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# Requirement for IL-2-receptor and B7/CD28 signaling in cytotoxic T cells

by

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Submitted in partial fulfillment of the requirements for the degree of

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The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "Requirement for IL-2-receptor and B7/CD28 signaling in cytotoxic T cells"

by Andrew Peter Makrigiannis

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## Abstract

In order for cytolytic T lymphocytes (CTL) to achieve optimal activation they must receive a variety of signals. These signals can be provided by cytokines, as well as by cell surface proteins present on antigen presenting cells. Signaling through the IL-2R of CTL is important for the induction of cytolytic gene expression and the acquisition of cytotoxicity. Rapamycin (RAP) is a drug which can inhibit the $p70^{s6k}$ pathway of IL-2R signal transduction. I have shown that RAP inhibits proliferation, cytotoxicity, granzyme B (Gzm B), and perforin gene expression in CTL induced with anti-CD3 mAb. Treatment with RAP plus rIL-2 was able to restore proliferation and cytolytic gene expression, but not cytotoxicity to control levels. This suggests that RAP-sensitive rather than RAP-resistant IL-2R signaling pathways are required for the acquisition of cytotoxicity. In addition to signaling through the IL-2R, anti-CD3-activated CTL also require CD28-derived costimulatory signals. Using blocking mAb specific for B7-1 and B7-2, I found that B7-2 is the major ligand for CD28 during anti-CD3 activation of CTL. On the other hand, B7-1-mediated costimulation is a very minor component. While B7-2 was expressed on both T and B cells, T cell-derived B7-2 did not contribute to costimulation. Blockade of B7-2/CD28 interactions led to decreased production of IL-2, IL-4, IL-6, IL-10, and IFN-gamma, as well as decreased expression of Gzm B. Exogenous rIL-2 and IL-12 were found to substitute for B7-2 costimulation, restoring cytolytic activity and Gzm B expression to control levels. IL-12-mediated costimulation was found to be IL-2-dependent and IFN-gamma-independent. In conclusion, these results indicate that RAP would be an effective therapy in pathologies mediated by the granule exocytosis pathway of CTL. In addition, B7-2 is the major costimulator for anti-CD3-induced CTL activation, suggesting that CTL activation *in vivo* may utilize this CD28 ligand.

## ABBREVIATIONS AND SYMBOLS

| | |
|---|---|
| ActD | actinomycin D |
| ADCC | antibody-dependent cell-mediated cytotoxicity |
| AP-1 | activating protein-1 |
| APC | antigen presenting cell |
| BCIP | 5-bromo-4-chloro-3-indolyl phosphate |
| BCR | B cell receptor |
| bp | base pairs |
| BSA | bovine serum albumin |
| CHO | Chinese hamster ovary |
| Con A | concanavalin A |
| CD28RE | CD28 responsive element |
| $^{51}Cr$ | $^{51}$chromium |
| CREB | cAMP responsive element binding (proteins) |
| cRPMI | complete RPMI 1640 medium |
| CsA | cyclosporin A |
| CTL | cytotoxic T lymphocyte |
| $ddH_2O$ | distilled and de-ionized water |
| EAE | experimental allergic encephalomyelitis |
| ELISA | enzyme-linked immunosorbent assay |
| FADD | Fas-associated death domain |
| FasL | Fas ligand |
| FCS | fetal calf serum |
| FcR | Fc receptor |
| FcγRIII | Fc-gamma receptor |
| FKBP12 | FK506-binding protein-12 |
| GAPDH | glyceraldehyde phospodehydrogenase |
| GM-CSF | granulocyte/macrophage colony stimulating factor |
| Gzm | granzyme |
| h | hour |
| HEPES | *N*-2-hydroxyethylpiperazine-*N*-2ethanesulfonic acid |

| | |
|---|---|
| HRP | horse-radish peroxidase |
| [$^3$H]-TdR | tritiated thymidine |
| IFN-γ | interferon-gamma |
| IFN-γR | interferon-gamma-receptor |
| IL-2 | interleukin-2 |
| IL-2R | interleukin-2-receptor |
| IL-4 | interleukin-4 |
| IL-6 | interleukin-6 |
| IL-10 | interleukin-10 |
| IL-12 | interleukin-12 |
| JAK | Janus kinase |
| kD | kilodalton |
| LAK | lymphokine activated killer cell |
| LCMV | lymphocytic choriomeningitis virus |
| LFA-1 | lymphocyte function in adhesion-1 |
| LPS | lipopolysaccharide |
| MAPK | mitogen-activated protein kinase |
| MHC | major histocompatibility complex |
| min | minute |
| mTOR | mammalian target of rapamycin |
| NBT | nitro blue tetrazolium |
| NK | natural killer |
| $p70^{s6k}$ | p70 S6 kinase |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |
| PBMC | peripheral blood mononuclear cells |
| PBL | peripheral blood lymphocytes |
| PCNA | proliferating cell nuclear antigen |
| PCR | polymerase chain reaction |
| PI3 | phosphatidylinositol 3 |
| PMA | phorbol 12-myristate 13-acetate |

| | |
|---|---|
| RAP | rapamycin |
| rIL-2 | recombinant interleukin-2 |
| RT | reverse transcriptase |
| sec | second |
| SDS | sodium dodecyl sulfate |
| STAT | signal transducers and activators of transcription |
| TAE | Tris acetate EDTA buffer |
| TBS | Tris-buffered saline |
| TCR | T cell receptor |
| Th | T-helper (cell) |
| TIL | tumor infiltrating lymphocyte |
| TMB | 3,3',5,5'-tetramethylbenzidine |
| TNF-α | tumor necrosis factor-alpha |
| TNFR | tumor necrosis factor receptor |
| TRAIL | TNF related apoptosis inducing ligand |

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## 1.0 Introduction

### 1.1 Activation of cytotoxic T lymphocytes

Eradication of intracellular pathogens and surveillance against malignant transformation are two of the main responsibilities of cytotoxic T lymphocytes (CTL) (1). CTL protect the host from infection and tumor growth by inducing the destruction of the affected cells through the delivery of a 'death' signal (1). Initial antigenic recognition by resting $CD8^+$ T cells is required for the activation and differentiation of precursor cells into active CTL effectors (2). The antigen receptor of T cells is comprised of the heterodimeric T cell receptor (TCR) in complex with the multiple protein chains of the signal transducing CD3 protein cluster (3). The TCR/CD3 complex of $CD8^+$ CTL recognizes peptide antigen on the surface of target cells in association with the protein products of the class I major histocompatibility complex (MHC) of genes (4). CTL precursors are usually $CD8^+$ T cells (2), but $CD4^+$ CTL have also been reported, although at a much lower frequency (5).

T cells also require a 'secondary' or 'costimulatory' signal in order to become activated and produce cytokines such as interleukin-2 (IL-2) which promote proliferation and differentiation. T cells from mice whose IL-2 gene expression has been disrupted have dramatically lowered proliferative responses to polyclonal activators (6). IL-2 is produced when CD28 on T cells is ligated by B7 proteins (7). However, target cells may not express B7 proteins (8), which restricts the self-sufficiency of CTL. Thus, an exogenous source of cytokines or B7 costimulation is required. Current hypotheses predict that T helper cells (Th), usually $CD4^+$ T cells, will produce IL-2 and other cytokines upon interaction with an antigen presenting cell (APC) constitutively expressing the necessary B7 molecules. Thus,

it is hypothesised that CTL borrow IL-2 from Th cells for their differentiation. However, different groups using antibody-mediated systemic depletion of $CD4^+$ T cells and CD4 gene-knockout mice have shown that CTL from both types of animals become activated normally in the absence of $CD4^+$ T cells (9,10). This suggests that CTL can either produce cytokines themselves or cytokines from other $CD4^-$ cells can help activate CTL.

Regardless of the source, IL-2 and other cytokines induce T cells to enter cell cycle (11) and cause CTL to produce cytolytic molecules associated with cytoplasmic granules (12). Gzm B and perforin are two granule-associated proteins whose expression is known to be upregulated by IL-2 (12). Gzm B and perforin comprise the granule exocytosis pathway of target cell destruction. Upon target cell recognition, a CTL can exocytose its granule contents onto a target cell resulting in its destruction (13). In addition, CTL can also employ membranolytic mechanisms to destroy target cells. Interactions of $FasL^-$ CTL with $Fas^+$ target cells, like Gzm B, also induces apoptosis (14). CTL may also employ a surface form of tumor necrosis factor (TNF) which is cytotoxic for target cells (15).

### 1.2 Polyclonal activation of CTL by anti-CD3 mAb

T cells can be activated by a wide variety of stimuli independently of specific antigen (16). Antibodies towards the epsilon chain of the mouse CD3 complex have been shown to activate T cells *in vitro* (16). T cells can also be activated with antibodies specific for the zeta-chain of the TCR/CD3 complex, although with differing patterns of tyrosine phosphorylation (17). An interesting side effect of this treatment is the loss of cell surface expression of the TCR/CD3 complex (18). The loss of antigen receptor is manifested in the

ability of anti-CD3-activated T cells from the thymus, spleen, lymph nodes, but not the bone marrow to kill certain tumor cells in an MHC-unrestricted manner (19). Such MHC-unrestricted murine CTL are able to kill both NK-sensitive (YAC-1) and NK-resistant (P815) target cells (19). In addition, MHC-unrestricted activity can also be elicited when other T cell surface antigens are cross-linked, such as CD4 (20). NK cells in mouse spleen cell preparations can also be activated due to the high levels of IL-2 elicited from T cells by the anti-CD3-treatment (19). Therefore, treatment with anti-CD3 mAb will activate T cells, as well as stimulate lymphokine-activated killer (LAK) cell activity. The action of anti-CD3 mAb is not restricted to the induction of IL-2 production and secretion. Activation of human peripheral blood mononuclear cells (PBMC) shows both an IL-2-dependent and IL-2-independent pathway of induction (21). This is supported by the finding that the transcription of a wide variety of cytokine genes is induced by anti-CD3-treatment (22).

