAM-1 was the critical factor in the TNF-induced increase in susceptibility to lysis of IgR39 cells.

CTL formed less efficient conjugates with the ICAM-1$^-$ cells as compared to ICAM-1$^+$ cells. Spontaneous and CD3-induced conjugate formation as well as CD3 mediated lysis of ICAM-1$^-$ tumor cells by CTL were enhanced by addition of anti-CD18 containing hetero-crosslinked mAb. This Ab-heteroconjugate mimicks LFA-1/ICAM-1 interactions between CTL and target cells. Soluble (noncrosslinked) anti-CD18 mAb inhibited CD3 mediated lysis of ICAM-1$^-$ target cells by CTL, without affecting their conjugate formation. Anti-CD18 mAb, when added postconjugate formation, still inhibited lysis of both ICAM-1$^+$ and ICAM-1$^-$ tumor cells. These findings suggest that the LFA-1/ICAM-1 interactions coactivate CD3/TCR mediated lysis by CTL on the one hand through enhanced CTL-target cell binding and on the other also through the delivery of postconjugate CD3-costimulatory signals.

Because TCR$^-$ NK cells lack the CD3/TCR signal transduction pathway, the question arose whether LFA-1/ICAM-1 interactions also affect tumor cell susceptibility to MHC-unrestricted lysis by TCR$^-$ NK (Chapter 6). Moreover, the role of LFA-1/ICAM-1 interactions on lysis by TCR$^-$ NK cells triggered via anti-CD16 mAb was investigated. Two melanoma derived cell lines, which express little or no ICAM-1, were relatively resistant to MHC-unrestricted lysis, as well as to anti-CD16 mAb triggered lysis by fresh or cloned TCR$^-$ NK cells. The ICAM-1$^-$ melanoma cells appeared also relatively resistant to MHC-unrestricted lysis by TCR$\gamma\delta^+$ clones. TNF induced ICAM-1 expression on ICAM-1$^-$ tumor cells, and thereby increased susceptibility of melanoma cells to MHC-unrestricted or to anti-CD16 mAb triggered lysis. This enhanced susceptibility to lysis was decreased again by anti-ICAM-1 mAb. These data demonstrate that LFA-1/ICAM-1 interactions are functionally and positively related to susceptibility to MHC-unrestricted and CD16 mediated cytolysis of tumor cells. Anti-CD18 mAb inhibited MHC-unrestricted as well as anti-CD16 mAb triggered lysis of ICAM-1$^+$ and ICAM-1$^-$ tumor cells. Anti-CD18 mAb expectedly blocked the formation of conjugates with ICAM-1$^+$ target cells. In spite of the inhibition of lysis, anti-CD18 mAb did not block formation of conjugates using ICAM-1$^-$ target cells. Thus LFA-1 also plays a postconjugate regulatory role in

MHC-unrestricted as well as anti-CD16 mAb triggered lysis.

In the last part of this thesis (Chapter 7) a number of parameters which determine tumor cell lysis *in vivo* are discussed.

SAMENVATTING

De in dit proefschrift gepresenteerde studies werden gestart om een rationale te definiëren voor immuuntherapie van kanker met behulp van cytotoxische lymfocyten in combinatie met bispecifieke mAb. Cytotoxische lymfocyten vormen de laatste verbinding in een keten van cellulaire interakties van het immuunsysteem. Zij zijn onder andere in staat tumorcellen te lyseren.

De voornaamste typen van cytotoxische lymfocyten, TCR^- NK cellen, $TCR\alpha\beta^+$ CTL en $TCR\gamma\delta^+$ CTL zijn beschreven in Hoofdstuk 1. Deze lymfocyten subpopulaties verschillen in hun membraanreceptor(en) voor herkenning van tumorcellen en membraanexpressie van zogenaamde helper moleculen. Tevens verschillen deze subpopulaties in type(n) van cytotoxische aktiviteiten. Lytische aktiviteit van $TCR\alpha\beta^+$ CTL is MHC-gerestricteerd, dat wil zeggen dat antigeen herkend wordt in combinatie met MHC determinanten. TCR^- NK celaktiviteit daarentegen is MHC niet-gerestricteerd. $TCR\gamma\delta^+$ CTL vertonen beide typen van aktiviteiten. In Hoofdstuk 1 wordt bovendien een kort overzicht gegeven van de meest bekende helper moleculen die geëxpresseerd worden op NK cellen of CTL en hun liganden die geëxpresseerd worden op tumorcellen. De rol van helper moleculen in lymfocyt/tumorceladhesie en in aktivatie van lymfocyten wordt bediscussieerd, alsmede de mechanismen via welke deze processen plaatsvinden.