Anti-CD3 mAb requires cross-linking either by $FcR^+$ accessory cells or with anti-isotypic antibody in order to induce T cell activation (18). In agreement with this report is the finding that anti-CD3 mAb cleaved of their Fc segment cannot activate T cells (23). In the absence of accessory cells, T cells can only be activated by plastic immobilized anti-CD3 mAb in combination with exogenous IL-2 and, to a lesser degree, IL-4 (24). Co-immobilized anti-CD28 mAb can also synergize with anti-CD3 mAb during *in vitro* activation of T cells (25). Because accessory cells such as B cells or macrophages do not make IL-2, this suggests that, in addition to FcR, accessory cells must also supply costimulation.

Attempts have been made to utilize the stimulatory ability of antibodies specific for

TCR/CD3 in activating host T cells to ameliorate disease. Administration of anti-CD3 mAb in conjunction with IL-2 has been shown to increase the survival time of mice with hepatic tumors, decrease secondary hepatic metastases and induce perforin, Gzm and TNF mRNA in hepatic tissues (26). Tumor infiltrating lymphocytes (TIL) are sensitive to activation by anti-CD3 mAb. Treatment of TIL with anti-CD3 mAb results in increased proliferation, IL-2 production (27), and become more cytotoxic to the tumor that they were originally isolated from (28). Therefore, the induction of CTL activity by anti-CD3 mAb can help to halt tumor progression and metastases, as well as provide a convenient *in vitro* tool to characterize CTL signaling requirements.

### 1.3 Mechanisms of cytotoxicity

Once a target cell is properly recognized, an activated CTL can then employ one or more mechanisms of cytotoxicity. The two best characterized mechanisms are the granule exocytosis pathway and the Fas-FasL pathway of apoptotic induction.

#### (a) Perforin and granule exocytosis

During activation, CTL generate intracellular vesicles termed granules which store cytolytic enzymes (29). Antigenic recognition of a target cell results in the reorganization of these granules towards the CTL-target cell interface, and the granules are then exocytosed (13). Residing within these granules are a wide array of proteins. The two best characterized are the pore-forming protein perforin and a family of proteases termed 'granzymes'. Perforin, a 70-75 kDa protein in its monomeric form (30), is found within the granules of CTL (30,31), and is related to the pore-forming proteins of the complement

membrane attack complex (32) which damages cells in a similar fashion to perforin. Once exocytosed, perforin binds to the target cell plasma membrane via phosphorylcholine molecules in a $Ca^{2+}$-dependent manner (33). Perforin monomers then polymerize into a barrel-like structure or pore (30,31). It is believed that this pore acts as an entrance for proteases/granzymes to enter the cytoplasm. Perforin expression is not restricted to CTL. Perforin is also found in natural killer (NK) cells and macrophages (34), and is expressed by some $CD4^+$ T cells (35). CTL and NK cells from animals in which the perforin gene has been disrupted have a drastically reduced ability *in vitro* to lyse tumor and virally-infected target cells (36-38). Residual cytotoxicity in perforin-less CTL is dependent on the Fas-induced apoptosis pathway (38). These mice also cannot erradicate lymphocytic choriomeningitis virus (LCMV) infection and have reduced effectiveness in combating tumor growth (36). Interestingly, there is a recent report of Gzm B inducing target cell destruction in the absence of perforin (39). However, another group has reported that FITC-labelled Gzm B crosses the outer plasma membrane of target cells in the absence of perforin, but only enters the nucleus and causes apoptosis in the presence of perforin (40). Nevertheless, these reports suggests that Gzm B has alternative methods of entering the cytoplasm of target cells.

**(b) Granzymes**

Introduction of granzymes into a target cell soon results in target cell death by apoptosis (41). The induction of apoptosis results in clumping of the chromatin, the loss of plasma membrane structural integrity and the concomitant membrane 'blebbing' of the affected cell (42). Meanwhile, activated nucleases destroy the cell's genome by cleaving

DNA between DNA-associated histone complexes (43). This results in the characteristic 'laddering' effect of 180 bp multiples when DNA from apoptotic cells is electrophoresed across an agarose gel (43).

The Gzm family is composed of various members. In humans and mice, there are two clusters of Gzm genes. Each of the genes within the cluster are homologous to each other, suggesting evolution of the different members through gene duplication events. In humans, these clusters consist of Gzm B, H, cathepsin G, and mast cell chymase on chromosome 14q11.2 (44) and Gzm A and K on chromosome 5q11-q12 (45). In mice, the gene duplication history is different with Gzm B, C, F, G, D, E, cathepsin G, and MMCP-2 being located (in that order) on chromosome 14 (46) and Gzm A and K tightly linked on chromosome 13 (47). Gzm A and K are tryptases (41,48), which cleave after a lysine or arginine residue, while Gzm B is an aspase (41), cleaving after aspartic acid. CTL express Gzm A, B, and C (49,50), while lymphokine-activated killer (LAK) cells express Gzm A, B, C, D, E, F, G, and H (46,51). NK cell lines have been reported to express Gzm A, B, C, D, F, H, and K (41,46,51). Cathepsin G is expressed in promyelocytes/promonocytes (51) and mast cell chymase is expressed in mast cells (52).

The most well characterized granzymes are Gzm A and B. Despite initial reports that Gzm A can induce apoptosis in target cells permeabilized with perforin (53), subsequent production of Gzm A knockout mice showed that CTL do not require the presence of this molecule to induce target cell apoptosis (54). Furthermore, Gzm A-deficient mice clear an LCMV infection as efficiently as wild-type animals (54). In contrast, it has been reported that Gzm A knockout mice cannot successfully combat

ectromelia virus infection as well as normal animals (55). This suggests that Gzm A plays some role in host defence, but one that is dependent on the type of infectious agent encountered.

Gzm B, unlike perforin, does not seem to be as necessary for target cell lysis, but is absolutely required for rapid DNA fragmentation induced by CTL (56) and NK cells (57). Specific $^{51}$Cr-release from target cells occurs at normal levels following longer incubation with Gzm B-deficient CTL, but DNA fragmentation is only partial (56,57). This suggests that Gzm B is necessary for the rapid induction of apoptosis in target cells, but during longer periods of association with CTL or NK cells alternative pathways exist which also contribute to DNA fragmentation.

A recent report has shed light on the reason for residual cytotoxicity found in Gzm B-deficient CTL. Mice crossbred for both Gzm B and FasL-deficiencies produce CTL which induce lower amounts of DNA fragmentation in target cells and require longer periods of CTL/target cell incubation for cytolysis to occur (58). The slow-acting, residual cytotoxicity of Gzm B-deficient CTL is perforin-dependent since CTL from perforin/FasL-deficient mice display no cytotoxicity (58). This suggests that other soluble molecules, perhaps other granzymes, use perforin to gain entrance to the cytoplasm of target cells. This hypothesis is supported by the finding that CTL from Gzm A and B double-knockout mice cannot mediate target cell DNA fragmentation (59). Single knockout animals induced target cell genome degradation, although Gzm B knockouts have a delayed effect, showing that, if given sufficient time, Gzm A can substitute for Gzm B.

In addition to Gzm A and B, other granzymes have been reported to induce

cytctoxicity. Like Gzm A, prolonged (>14 h) exposure to purified rat Gzm K has been found to induce DNA fragmentation in target cells (41). A separate group studying a cloned CTL line, which specifically lyses renal epithelial cells, showed decreased Gzm C mRNA levels and cytotoxicity towards renal target cells when CTL were pre-incubated with an anti-sense oligonucleotide specific for Gzm C (60). Furthermore, kidney damage in mice resulting from injection of this CTL line was lessened if the CTL were treated with anti-sense oligonucleotide prior to injection (60). However, this study did not show that Gzm B levels were unaffected in these cells. Considering the high degree of homology which Gzm C shares with Gzm B and the other genes in the gene cluster, it would not be surprising if the saturating amounts of anti-sense oligonucleotides used to block Gzm C synthesis also bound to the Gzm B gene, resulting in decreased cytotoxicity due to lower Gzm B levels.

Once introduced into the cytoplasm, Gzm B specifically cleaves proteases whose role in apoptosis was first characterized in the genetic studies of the flatworm *Caenorhabditus elegans*. Gzm B has been shown to cleave CPP32/caspase-3 (61), a member of the ICE/CED family of proteases, which in turn processes many other protein substrates known to be cleaved during the induction of apoptosis (62). Multiple members of the ICE family have been identified in the proteolytic cascade induced by Gzm B and have been renamed as 'caspases' due to internal cysteine residues and the ability to cleave after aspartic acid. An intermediary step in the induction of apoptosis by caspases is the release of mitochondrial membrane proteins such as cytochrome c and apoptosis inducing factor. These molecules, along with caspase 9, help to form a complex termed the

apoptosome which helps to activate pivotal enzymes such as caspase 3 (62a). Once activated, these proteases are responsible for the cleavage of cytoskeletal molecules such as actin (63) and the processing and activation of latent endonucleases with the nucleus (64). The CPP32-caspase cascade also seems to be specific for activation by Gzm B and Fas (65), since inhibition of CPP32 protease activity does not inhibit Gzm A-induced cytotoxicity (65). Recently, CPP32 or caspase 3 has been found to be responsible for directly activating a latent endonuclease in the cytoplasm which then travels to the nucleus and degrades chromosomal DNA (64). Caspase 3 has been shown to activate the caspase-activated deoxyribonuclease (CAD) by cleaving its chaperone inhibitor protein ICAD (64). It should be noted, however, that genome fragmentation does not seem to be a necessary consequence of CTL attack. It has been shown that cells can be lysed by CTL just as efficiently in the absence of DNA digestion (66). Similarly, in the presence of global inhibitors of caspases, target cells are still lysed by granule exocytosis (67). Thus, there are still unelucidated lytic pathways induced by the introduction of granzymes into target cells.