In vitro studies aan gescheiden subpopulaties van lymfocyten vereisen grote aantallen van gezuiverde (gekloneerde) cellen. Daarom werden kweekcondities vastgesteld voor de preferentiële en snelle vermeerdering van óf TCR^+ lymfocyten óf TCR^- NK cellen. Tevens werden condities bepaald voor de niet-selectieve uitgroei van beide subpopulaties uit perifeer bloed lymfocyten (PBL) van gezonde donoren (Hoofdstuk 2). Optimale proliferatie van TCR^- NK cellen werd verkregen door een combinatie van bestraalde allogene PBL en bestraalde Epstein Barr virus (EBV) getransformeerde lymfoblastoïde B-cellijnen (B-LCL) plus interleukine 2 (IL2). Additie van 1 $\mu g/ml$ van het lectine leucoagglutinine aan het kweekmedium induceerde een preferentiële uitgroei van TCR^+ T

lymfocyten. Hoewel de proportie van TCR^- NK cellen verlaagd was tot 5% of minder, werd een 2000-voudige vermeerdering verkregen van NK cellen tot aan dag 13. Zonder leucoagglutinine werd een 1000-voudige vermeerdering verkregen van ongeveer 70% zuivere TCR^- NK cellen tot aan dag 13.

De meerderheid van gekloneerde PBL hadden het immunofenotype $TCR\alpha\beta^+/CD4^+$. Nu en dan werden $TCR\gamma\delta^+$ klonen verkregen, die gedurende enige maanden in kweek gehouden konden worden. Ofschoon de levensduur van de meeste TCR^- NK klonen beperkt is tot ongeveer 4 weken, hadden een aantal NK klonen een levensduur van 2-3 maanden.

In Hoofdstuk 3 zijn de populatiesamenstelling en cytotoxische kenmerken van PBL en tumor-infiltrerende lymfocyten (TIL) afkomstig van longtumorpatiënten beschreven. TIL, maar niet PBL van longtumorpatiënten bevatten een lagere proportie NK cellen en vertonen een lagere NK- en antilichaam-afhankelijke cytotoxische aktiviteit (ADCC) dan PBL van gezonde donoren. Na in vitro vermeerdering, gebruikmakend van de condities voor selectieve expansie zoals beschreven in Hoofdstuk 2, vertoonden de voor NK-cellen-verrijkte bulkkweken een hoge NK en ADCC aktiviteit. Klonering van PBL en TIL demonstreerde een verlaagde proliferatieve capaciteit van TIL met cytotoxische aktiviteit. Echter de cytotoxische aktiviteit van de enkele groeiende klonen was gelijk aan die van gekloneerde PBL afkomstig van dezelfde patiënt of van normale donoren.

De vereisten voor aktivatie van het cytotoxisch mechanisme van lymfocyten subpopulaties via het aktivatiemolecuul CD2 werden onderzocht met behulp van bispecifieke Ab-heteroconjugaten (Hoofdstuk 4). Hiervoor werd een Ab-heteroconjugaat gebruikt bestaande uit een anti-CD2 mAb gekoppeld aan een anti-DNP mAb en TNP-behandelde tumorcellen. Op deze manier ontstaat een brugverbinding tussen lymfocyt en tumorcel, wat tot aktivatie kan leiden van de lymfocyt. In tegenstelling tot $TCR\alpha\beta^+$ CTL kan cytotoxische aktiviteit van TCR^- NK celklonen en $TCR\gamma\delta^+$ CTL klonen worden geïnduceerd via één anti-CD2 (anti-T11.1) mAb-bevattend heteroconjugaat. Aktivatie van $TCR\gamma\delta^+$ CTL via CD2 is onafhankelijk van Ab-heteroconjugaatbinding aan CD16 ($Fc\gamma RIII$) omdat Ab-heteroconjugaten gemaakt van Fab fragmenten eenzelfde nivo van lysis induceerden. Bovendien werd de aktivatie van $TCR\gamma\delta^+$ CTL via CD2 niet geremd door anti-CD16 mAb. In TCR^- NK cellen echter was dit wel het geval en werd bovendien een aanzienlijk lager nivo van lysis geïnduceerd door Ab-heteroconjugaat gemaakt van Fab fragmenten. In TCR^- NK

cellen is de inductie van cytotoxiciteit via CD2 dus mede afhankelijk van een wisselwerking met CD16.