One possible role for activated endonucleases may be to destroy viral nucleic acid present in virus-infected cells from becoming infected. This would protect neighbouring cells and phagocytes which absorb apoptotic debris from viral infection. Studies with a serine protease inhibitor provide evidence for this idea. Introduction of Gzm B, but not Gzm A, to cells infected with vesicular stomatitis virus resulted in viral RNA degradation, which could be blocked with a serine protease inhibitor (39).

**(c) The Fas receptor and Fas ligand-induced apoptosis**

One of the best characterized ligand-receptor systems which induces apoptosis in

the absence of CTL degranulation is the Fas/FasL system. This system of interactions has gone through an evolution in its classification by immunologists. First thought to be a mechanism by which CTL lyse target cells, the Fas/FasL cytolytic pathway is now considered to be primarily a regulator of T cell expansion. FasL may also play a role in the protection of certain 'immunologically privileged' tissues from T cells.

Fas is a type I cell surface molecule with a molecular weight of 45 kDa (68) and is expressed in very many cells types including myocytes, splenocytes, thymocytes, hepatocytes and ovarian tissue (69). Ligation of cell-surface Fas by specific antibody was found to induce apoptosis in treated cells (70). Mice injected with as little as 10 μg of anti-Fas mAb Jo2 was adequate to kill 5 of 10 mice within 8 h (71). Autopsy results showed that these mice died of liver destruction, which is not surprising considering that the liver is known to constitutively express Fas mRNA (69). The damaged livers displayed many areas of focal haemorrhage and necrosis, and most hepatocytes displayed the characteristic cytoplasmic condensation seen in apoptotic cells (71).

Sequencing of the mouse Fas antigen showed that it is a member of tumor necrosis factor receptor (TNFR) family (69). Other members of this family, such as TNF-α, can also signal cells to undergo apoptosis (70). Naturally occurring mutations in the Fas gene (*lpr*) lead to lymphadenopathy, splenomegaly and autoimmune disease (72). The dramatic increase in the sizes of the lymphoid organs of *lpr* mice is due to the accumulation of huge numbers of $CD4^-$ $CD8^-$ T cells (72). Mice with the *lpr* mutation also suffer from systemic lupus erythematosus, producing autoantibodies to single and double-stranded DNA (73). However, the *lpr* mutation does not constitute a complete loss of Fas expression and is

therefore slightly 'leaky' (74). Fas-knockout mice, where Fas expression is totally absent, develop lymphadenopathy and splenomegaly more rapidly and severely than *lpr* mice and also suffer from lymphocytic infiltration into the lungs and liver (75,76). The majority of T cells from $Fas^{-/-}$ mice express the unusual surface phenotype also found with *lpr* mice, i.e., $Thy1^{+}$, $B220^{+}$, $CD4^{-}$, and $CD8^{-}$ (75). Humans with a mutation in the Fas gene develop symptoms similar to those of *lpr* mice (77).

Fas is known to be expressed by a wide variety of nonhematopoietic tumors including colonic carcinomas, epidermoid carcinomas, glioblastomas, melanomas, osteosarcomas, pancreatic adenocarcinomas, and prostatic adenocarcinomas (78). However, expression of Fas by these cell lines does not always correlate with sensitivity to an apoptosis-inducing anti-Fas mAb. In fact, in one particular study only 4 of 10 $Fas^{+}$ tumor cell lines were killed by anti-Fas antibody treatment (78). This suggests that the genes controlling Fas expression and the intermediate cytoplasmic proteins involved in the signal cascade leading to apoptosis are disregulated during malignant transformation. Perhaps this acts as a survival mutation for the neoplastic cell.

The mechanism by which Fas signaling induces apoptosis in cells has much in common with the Gzm B protease. Like Gzm B (61), the distal arm of the signal cascade induced by Fas is mediated by the caspase proteolytic cascade (79). The connection between the Fas cytoplasmic complex and the caspase cascade is mediated by the 'adaptor' molecule Fas-associated death domain protein (FADD) (80). FADD associates with both the cytoplasmic death domain of Fas and the prodomains of caspase family members (80). Interestingly, FADD is essential for embryo development as $FADD^{-/-}$ mice do not survive

embryogenesis (81), while $Fas^{-/-}$ mice display normal embryonic development (75). This suggests that the downstream signal cascade used by Fas is also used by other receptors which are needed during embryogenesis.

**(d) Fas ligand (FasL)**

Fas ligand (FasL) is a 40 kDa type II surface protein that belongs to the TNF family of growth factors (14,82). FasL is expressed in all lymphoid organs including the thymus, lymph nodes, and spleen, as well as the lung, small intestine, and testis (83). FasL is expressed by T cells of both the $CD4^+$ and $CD8^+$ subsets, although $CD8^+$ T cells express FasL at much higher levels than $CD4^+$ T cells (83). Among $CD4^+$ cells, FasL is expressed by Th1, Th2 and Th0 cells (83). FasL expression in T cells can be increased by mitogenic stimulation via phorbol 12-myristate 13-acetate (PMA)/ionomycin, concanavalin A (Con A), or anti-CD3 mAb, as well as IL-2 treatment (83). FasL has recently been detected in murine B cells activated with lipopolysaccharide (LPS) or PMA/ionomycin (84). This expression is functional as LPS activated B cells from normal mice, but not FasL-deficient mice, were able to kill Fas-sensitive target cells (84). Constitutive FasL expression has also been reported in murine NK cells (85). In humans, NK cells have been found to express FasL only when activated by ligation of FcγRIII (86).

At least in mice, FasL exists as two different, yet functional alleles. Differences in recognition by anti-FasL mAb led to the discovery of mFasL.1 (Thr-184, Glu-218) and mFasL.2 (Ala-184, Gly-218) (87). The amino acid differences of these two proteins lie in the extracellular region and so may affect antibody binding. mFasL.1 is expressed in B6, C3H, MRL, SJL, NOD, NZB, and NZW mice, while mFasL.2 is found in BALB/c, DBA/1,

and DBA/2 mice (87). Interestingly, mFasL.2 is more efficient than mFasL.1 in inducing apoptosis when transfected into COS cells (87). This shows that, depending on the genetic background, certain mice may have relatively weak FasL function. This may explain why the severity of autoimmune disease can be several orders of magnitude greater when the leaky *lpr* mutation is bred onto different mouse strains (88).

**(e) Fas/FasL interactions in immune responses and pathogenesis**

A phenotype similar to that of *lpr* mice is seen in *gld* mice (72), which have a mutation in the FasL gene. In *gld* mice FasL is improperly folded due to a point mutation and cannot bind to Fas (89). There is evidence that Fas/FasL interactions may play some role in host resistance to viral infections. β-2-Microglobulin-deficient mice, which are deficient in $CD8^+$ T cells, develop $CD4^+$ CTL in response to LCMV infection (90). These CTL were shown to destroy virally-infected target cells in a manner which could be inhibited by a soluble Fas-Ig fusion protein (90). However, these CTL were not sufficient to overcome the infection and the animals subsequently died. A role for FasL in the pathogenesis of the infection was shown when CTL from LCMV-infected β-2-microglobulin-deficient mice were adoptively transferred to irradiated infected mice. Although these mice died, transfer into similarly treated *lpr* mice did not cause death (90). This suggests that an uncontrolled FasL-mediated cytotoxicity can have dire consequences and is in agreement with the lethal effects of injection anti-Fas mAb into mice (71).

Non-hematopoietic cells can also express FasL. FasL expression has been detected on human epithelial colon adenocarcinoma cells (91). These $FasL^+$ carcinoma cells are able to kill Jurkat cells in a Fas-dependent manner, but are themselves immune to Fas-killing

(91). This suggests that abherant expression of genes such as FasL by neoplastic cells are one way of destroying tumor-specific T cells. *In vivo* evidence for this theory has been provided in a malignant melanoma model. Injection of FasL$^+$ mouse melanoma cells into normal mice led to tumor growth, whereas when FasL$^+$ mouse melanoma cells were injected into Fas-deficient-*lpr* mice tumor growth was delayed (92).

Fas/FasL interactions may also play a role in the resolution of granulocytic infiltration in a site of antigen challenge. Eosinophils recruited into the lung after aerosolized antigen challenge express the Fas receptor and are sensitive to anti-Fas mAb killing (93). This supports the idea that Fas is responsible for the downregulation of leukocyte numbers, either locally or systemically, after antigen-induced white blood cell expansion or aggregation.

### 1.4 The role of CTL in graft rejection

CTL not only destroy virally-infected and transformed cells, but have also been implicated in attacking allografts. Human infiltrating leukocytes isolated from grafts undergoing acute rejection have been shown to lyse proximal tubular epithelial cells of donor origin in a TCR/CD3 and MHC class I-dependent fashion (94). Gzm B and perforin expression are associated with human cardiac and renal transplants undergoing severe acute rejection (95,96). In rats, perforin and Gzm B mRNA are upregulated during the rejection of small intestine allografts (97). Gzm B mRNA is also increased in muscle biopsies after allogeneic myoblast transplantation in mice (98). It should be noted that perforin may not be absolutely required for allograft rejection, since perforin-deficient mice are able to reject

allogeneic tumor cells as efficiently as normal mice (99). Possible rejection through Fas and TNF-α-dependent pathways were also excluded as the tumor cells were insensitive to both of these cytotoxic mediators. This seems to indicate that CTL are not required for allograft rejection. However, in the same study, mice depleted of $CD8^+$ cells could not control allogeneic tumor growth (99). This suggests that while perforin is not required for allograft rejection, other molecules produced by $CD8^+$ T cells are necessary for responses against allogeneic target cells.

Because cardiac tissue has long been known to constitutively express Fas mRNA (69), the role of Fas/FasL interactions in allogeneic cardiac rejection was addressed. It was found that FasL-deficient *gld* mice rejected normal allogeneic hearts at control rates, and Fas-deficient *lpr* donor hearts were also rejected quickly by normal mice (100). This suggests that rejection of cardiac allografts is probably being carried out by granule exocytosis. However, a separate study found that perforin-deficient mice can also reject allogeneic hearts from *lpr* donor mice at normal rates (101). This suggests that either other cell types or other T cell cytotoxic products can also mediate graft rejection in the absence of either perforin or FasL function. These findings seem to contradict initial reports of CTL/NK cells from perforin-knockout animals which could not lyse target cells *in vitro* (36,37). This may indicate that alternative pathways exist *in vivo* which can replicate the membrane-damage induced by perforin. At least one study has shown that alternative perforin-like proteins exist (102) which may also be expressed and utilized by perforin-deficient CTL. In addition, it is known that other membraneolytic mechanisms distinguishable from FasL and TNF-α are present on the surface of some cells. One such

mechanism involves the action of TNF related apoptosis inducing ligand (TRAIL) (103).

### 1.5 Cytokines involved in CTL activation

#### (a) IL-2

The presence of a T cell growth factor was first described in mixed lymphocyte reactions and in the supernatants of T cell cultures stimulated with mitogens (104). This factor was later renamed interleukin-2. IL-2 is a small molecule of approximately 15 kDa (105). Once bound to its receptor, IL-2 induces the activation of several growth and differentiation pathways which eventually result in cell proliferation and the development of cytolytic capability in cytotoxic lymphocytes through upregulation of Gzm B and perforin gene expression (106,107). IL-2 will stimulate NK cells to kill tumor cells and, after an extended period of culture with IL-2, NK cells differentiate into LAK cells which are able to recognize a wider range of targets (108-110). In addition, IL-2 induces B cell proliferation and immunoglobulin production (111), increases IFN-γ production by NK cells (112), and increases IL-6 production by monocytes (113).

IL-2 promotes the activation and differentiation of resting $CD8^+$ precursors into mature CTL, but is not absolutely required for this process (114). Primary CTL isolated from virally-infected $IL\text{-}2^{-/-}$ mice display cytotoxicity to virally-infected target cells comparable to wild-type mice (114). However, virus-specific $IL\text{-}2^{-/-}$ CTL re-stimulated *in vitro* with virally infected stimulator cells displayed little cytotoxicity (114). Interestingly, proliferative responses of the restimulated $IL\text{-}2^{-/-}$ CTL proceeded normally (115). These results suggest that other cytokines promote CTL activation *in vivo*, but induction of CTL

cytotoxicity *in vitro* is strictly dependent on the presence of IL-2.

**(b) IL-2R signal transduction pathways**

The IL-2R is present on the surface of T cells as a heteromorphic combination of at least three subunits: the α, β, and γ subunits (116,117). The αβγ trimeric structure is the receptor with the highest affinity for IL-2 (118). The βγ complex will also bind IL-2, but with less affinity (118). Other combinations of subunits will bind IL-2 but do not transduce a signal (118). The γ chain of the IL-2R is also utilized by a number of other cytokine receptors such as those for IL-4, IL-7, IL-9, and IL-15 (119,120). The γ chain is expressed constitutively on lymphoid cells (121), including both resting and activated T cells (116), while the α chain is expressed only upon T cell activation (117). The β chain is constitutively expressed on $CD8^+$ T cells (117), and is induced on $CD4^+$ T cells following activation (117,118). Several signaling pathways are known to emanate from the IL-2R, resulting in the indirect activation of transcription factors c-*fos*/c-*jun* (122) by the src-family kinase *lck* (123), c-myc activation (122) and induction of the cell-survival protein *bcl-2* (124). Another immediate consequence of IL-2 binding to the IL-2R is the activation of p70 S6-kinase ($p70^{s6k}$) (125), which is involved in cell cycle control, transcription and translation initiation (126). The intermediate step between cytokine receptor binding and $p70^{s6k}$ phosphorylation is the activation of the mammalian target of rapamycin (mTOR) kinase (126). mTOR has been shown to be directly responsible for the activation of, among other proteins, $p70^{s6k}$ (127). Activated $p70^{s6k}$ phosphorylates a family of transcription

elements known as the cyclic adenosine monophosphate responsive element (CRE) modulator proteins which are required for the expression of many different genes (128).

The IL-2R is also known to activate the Janus-kinase (JAK)-signal transducers and activators of transcription (STAT) family of proteins (129,130). JAK kinases are a family of proteins usually associated with the cytoplasmic domains of cytokine and other growth-hormone receptors (131). Upon receptor ligation, the JAKs become phosphorylated which in turn leads to their activation and the phosphorylation of cytoplasmic STAT proteins (132). Once STAT monomers become phosphorylated they homo- or hetero-dimerize with either identical or related STAT proteins and migrate to the nucleus, whereupon entering, they act as transcription-inducing DNA-binding factors (133). The importance of JAK-STAT activation pathways in T cell activation is seen in the phosphorylation and activation of STAT5 after IL-2R-binding (129). Activated STAT5 binds to a portion of the human IL-2R$\alpha$ gene and induces its transcription (134). Furthermore, JAK3 knockout mice have a drastic defect in T cell development (135). JAK3 is associated with the $\gamma$ chain of the IL-2R (133).

Surprisingly, a structurally and sequentially unrelated cytokine, IL-15, has been shown to bind the $\beta$ and $\gamma$ subunits of the IL-2R (120,136). IL-15 was first identified as a molecule released by a simian kidney epithelial cell line which could stimulate the growth of the murine CTLL T cell line (136). IL-15, not surprisingly, can substitute for IL-2 in the activation of killer cells and induce the transcription of mRNAs for perforin, Gzm A and Gzm B (137). Interestingly, despite being comparable to IL-2 in terms of the ability to upregulate cytolytic mediators, IL-15 was not as effective in inducing proliferation or

cytotoxicity (137). The existence of IL-2-like cytokines such as IL-15 may explain why IL-2-deficient mice are able mount an effective anti-viral response.

**(c) IL-12**

APC also produce cytokines which activate CTL. Although IL-12 is secreted by a wide variety of professional and non-professional APC, including B cells (138), monocytes/macrophages (139), dendritic cells (140), Langerhans cells (141) and keratinocytes (142), macrophages seem to be the major source of IL-12 (143). IL-12 has been shown to increase by 10-20 fold the cytotoxicity of anti-CD3 activated peripheral blood human $CD8^+$ T cells (144). IL-12 upregulates mRNA expression of the cytolytic mediators Gzm B and perforin (145). IL-12 also activates NK cells to recognize $B7^+$ target cells (146). Transfection of tumor cells with both IL-12 and B7-1 as opposed to just B7-1 alone dramatically improved the ability of mice to remain tumor-free (147). IL-12 is a heterodimeric molecule in its bioactive form, composed of p40 and p35 chains (138,148). Dimerized p40 is able to bind to the IL-12R and block the productive binding of the bioactive p70 heterodimer (149). Among its many effects, IL-12 induces interferon (IFN)-$\gamma$ production in peripheral blood lymphocytes (148) and $CD30^+$ T cells (150), suppresses IL-4-induced IgE production (151), induces Th1 responses (152), and helps to cure *Listeria* and *Leishmania major* infections (153,154). The IL-12R, composed of IL-12R$\beta$1 and IL-12R$\beta$2 subunits, has been found on both $CD4^+$ and $CD8^+$ T cells, as well as NK cells (155). The IL-12R$\alpha$ nomenclature is reserved for the p40 subunit of IL-12 due to its homology with IL-6R$\alpha$.

**(d) IFN-γ**

Secretion of IFN-γ by Th1 cells suppresses Th2 cell proliferation (156). IFN-γ has also been reported to increase the expression of class I and II MHC on macrophages, B cells, epithelial cells, dendritic cells, and endothelial cells (157-159). Murine B cells proliferate and differentiate into IgG2a and IgG3-secreting plasma cells in the presence of IFN-γ (160). In addition, IFN-γ increases tumor cell sensitivity to monocyte-mediated cytotoxicity (161), and has been reported to promote the activation of CTL and LAK cells by increased expression of IL-2Rα (162). Although IFN-γR-knockout mice are more susceptible to vaccinia virus infection, vaccinia virus-specific CTL from these mice display cytotoxicity comparable to wild-type CTL against vaccinia-infected target cells (163). This suggests that IFN-γ is not required *in vivo* for CTL activation.

## 1.6 Rapamycin and its effects on CTL function

### (a) Blockade of cell cycle transition from $G_1$ to S phases

Rapamycin (RAP) is an immunosuppressive macrolide produced by the soil bacterium *Streptomyces hygroscopicus* obtained from the island of Rapa Nui or Easter Island (164). The chemical structure of RAP is complex, $C_{51}H_{79}NO_{13}$, with a molecular weight of 914 Daltons (165). Initially, RAP was shown to act as a fungal growth inhibitor, with specific toxicity towards *Candida albicans* (164). Subsequent studies have shown RAP to also delay T and B cell proliferation (166). RAP inhibits T cell activation by delaying the transition from the G1 to S phase of cell cycle (167). This is attributed, in part,

to a delay in protein synthesis caused by an inhibition of ribosomal and ribosome-associated protein mRNA translation (167-169). RAP has been shown to suppress the proliferation of cell lines in response to IL-2 (166), IL-4 (170), and IL-6 (171).

RAP mediates G1 arrest, in part, by inducing the expression of $p27^{kip1}$, a cyclin/ckds inhibitor which is eliminated when quiescent cells are activated by mitogenic signaling pathways (172). $p27^{kip1}$ protein may be a target of degradation in mitotic cells. The degradation of most cellular proteins is mediated by large protease complexes termed proteasomes. RAP has been found to specifically inhibit the mRNA expression of the β subunit of the proteasome activator (173). This suggests that $p27^{kip1}$ could be protected by RAP and therefore delay cell division. RAP has also been found to block signaling from the B cell receptor (BCR) by inhibiting the binding of the BCR-associated protein α4 to protein phosphatase 2A which is involved in multiple signal transduction pathways (174). Finally, RAP has also been shown to block the transcription of *bcl-2* mRNA (124). *bcl-2* is a proto-oncogene (175) which inhibits apoptosis (176). Some or all of these pathways may be involved in the delay of cell cycle progression induced by RAP.