Anti-CD3 mAb blokkeerde de anti-CD2 x anti-DNP Ab-heteroconjugaat geïnduceerde cytotoxiciteit van $TCR\gamma\delta^+$ CTL, hetgeen wijst op een funktionele relatie tussen CD2 en CD3 op deze cellen. De konklusie is dat de vereisten voor inductie van cytotoxiciteit via CD2 kwalitatief verschillend zijn voor TCR^- NK cellen, $TCR\alpha\beta^+$ en $TCR\gamma\delta^+$ CTL.

De voornaamste aktivatieroute van T cellen verloopt via het TCR/CD3 complex, dat funktioneel in verband staat met verscheidene helper moleculen. De rol van één van deze helper moleculen, LFA-1, in de CD3/TCR-gemedieerde lysis door $TCR\alpha\beta^+$ of $TCR\gamma\delta^+$ CTL is beschreven in Hoofdstuk 5. LFA-1 wordt onder andere geëxpresseerd op de celmembraan van NK cellen en CTL en kan binden aan ICAM-1, een molecuul dat door een aantal verschillende celtypen geëxpresseerd wordt. Daarom werden TNF-behandelde $ICAM\text{-}1^+$ of $ICAM\text{-}1^-$ tumorcellijnen gebruikt als "target" cellen. Anti-CD3 en anti-CD18 (LFA-1β) bevattende heteroconjugaten werden gebruikt om tumorcellen aan CTL te binden en om CTL te aktiveren. De $ICAM\text{-}1^-$ melanoom-afgeleide cellijn IgR39 bleek relatief resistent tegen CD3-gemedieerde lysis door zowel $TCR\alpha\beta^+$ als $TCR\gamma\delta^+$ CTL, in vergelijking tot $ICAM\text{-}1^+$ cellijnen. Induktie van ICAM-1 expressie op de membraan van IgR39 cellen door TNF maakte deze cellen gevoeliger voor CD3-gemedieerde lysis. Anti-ICAM-1 mAb remde deze verhoogde gevoeligheid voor lysis, aangevend dat de induktie van ICAM-1 expressie de kritieke faktor was in de TNF-geïnduceerde toename in gevoeligheid voor lysis van IgR39 cellen.

Het percentage gevormde conjugaten tussen CTL en $ICAM\text{-}1^-$ cellen was lager dan het percentage conjugaten tussen CTL en $ICAM\text{-}1^+$ cellen. Spontane en anti-CD3 x anti-DNP Ab-heteroconjugaat geïnduceerde conjugaatformatie alsmede CD3-gemedieerde lysis van $ICAM\text{-}1^-$ tumorcellen door CTL werden verhoogd door toevoeging van een anti-CD18 (LFA-1β) bevattend heteroconjugaat. Dit anti-CD18 x anti-DNP Ab-heteroconjugaat bootst de LFA-1/ICAM-1 interaktie tussen CTL en tumorcel na, wat resulteert in toename van tumorcellysis. Anti-CD18 mAb niet gekoppeld aan een anti-DNP Ab remde de CD3-gemedieerde lysis van $ICAM\text{-}1^+$ tumorcellen door CTL, alsmede de conjugaatformatie, door blokkering van de LFA-1/ICAM-1 interaktie. Echter, anti-CD18 mAb remde ook de CD3-gemedieerde lysis van $ICAM\text{-}1^-$ tumorcellen door CTL, zonder de conjugaatformatie te beïnvloeden. Zelfs wanneer anti-CD18 mAb werd toegevoegd na conjugaatformatie werd de lysis van zowel $ICAM\text{-}1^+$ als $ICAM\text{-}1^-$ tumorcellen geremd.

Deze bevindingen suggereren dat de LFA-1/ICAM-1 interakties de CD3/TCR-gemedieerde lysis door CTL co-aktiveren. Dit vindt plaats door zowel een verhoogde CTL-tumorcel binding als door het leveren van een CD3-costimulatoir signaal nadat de conjugaatformatie heeft plaatsgevonden.