**(b) Blockade of IL-2R signal transduction by RAP**

The mechanism by which RAP inhibits cell division is dependent on its intracellular binding to a small (12 kDa) polypeptide termed FK506-binding protein-12 (FKBP12) (177). FKBP was initially identified on the basis of its ability to bind another immunosuppressive molecule, FK506, which bears structural similarity to RAP (178). Despite binding to the same intracellular receptor, FK506 and RAP mediate immunosuppression through distinct mechanisms. FK506, like cyclosporin A (CsA),

inhibits the production of IL-2, IL-3, IL-4, GM-CSF, TNF-α, and IFN-γ mRNA after TCR-stimulation (179). Unlike FK506 and CsA, RAP does not affect IL-2 production (177) or IL-2R expression (170). However, one report has claimed that in human PBMC RAP blocks surface expression of the IL-2Rα-chain (180). RAP also does not inhibit the production of IL-3, IL-4, IFN-γ and TNF-α by T cells (179). RAP, unlike FK506 and CsA, inhibits IL-2 (166) and IL-4 (170) stimulation of murine T cell lines, the activation of human peripheral blood T cells by phorbol ester and anti-CD28 mAb, as well as the activation of murine B cells by lipopolysaccharide (166). Therefore, RAP blocks lymphocyte activation farther downstream than FK506 or CsA.

FKBP12 is a *cis-trans* peptidyl-prolyl isomerase similar to the CsA-binding protein cyclophilin (178). FKBP12 has been found to interact with intracellular calcium release channels in skeletal muscle ryanodine receptors involved in contraction (181). Despite being very highly conserved between yeast and mammals, the FKBP or cyclophilin families are not essential for survival of yeast cells (182). In contrast, when the FKBP12 gene is disrupted in mice, most mice die during embryogenesis or soon after birth (183). Live mutant pups display heart defects and altered calcium release of ryanodine receptors from skeletal and cardiac muscle tissue (183). Thus, FKBP12 has an essential role in mammalian embryogenesis.

The RAP-FKBP12 complex binds to a 210 kDa protein originally given the names FRAP (184), RAPT1 (185), RAFT1 (186) and SEP (187). Due to its strikingly high homology with the yeast protein target of rapamycin (TOR), the mammalian RAP-FKBP12-binding protein has now been designated as the mammalian homologue of the

yeast target of rapamycin or mTOR. The double-binding of mTOR and FKBP12 by RAP requires the simultaneous occupation of a hydrophobic pocket in both proteins (188). Crystallography studies show that there is minimal interaction between mTOR and FKBP12 proteins (188). Therefore, RAP acts as a 'gain of function' drug bringing together two proteins which normally would not interact. This is similar to the effects of CsA on the proteins cyclophilin and calcineurin, and the action of FK506 on FKBP12 and calcineurin (189).

IL-2 binding to its receptor results in mTOR activation which leads to the activation of $p70^{s6k}$. This process is inhibited by RAP (190). RAP interferes with $p70^{s6k}$ activation normally induced by IL-2R binding in T cell (125) and B cell lines (191). Dephosphorylation of $p70^{s6k}$ at position $Thr^{229}$ in the presence of RAP is thought to be the mechanism of $p70^{s6k}$ inactivation (192). The Swiss 3T3 fibroblast cell line can also be delayed in its entry into the S phase of cell cycle by a RAP-mediated blockade of serum-induced $p70^{s6k}$ activation (193). This suggests that $p70^{s6k}$ is ubiquitously activated in cells entering cell cycle.

$p70^{s6k}$, a serine/threonine-specific kinase, is normally involved in phosphorylating a wide array of proteins involved in cell-cycle control, transcription and translation initiation (126). For example, one of the subfamilies of cAMP-responsive element (CRE)-binding proteins, CRE-modulator (CREM), whose DNA binding ability is activated in T cells activated with IL-2 (194), is directly phosphorylated by $p70^{s6k}$ *in vitro* on a serine residue at position 117 (128). Phosphorylation of CREM allows it to induce the transcription of genes. This can be blocked by RAP treatment (128). One of the consequences of inhibiting

CRE-binding protein activation by RAP is the suppression of proliferating cell nuclear antigen (PCNA) expression (195). Induction of PCNA gene expression is required for DNA replication (196). This suggests the delay in the G1 phase of cell cycle caused by RAP may be attributable, in part, to slower DNA replication.

The inhibition by RAP of mTOR function has other deleterious effects on cellular activation. Treatment of NIH 3T3 cells with RAP results in the dephosphorylation and activation of the protein 4E-BP1/PHAS-I which is a repressor of the 5' mRNA cap binding protein eIF-4E (197). Cap-dependent translation constitutes the majority of eukaryotic mRNA translation (198). The PHAS-I repressor is inactivated by mTOR-mediated phosphorylation of Ser/Thr-Pro motifs (199). These motifs of the PHAS-I repressor are rapidly dephosphorylated in the presence of RAP (199). The inhibition of cap-dependent synthesis by RAP mirrors the characteristics of certain members of the picornavirus family which can shut off host-protein synthesis by inhibiting proper cap-assembly (200). Presumably, this frees the cell's ribosome population for the production of mainly viral proteins which utilize cap-independent translation. Consistent with this model, one study has shown that the presence of RAP in virus-infected cells increases viral protein translation (201), presumably by helping to inhibit cap-dependent translation of host mRNAs. This suggests that, in addition to the immunosuppression induced by RAP, the lack of a protective T cell-mediated immune response against viruses is compounded by the increased efficiency of viral replication in RAP-treated individuals.

The block in translation mediated by RAP seems to be two-fold. In addition to the inhibition of 5' cap-assembly as discussed above, RAP also specifically suppresses the

translation of 'polypyrimidine tract'-containing mRNAs (168). Polypyrimidine tract-containing mRNAs include ribosomal and elongation factor species, whose translation is specifically inhibited by RAP (169). The mechanism of translation for these mRNAs is dependent on the activation of $p70^{s6k}$ (202). Thus, RAP-inactivated $p70^{s6k}$ is implicated not only in transcriptional repression but also translational inhibition as well.

Although RAP has profound effects on the $p70^{s6k}$ signaling pathway, there are several reported RAP-insensitive pathways. One such RAP-insensitive pathway involves STAT5a and STAT5b activation (203). Another reported RAP-insensitive pathway is the mitogen activated protein kinase (MAPK) signaling pathway (204). This agrees with the finding that activating protein-1 (AP-1, c-fos/c-jun) function is not affected by RAP-treatment (124). AP-1 is one of the downstream transcription factors activated by the MAPK signaling pathway (205).

**(c) Inhibition of allograft-specific-CTL activation by RAP**

Gzm B and perforin downregulation was correlated with graft stabilization and was also observed in patients taking immunosuppressive corticosteroids (95). Similarly, RAP treatment of rats receiving allogeneic cardiac transplants resulted in decreased levels of intragraft Gzm B (206). RAP is a very potent immunosuppressant which can dramatically extend the survival of allogeneic and xenogeneic skin grafts in mice (207). For example, RAP prolonged allograft survival two to three times longer than CsA with doses which were 75 fold lower than CsA (207). Due to the different facets of lymphocyte activation which RAP and CsA/FK506 inhibit, combined treatment with CsA and RAP, or FK506 and RAP, has been shown to synergize in the extension of allograft survival (208,209). RAP

has also been found to have a synergistic effect with CTLA4-Ig in delaying skin allograft rejection (210). Depending on the species of animal treated, RAP has wide range of side effects which range from very severe in dogs, to very mild in rodents and humans (211,212).

*In vitro* studies show that RAP increases the effectiveness of CsA inhibition of CTL maturation in mixed lymphocyte reactions (171). In addition to inhibiting the development of CTL activity, 24 h pre-treatment with RAP has been shown to inhibit adherent cell-depleted human PBMC killing of K562 cells, an NK-sensitive target cell line (213). Similarly, LAK cells activated in the presence of RAP have decreased cytotoxicity to Daudi cells (213). Finally, antibody dependent cellular cytotoxicity (ADCC) of PBMC was also inhibited by RAP pre-treatment (213). In comparison, pre-treatment with CsA or addition of CsA during the effector phase did not affect human NK or ADCC killing (214). These reports suggest that RAP affects signaling mechanisms common to NK, ADCC and CTL, while the inhibition mediated by CsA is specific for CTL activation.

### 1.7 B7-CD28 costimulation

#### (a) The CD28/CTLA4 family

CD28 is constitutively expressed on all mature murine T cells (215). While almost all human $CD4^+$ T cells are also $CD28^+$, only about 50% of human $CD8^+$ T cells express CD28 (216). The state of differentiation seems to govern CD28 expression in human $CD8^+$ T cells. For example, naive and memory $CD8^+$ T cells express CD28 while effector $CD8^+$ T cells do not (217). This finding is explained by a report showing that the telomeres of

$CD28^-$ human T cells are much shorter than the telomeres of $CD28^+$ T cells (218). Telomeres consist of a 6 base pair motif repeated thousands of times on the ends of chromosomes, and each replication during cell division results in a portion of these telomeres being lost (219), suggesting that $CD28^-$ T cells have undergone more cell cycles of proliferation. This agrees with the previous finding that effector T cells are $CD28^-$ since they would be dividing and hence have shorter telomeres.

The production of IL-2 depends on signaling mediated by the TCR-CD3 complex (220) and costimulation through CD28 (7). Purified T cells can be activated and induced to produce IL-2 without exogenous APC, in the presence of co-immobilized anti-CD3 and anti-CD28 mAb (25). It has also been reported that certain anti-CD28 mAbs alone can initiate T cell activation both *in vitro* and *in vivo*, as measured by proliferation (221). However, binding of CD28 via antibodies and via its natural ligands (B7 family members) are known to lead to activate distinct signal transduction pathways (222). This suggests that the effects of antibody crosslinking are not always indicative of the *in vivo* function of cell surface molecules.