TCR^- NK cellen missen het CD3/TCR signaal transduktie mechanisme. Daarom rees de vraag of LFA-1/ICAM-1 interakties ook de gevoeligheid van tumorcellen voor MHC niet-gerestricteerde lysis door NK cellen beïnvloeden (Hoofdstuk 6). Bovendien werd de rol van LFA-1/ICAM-1 interakties onderzocht op de lysis van NK cellen die geaktiveerd zijn door een anti-CD16 mAb. Twee melanoom-afgeleide cellijnen met zwakke ICAM-1 expressie bleken relatief resistent tegen MHC niet-gerestricteerde en anti-CD16 mAb geïnduceerde lysis door verse of gekloneerde TCR^- NK cellen. De $ICAM\text{-}1^-$ melanoomcellen bleken ook relatief ongevoelig voor MHC niet-gerestricteerde lysis door $TCR\gamma\delta^+$ klonen. TNF induceerde ICAM-1 expressie op $ICAM\text{-}1^-$ tumorcellen en verhoogde daardoor de gevoeligheid van deze cellen voor MHC niet-gerestricteerde en anti-CD16 mAb geïnduceerde lysis. Deze toegenomen gevoeligheid voor lysis werd weer verlaagd door anti-ICAM-1 mAb. Deze data laten zien dat LFA-1/ICAM-1 interakties funktioneel en positief gecorreleerd zijn met gevoeligheid voor MHC niet-gerestricteerde en CD16-gemedieerde lysis van tumorcellen. Anti-CD18 mAb remde zowel MHC niet-gerestricteerde lysis als anti-CD16 mAb geïnduceerde lysis van $ICAM\text{-}1^-$ en $ICAM\text{-}1^+$ tumorcellen. Zoals verwacht remde anti-CD18 mAb de conjugaatformatie met $ICAM\text{-}1^+$ tumorcellen. Ondanks de remming van lysis werd de conjugaatformatie met $ICAM\text{-}1^-$ tumorcellen niet geremd. Dus LFA-1 speelt een post-conjugaat regulatoire rol in MHC niet-gerestricteerde lysis en anti-CD16 geïnduceerde lysis.

In het laatste Hoofdstuk (Hoofdstuk 7) worden een aantal parameters bediscussieerd die de lysis van tumorcellen in vivo bepalen.

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LIST OF ABBREVIATIONS

ADCC	- antibody dependent cellular cytotoxicity
AK	- activated killer
B-LCL	- lymphoblastoid B cell line
CD	- cluster designation
CTL	- cytotoxic T lymphocyte(s)
EBV	- Epstein Barr virus
E/T	- effector/target
FACS	- fluorescent activated cell sorter
FCS	- fetal calf serum
FITC	- fluorescein isothiocyanate
HLA	- human leucocyte antigen
ICAM	- intercellular adhesion molecule
IFN	- interferon
Ig	- immunoglobulin
IL2	- interleukin 2
LDCC	- lectin dependent cellular cytotoxicity
LFA	- leucocyte function associated antigen
mAb	- monoclonal antibody (ies)
MHC	- major histocompatibility complex
Mw	- molecular weight
NK	- natural killer
PBL	- peripheral blood lymphocyte(s)
PE	- plating efficiency
PHA	- phytohemagglutinin
PMA	- phorbol myristate acetate
SPDP	- N-succinimidyl-3-(2-pyridyldithiol propionate)
SRBC	- sheep red blood cell(s)
TAA	- tumor associated antigen
TCR	- T cell receptor
TIL	- tumor infiltrating lymphocyte(s)
TNF	- tumor necrosis factor
TNP	- trinitrobenzene sulfonate

CURRICULUM VITAE

De auteur van dit proefschrift werd op 19 augustus 1960 geboren te Rotterdam. In 1978 behaalde hij het Atheneum-B diploma aan het Christelijk Lyceum Delft. In datzelfde jaar werd een aanvang gemaakt met de studie biologie aan de Rijksuniversiteit Leiden. In juni 1985 behaalde hij zijn doctoraal examen met als hoofdvak biochemie (Dr. J. Bol en Prof. Dr. P. van Knippenberg) en als bijvakken celbiologie (Prof. Dr. T. Konijn) en immunologie (Dr. S.O. Warnaar).

Vanaf september 1985 tot aan januari 1989 was hij werkzaam op de afdeling Immunologie van de Dr Daniël den Hoed Kliniek, op een door de Nederlandse Kankerbestrijding "Koningin Wilhelmina Fonds" gefinancieerd project. Onder leiding van Dr. R.L.H. Bolhuis en Prof. Dr. D.W. van Bekkum (Radio Biologisch Instituut, TNO) werd het in dit proefschrift beschreven onderzoek verricht.

Vanaf augustus 1989 is hij werkzaam op de afdeling "Clinical and Medical Oncology" van de "University of Southern California" in Los Angeles onder begeleiding van Dr. M.S. Mitchell.