Costimulation by CD28 results in a synergistic increase in IL-2 transcription due to the activation of transcription factors which bind to the IL-2 gene regulatory region (223). CD28-signaling also results in the stabilization of IL-2 mRNA (224). The net effect of both mechanisms is the increased production of IL-2. The importance of CD28 signaling in immune function is seen in $CD28^{-/-}$ mice which, despite having normal B and T cell development, have very low overall levels of immunoglobulins with specific decreases in the amounts of $IgG_1$ and $IgG_{2b}$ that are present (225). Class switching is also dramatically

inhibited in $CD28^{-/-}$ mice after infection with vesicular stomatitis virus (225). Finally, the T cells from $CD28^{-/-}$ mice are hyporesponsive to polyclonal activators (225).

The ligands for CD28 are the B7-family of proteins. The B7-family is composed of B7-1 (8) and B7-2 (226), although other CD28 binding proteins have been described (227). Although signaling through CD28 results in the increased transcription and prolongation of IL-2 mRNA half-life which eventually leads to high levels of IL-2 secretion (224), it is evident from IL-2-deficient mice that B7 costimulation also results in IL-2-independent proliferation (228). Despite lower proliferation levels in B7-stimulated $IL\text{-}2^{-/-}$ T cells, B7 costimulation most likely results in the production of other T cell growth factors (228).

B7 family members have also been shown to bind to the T cell activation marker CTLA4 (229). CTLA4 is related to CD28. The genes show modest sequence identity and are closely linked in the genome (230). However, CTLA4 binds B7 proteins with higher affinity than CD28 (229). CTLA4 is expressed after T cells become activated (231), whereas CD28 expression by T cells is constitutive (232). Recently, observations of T cell development in $CTLA4^{-/-}$ mice have suggested that CTLA4 is a negative signaling molecule (233). $CTLA4^{-/-}$ animals have very high numbers of T cells in the liver, heart, lung and pancreas and these T cells are hyperresponsive to activating stimuli (233). CTLA4-deficient mice soon die of general organ failure due to tissue destruction by massive lymphocytic infiltration. This lethal effect can be countered with CTLA4-Ig fusion protein that binds both B7-1 and B7-2 molecules and blocks their costimulatory ligation of CD28 (234). This suggests that CTLA4 interactions normally function to inhibit lymphocyte proliferation. The symptoms of CTLA4-deficient mice are reminiscent of *lpr* or *gld* mice

which are deficient in Fas and FasL function, respectively. The activated T cells of *lpr* mice do not die quickly enough and accumulate in the lymphoid organs (72). Perhaps T cells from CTLA4$^{-/-}$ mice are dividing faster than the Fas/FasL homeostatic mechanisms can deal with.

The negative signaling mediated by CTLA4 may even be detrimental to host immunity. Mice injected with colon carcinoma cells cannot prevent tumor growth and eventually have to be euthanized by day 35 after innoculation. However, mice injected with anti-CTLA4 mAb shortly after tumor introduction showed no tumor growth (235). Interestingly, pre-established tumor growth was also inhibited by late treatment with anti-CTLA4 mAb (235). These results suggest that some tumors may be allowed to grow uncontrollably because T cells become anergic to tumor antigens through a CTLA4-dependent mechanism.

Despite the overwhelming evidence of that CTLA4 is a negative regulatory molecule, there is also strong evidence that CTLA4 can function as a costimulator of T cell activation in certain circumstances. A recent study has shown that B7-1 transfected Chinese hamster ovary (CHO) cells can costimulate, along with immobilized anti-CD3 mAb, the proliferation of CD4$^{+}$ T cells from CD28$^{-/-}$ mice (236). Surprisingly, the B7-1 costimulation could be inhibited with intact and Fab fragments of anti-CTLA4 mAb (236).

**(b) The B7-family**

B7-1 or CD80 was the first CD28 ligand to be discovered (8). B7-1 is expressed on activated antigen presenting cells (237), as well as activated T cells (238). At least two splice-variants of B7-1 exist in mice (239). B7-1a lacks the second Ig-like domain coded

by exon 3 of the B7-1 gene in activated murine B cells (239). B7-1a cannot costimulate the activation of resting T cells but can support the continued proliferation of pre-activated T cells (239).

The existence of an alternative CD28-binding molecule, B7-2, was established after B7-1-deficient mice were engineered. It was found that residual alloantigen responses in T cells from these animals could be blocked by CTLA4-Ig (226). B7-2, unlike B7-1, is expressed constitutively at low levels on macrophages, dendritic, B, and T cells (238,240,241). Also, upon Ig-receptor binding by antigen or anti-Ig mAb, B7-2 is quickly expressed on B cells, while B7-1 is not responsive to Ig-receptor stimulation and displays a slower surface appearance in response to the more general mitogenic stimulus LPS (242). During the activation of APC and T cells, B7-2 expression is enhanced on APC (240), but is diminished on murine T cells (238). A somewhat contradictory study showed that splenic $CD4^+$ T cells sorted into naive ($CD44^{low}$) and memory ($CD45RB^{low}$) populations demonstrated B7-2 expression only on the memory T helper cell subset (243). B7-1 and B7-2 show limited homology to each other at the amino acid level, and have been mapped to the human chromosome 3q13.3-q21 and 3q13-q23, respectively (244). This suggests that a gene duplication event might be responsible for the existence of B7-1 and B7-2, despite their poor sequence identity. Both B7-1 and B7-2 bind to CTLA4 with a 20-100 fold higher avidity than they do to CD28 (245). However, B7-1 has a slightly higher avidity for CD28 than B7-2 (245). In addition, B7-2 dissociates more rapidly from CTLA4 than does B7-1 (245).

Alternative CTLA4 binding molecules have been characterized for murine B cells.

A 130-kDa disulfide-linked protein was identified on the mouse immature B cell line WEHI231 which could costimulate for T cell proliferation in the presence of anti-B7-1 and anti-B7-2 blocking antibodies but not CTLA4-Ig (227). A protein homologous to B7-1 termed MRC OX-2 has also been isolated in the rat (246). MRC OX-2, when expressed in CHO cells, will costimulate murine $CD4^+$ T cell proliferation, but without the concomitant production of IL-2, IL-4 and IFN-γ as seen with B7-1 (246). Furthermore, MRC OX-2 does not bind mouse CD28 or CTLA4, suggesting that it is one component of an alternative T cell costimulatory pathway (246). Whether MRC OX-2 binds to rat CD28 or CTLA4 remains to be determined.

**(c) B7 costimulation of Th1 and Th2 responses**

It has been proposed that B7-1 and B7-2 have the capacity to differentially signal through CD28. Evidence for this theory has come from various disease models which have been previously assigned a Th1 or Th2 phenotype. It is clear that antibodies to B7-1 and B7-2 have different effects in various disease states. For example, injection of anti-B7-1 mAb into mice artificially predisposed to develop experimental allergic encephalomyelitis (EAE) blocked disease onset while injection with anti-B7-2 mAb accelerated and increased disease severity (247). EAE is thought to be mediated by Th1 cells, and recovery is accompanied by Th2 cytokine production (248). Thus, it would appear that in this model system B7-1/CD28 interactions result in Th1 responses while B7-2/CD28 binding results in Th2 cytokines being produced. Differences in B7-1 and B7-2 binding function have also been observed in a murine diabetes model. Nonobese diabetic mice are thought to develop autoimmune diabetes due to a T cell-mediated attack against antigens expressed by islet

cells (249). Administration of anti-B7-2 mAb to NOD mice was shown to block disease development, while anti-B7-1 mAb enhanced disease onset and severity (250). If one assumes that diabetes is mediated by a type 1 response then this study shows the opposite signaling patterns for B7-1 and B7-2 in comparison to the murine EAE model. This suggests that either the etiology of NOD or EAE with respect to Th1 and Th2 immune responses is misunderstood, or that the anti-B7 antibodies used in these studies may be mediating non-specific effects.

It has been suggested that B7-1 and B7-2 interactions with CD28 lower the activation threshold of human T cells. CHO cells transfected with human MHC class II and either B7-1 or B7-2 were used as APC for a human T cell line which is specific for a myelin basic protein peptide fragment. B7-1 and B7-2 transfectants induced similar levels of T cell proliferation in response to the normal peptide fragment, yet a weak peptide agonist of MBP only costimulated T cell proliferation to normal levels with B7-1 and not B7-2$^+$ CHO cells (251). This suggests that B7-2 costimulation is only available to T cells whose TCR strongly recognize antigen/MHC complexes, while B7-1 allows T cells to be more promiscuous in their antigenic binding.

*In vitro* studies on the effect of blocking B7-1 or B7-2 during the activation of T helper cells showed that B7-1 and B7-2 have overlapping roles during cytokine production. Murine T cells activated in the presence of B7-1$^+$/B7-2$^+$ peritoneal exudate macrophages along with anti-CD3 mAb and either blocking anti-B7-1 or anti-B7-2 mAb were tested for the production of various cytokines by ELISA. Blockade of B7-2 costimulation resulted in decreased levels of IL-2, IL-4, IL-5 and IFN-γ, while blockade of B7-1 costimulation

resulted in decreased levels of IFN-γ production only (252). It is not clear why B7-1 costimulation could not substitute for B7-2 costimulation or why B7-2 could not costimulate IFN-γ production when B7-1 was blocked. It is clear, however, that *in vitro* B7-2 interactions signal for the production of both Th1 and Th2 cytokines.

The now-widely used technology of genetically engineered transgenic and knockout mice have been used to try to answer the question of whether Th1 or Th2 responses are governed by different B7 molecules. In one study, T cells from transgenic mice expressing an ovalbumin-peptide-specific TCR were stimulated by syngeneic splenic APC from B7-1$^{-/-}$ or B7-2$^{-/-}$ animals. It was found that both B7-1 and B7-2 molecules could costimulate the production of IL-2, IFN-γ and IL-4 (253). It would be interesting to observe the reaction of these mice to infections inducing or requiring a particular Th response for clearance. This report contradicts prior studies and cautions against using antibodies to assign a role for B7-1 and B7-2 during an immune response. Detection of both Th1 and Th2 cytokines in the genetically-assured absence of either B7-1 or B7-2 is a powerful argument against assigning a role for these costimulators in Th1/Th2 development.

**(d) Signal transduction through CD28 and B7**

In an effort to differentiate the signal induced through CD28 by ligation with B7-1 or B7-2 molecules researchers have characterized certain elements of the signal transduction machinery associated with the cytoplasmic portion of CD28. Studies have shown that CD28 ligation induced an increase in intracellular $Ca^{2+}$ levels (254). It was subsequently shown that CD28 signaling was through both CsA-sensitive and -insensitive signal transduction pathways (25). Following B7-CD28 ligation, phosphatidylinositol 3 (PI3)

kinase associates with the cytoplasmic domain of CD28 (255). CD28 signaling also involves activation of phospholipase Cγ1 (256), Tec family kinases (257), $p21^{ras}$ (222), and Jun kinase (258). The association of CD28 with PI3 kinase and ITK are regulated by $p56^{lck}$ and $p59^{fyn}$ (259). It has also been shown that the formation of reactive oxygen intermediates are required for CD28-mediated IL-2 gene expression (260). The eventual result of CD28 ligation is the activation and binding of transcription factors to the 5' regulatory region of the IL-2 gene known as the CD28 responsive element (CD28RE) (223). Members of the NF-κB family have been identified as binding to the CD28RE (261). Interestingly, like the IL-2R, CD28 binding leads to the cytokine-independent activation of $p70^{s6k}$ (262). It is not known whether CD28-mediated activation of $p70^{s6k}$ can be inhibited by RAP.

Mitogen-activated protein (MAP) kinase responses after CD28 ligation, either through B7-1 or B7-2, seem to be identical with respect to the Raf-1/ERK2 activation portion of the cascade, which is not stimulated by either B7 protein, and to Jun kinases, which are activated by both B7 members (263). Studies have also shown that that the global patterns of tyrosine phosphorylation induced in the Jurkat human T cell line by B7-1 or B7-2-transfected L cells are identical (264). Similarly, association of PI3-kinase with CD28 is seen after ligation with either B7-1 or B7-2 (265). Thus, these results corroborate recent findings with B7-1 and B7-2 knockout mice that T cell differentiation is unaffected by the type of B7 ligand encountered by CD28 (253).

Despite the lack of biochemical evidence that differences exist between B7-1 and B7-2-induced signals, there is evidence that CD28-signaling differs between $CD4^+$ and $CD8^+$ T cells (254). Ligation by anti-CD28 mAb induces an elevation in $Ca^{2+}$ levels, IL-2R

and CD69 expression and proliferation in $CD4^+$ T cells but not to the same extent as in $CD8^+$ T cells (254). The differences in signal transduction cannot be accounted for by lower constitutive levels of CD28 expression by $CD8^+$ T cells. Additionally, B7-1-mediated costimulation results in a quick proliferative burst by $CD8^+$ T cells which then declines rapidly along with cell viability. In contrast, $CD4^+$ T cells enter cell cycle at a later stage and divide for a longer time while viability actually increases in response to B7-1 costimulation (266). $CD4^+$ T cells also produce much more IL-2 in response to CD28/B7-1 interactions (266). In agreement with these studies, it has been found that PI3-kinase associates with CD28 after B7 ligation in $CD4^+$ T cells but not $CD8^+$ T cells (265). This suggests an immediate downstream signal variation which could account for the different responses by $CD4^+$ and $CD8^+$ T cells to costimulate through CD28.

Like other receptor/ligand interactions, such as CD40L/CD40, there is some evidence that signaling is bi-directional in the case of B7-1. Co-immobilized anti-CD3 mAb and anti-B7-1 mAb have been shown to costimulate pre-activated, but not resting, human T cells to proliferate (267). Furthermore, anti-B7-1 mAb increases proliferative responses to allogeneic B lymphoma cell lines and induces a protein tyrosine phosphorylation pattern in T cells distinct from that induced by anti-CD3 or anti-CD28 mAb (267). However, isotype-specific effects were also seen in the controls, suggesting that the data could be artifactual. Independent confirmation is therefore required. When the murine B7-1 molecule was initially characterized, it was described as having a short cytoplasmic tail, making it unsuitable for transducing signals. However, an additional exon has been discovered for the murine B7-1 gene whose transcription and alternative mRNA

splicing results in a second B7-1 protein isoform with a longer cytoplasmic tail (268). Thus, it is possible that murine B7-1 may also have the signaling abilities claimed for its human counterpart.

**(e) Blockade of B7/CD28 interactions and allograft survival prolongation**

The most valuable knowledge obtained from the study of B7 costimulation of T cell activation has been the indispensable role of B7 costimulation for foreign tissue rejection responses. One of the most impressive experiments has shown that B7/CD28 blockade can lead to the long-term acceptance of xenogeneic islet cells (269). Similarly, rat renal allografts are normally rejected within 15 to 20 days post-implantation, but when rats are injected with CTLA4-Ig fusion protein 2 days post transplantation the grafts survived for >100 days (270). The acceptance of the allograft was associated with increased levels of Th2 cytokines, while a normal rejection response is characterized with increased IL-2 and IFN-γ (270). Thus, it appears that B7 costimulation of allograft-specific T cells leads to the preferential activation of Th1 cytokines. This agrees with *in vitro* studies where purified T cells which were activated with anti-CD28 mAb produced high levels of IFN-γ and IL-2, while IL-4 and IL-5 were secreted at a much lower level (252).

Cardiac allograft rejection in mice can be induced by the expression of B7-1 on either the graft or cells of the host as shown with the use of B7-1-deficient animals (271). Treatment with anti-B7-2 mAb mirrored CTLA4-Ig-induced long term graft acceptance only if both the graft and recipient were B7-1$^{-/-}$ (271). This study also proves that both B7-1 and B7-2 are the only ligands required for the induction of murine cardiac allograft rejection.

**(f) B7 costimulation of CTL induction and anti-tumor immunity**

Both human and murine CTL can kill target cells more efficiently if the targets express either B7-1 (272) or B7-2 (273). In addition, NK cells activated by IL-12 become very potent killers of B7-1$^+$ or B7-2$^+$ target cells, (146). However, the main value of B7 molecules for CTL function seems to be in the induction phase. It is known that CTL precursors can be activated by target cells which bear CD28-binding ligands and that this activation can be inhibited by the CTLA4-Ig fusion protein (274). These CTL acquire the ability to produce IL-2 for proliferation, and can synthesize Gzm B independently of cytokines secreted by CD4$^+$ T cells (274). Similarly, CTL activated by B7-1$^+$ CHO cells acquire the ability to produce IL-2 and, in conjunction with LFA-3-transfection of CHO cells, the additional production of TNF-$\alpha$ and IFN-$\gamma$ was also observed (275). This suggests that CD8$^+$ T cells can stimulate their own activation independently of CD4$^+$ Th cells if a target cell supplies the necessary CD28 ligand. This is consistent with past reports of CTL becoming activated and protecting the host in the absence of CD4$^+$ T cells (9).

Although it is accepted that CTL can utilize CD28 to become activated, there is some controversy as to whether both B7-1 and B7-2 can stimulate CTL responses to tumor cells. When transfected into acute myeloid leukemia cells, B7-1 was able to immunize mice against subsequent challenge with the wild-type tumor, while B7-2 transfected tumor cells did not generate as vigorous a memory response (276). In support of this study, it has been shown that CTL raised in a syngeneic or allogeneic mixed lymphocyte-tumor culture using B7-2 transfected P815 mastocytoma as stimulator cells, were not able to kill wild-

type P815 whereas CTL raised against B7-1 transfected P815 lysed wild-type P815 efficiently (277). The transfected tumor cells appear to have been activating CTL directly because a separate study reported that $CD8^+$ CTL could be induced against B7-1 transfected P815 in the absence of $CD4^+$ T (278).

Despite the plethora of studies suggesting that B7-1 costimulation can effectively immunize against B7-1-deficient parental tumors, at least one study has shown that immunization with a B7-1 transfected plasmacytoma does not offer protection against the parental tumor line (272). There is also strong evidence showing that immunization with B7-2-transfected P815 protects against challenge with wild-type P815 (279). Furthermore, this protection was lost if $CD8^+$ T cells were depleted from mice before tumor innoculation, suggesting CTL were activated by the initial challenge with $B7\text{-}2^+$ P815 (279). These results were confirmed in a separate study which showed that $B7\text{-}2^+$ P815 immunized as well as or better than $B7\text{-}1^+$ P815 against subsequent wildtype challenge (280). In this study, the immune response elicited by $B7\text{-}2^+$ P815 was also shown to retard established P815 tumor growth (280). However, eradication of the pre-existing tumor could not be achieved.

Despite the apparent importance of CD28-signaling for CTL induction, CTL responses were normal after CD28-deficient mice were injected with lymphocytic choriomeningitis virus (225). This suggests that other pathways are available to CTL for their activation. These alternative pathways may include CD28-independent production of cytokines and/or activation through CTLA4, which has been shown to be a positive signaling molecule in $CD28^{-/-}$ mice (236). Other costimulatory molecules such as CD2 may

also substitute, at least in part, for CD28 (275).

**(g) The role of B7 molecules on T cells**

Differences in the way that B7-1 and B7-2 can costimulate T cell activation have also been proposed on the basis of the type of cell which expresses B7-1 or B7-2. B7-1 expressed by murine CTL clones appears to be costimulatory for proliferation. The mouse CTLL-2 IL-2-dependent T cell line expresses both CD28 and B7-1. The proliferation of these cells in response to exogenous IL-2 addition can be blocked with CTLA4-Ig and anti-B7-1 mAb (281). Recently, it has been reported, in mice, that CTLA4 can bind to B7-2 expressed by T cells, while CD28 cannot (282). A possible mechanism for this has been proposed in light of recent findings showing that human T cells are able to express an altered form of B7-2 (283). This form of B7-2 is different from that expressed on B cells in that it is hypoglycosylated (283). Further evidence that T cells express a unique form of B7-2 comes from RT-PCR studies of murine B7-2 pre-mRNA splicing events. The murine B7-2 locus is made up of 12 exons, whose post-transcriptional rearrangement results in at least 5 detectable mRNA splice variants. At least one of these splice variants has been found to be peculiar to unstimulated T cell clones (284). In contrast, there is also evidence to indicate the B7-2 on T cells may in fact bind to CD28 on other T cells and lead to proliferation. Recently, a B7-2 transgenic mouse has been engineered that expresses high, constitutive levels of B7-2 on T cells. The T cells from this transgenic animal were shown to be activated with anti-CD3 mAb at three times the level of T cells from normal mice in the absence of costimulatory APC (285). Furthermore, this increased activation could be reduced to normal levels by the addition of anti-B7-2 mAb (285). However, the B7-2

transgene in these T cells was under the control of an artificial promoter. It is possible that T cell-specific transcription factors may interact with the endogenous B7-2 gene in a way that results in the production of a different isoform of B7-2. In support of this report, a separate group has found that B7-2 expressed on memory $CD4^+$ T cells can also costimulate naive T cells which receive the primary activation signal through contact with immobilized anti-CD3 mAb (243).

### 1.9 Objectives

The objectives of this study are two-fold. Although the role of RAP in the suppression of lymphocyte activation and the downregulation of the immune response has been well documented, the specific effects of RAP on CTL activation, cytotoxicity, and gene expression have not yet been elucidated. Due to the important role of CTL in the rejection of allografts, the specific effects which RAP exerts on CTL to prolong of transplant survival is a paramount question. Furthermore, such a study would also provide valuable insight into the kinds of signals required by CTL precursors in order to properly differentiate into effector cells. I hypothesized that RAP downregulates CTL activation by inhibiting lytic molecule production. I, therefore, studied the effects of RAP during anti-CD3-induced CTL activation from the point of view of transcriptional and translational regulation of cytolytic genes, as well as cytotoxicity.

Secondly, I have characterized the costimulatory signals that CTL precursors require in order to become maximally activated. I chose to focus on CD28/B7 interactions because of the controversy surrounding the role of B7 family members in the development of T cell-

mediated immunity. The identity of the CD28 ligand(s) required for CTL induction in response to anti-CD3 mAb were identified with the use of blocking mAb towards B7 proteins. As murine CTL express CD28, B7 proteins bind to these directly for optimal activation of cytotoxicity and lytic gene expression. The effect of costimulation blockade was assessed at the level of cytokine production, as well as transcriptional activation of CTL genes such as Gzm B, perforin and FasL. Finally, the ability of exogenous cytokines to substitute for ligand/receptor-based costimulation was tested.

## 2.0 Methods and Materials

### 2.1 Mice

Female wild-type and $CD4^{-/-}$ C57BL/6 mice (6-10 weeks old) were purchased from Jackson Laboratories, Bar Harbour, ME. Mice were maintained on standard laboratory chow and water supplied *ad libitum* in the Sir Charles Tupper Building animal care facilities.

### 2.2 Culture medium

RPMI 1640 medium (ICN Biomedicals Canada Ltd., Mississauga, Ontario), hereafter referred to as complete RPMI 1640 medium (cRPMI), was supplemented with 10 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin (all from ICN Biomedicals), 5 μM HEPES (Sigma Chemical Co., St. Louis, MO; pH 7.4) and 5% heat-inactivated (at 56°C for 30 min) fetal calf serum (FCS) (Life Technologies, Burlington, ON). The medium was filter-sterilized using Sterivex GS 0.22 μm filter units (Millipore Corp., Bedford, MA) and placed in 250 ml sterile polystyrene tissue flasks (Sarstedt, St. Laurent, PQ) and stored at 4°C. *E. coli* transfected with plasmids carrying a Gzm B or β-actin cDNA probe were grown in YT broth (1.6 g tryptone, 0.5 g NaCl, 1.0 g yeast extract, 100 ml $ddH_2O$ and supplemented with 100 μg/ml ampicillin; heat sterilized and stored at room temperature). Unless stated otherwise, all cell washes were performed with 5 ml of phosphate buffered saline (PBS).

## 2.3 Antibodies, Cytokines and Reagents

Human rIL-2 (active in the murine system) was obtained from Cetus Corp. (Emeryville, CA). Specific activity is expressed as Cetus units per milliliter. Mouse rIFN-γ and mouse rTNF-α were purchased from Genzyme Corp. (Cambridge, MA). Specific activity is expressed as Genzyme units per milliliter. Mouse rIL-6 was a kind gift from Dr. A. Stadnyk (Dalhousie University, Halifax, NS) and mouse rIL-12 was generously provided by Dr. J. Marshall (Dalhousie University, Halifax, NS). All cytokines were aliquoted and stored at -70°C. A stock solution of 1 μg/ml RAP (Research Biochemicals International, Natick, MA), was prepared in DMSO and stored at -20°C. Actinomycin D (ActD), phorbol-myristate-acetate (PMA), and ionomycin were purchased from Sigma Chemical Co. ActD and PMA were dissolved in ethanol, and ionomycin was dissolved in DMSO. ActD, PMA, and ionomycin stock solutions were stored at -20°C. Blocking Anti-B7-1 mAb (clone RM80, rat $IgG_{2a}$) and blocking anti-B7-2 mAb (clone PO3, rat $IgG_{2b}$) were kind gifts from Dr. K. Okumura (Juntendo University, Tokyo, Japan) (286). Anti-mouse IFN-γRα chain, anti-B7-1 mAb (clone 1G10, rat $IgG_{2a}$), rat $IgG_{2a}$ isotype controls clone LO-DNP-16 (DNP-specific) and clone R35-95 (unknown specificity) were purchased from Pharmingen (Mississauga, ON). Rat anti-mouse CD25 (IL-2Rα), hamster anti-mouse CD28 mAb, rat anti-mouse CD8α, and Low-Tox rabbit complement were purchased from Cedarlane (Hornby, ONT). Anti-mouse perforin mAb (rat $IgG_{2a}$, clone KM585/P1-8) was purchased from Kamiya Biomedical Co. (Tukwila, WA). Whole rat IgG, hamster serum, FITC-conjugated AffiniPure F(ab')2 fragment mouse anti-rat IgG (H+L), FITC goat F(ab')2

fragment anti-hamster IgG (H+L), and alkaline phosphatase-conjugated AffiniPure mouse anti-rat IgG (H+L) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Rabbit anti-mouse IL-2 was purchased from Collaborative Biomedical Products (Bedford, MA). Rat anti-mouse IFN-γ was purchased from Upstate Biotechnology (Lake Placid, NY). Purified antibodies were stored at 4°C while antibody containing hybridoma supernatants were aliquoted into 4 ml volumes and stored at -70°C. dATP-$\alpha^{32}$P, $Na_2{}^{51}CrO_4$ and thymidine [methyl-$^3$H([$^3$H]-TdR)] were purchased from ICN Radiochemicals (Irvine, CAL). Polyclonal rabbit anti-mouse asialoGM1 antiserum, (Waco Chemicals, Richmond, VA) was reconstituted with $ddH_2O$ and stored at 4°C.

**2.4 Cell lines**

P815 murine mastocytoma cells (H-2$^d$) (ATCC clone TIB64) were obtained from ATCC (Rockville, MD). The hybridoma (clone 145-2C11) that produces hamster anti-mouse CD3 mAb (16) was provided by Dr. J. Bluestone (University of Chicago, Chicago, IL). The hybridoma (clone GL1) that produces rat $IgG_{2a}$ anti-mouse B7-2 mAb was a generous gift from Dr. K. Hathcock (NCI, Bethesda, MD) (240). Hybridoma clone 16-10A1, which produces hamster anti-mouse B7-1 mAb, and clone FD441.8 which produces rat anti-mouse LFA-1 were both purchased from ATCC. All cells lines were grown in cRPMI (5% FCS) at 37°C/5% $CO_2$ in a humidified atmosphere. Tumor cells were diluted 1/10 with fresh cRPMI twice a week and flasks were changed at least once a week.

**2.5 T cell Isolation**

Female wild-type and $CD4^{-/-}$ C57BL/6 mice (6-10 weeks old) were sacrificed by cervical dislocation. Spleens were aseptically removed and dissociated in 4 ml PBS using a tissue homogenizer. A maximum of 3 spleens were homogenized at any one time. PBS was added to a total of 10 ml and the homogenate was allowed to settle. Cell suspensions were decanted from splenic debris and cells were pelleted by centrifugation at 500 x *g* for 10 min. The supernatant was discarded and erythrocytes were lysed by osmotic shock. Briefly, cells were resuspended in 3 ml 0.2% NaCl and mixed by inversion for 15 sec. Then 3 ml of 1.6% NaCl was added, followed by 4 ml of PBS and additional mixing by inversion. Erythrocyte debris was allowed to settle and the resulting cell suspension was decanted, centrifuged at 500 x *g* for 10 min, resuspended in 5 ml of cRPMI and counted. Whole splenocytes were depleted of B cells and macrophages by adherence to sterile nylon wool at 37°C and 5% $CO_2$ in a humidified environment. One gram of sterile nylon wool was prepared for splenocyte adherence by repeatedly pulling the fibres apart with sterile forceps, then pre-incubating in 9 ml of cRPMI in a 10 cc syringe attached to a clamped butterfly needle for 30 min at 37°C and 5% $CO_2$ in a humidified atmosphere. The column was washed with cRPMI prior to addition of spleen cells. No more than 3 spleen equivalents were loaded onto each column. Splenocytes were allowed to adhere to the nylon wool for 1 h at 37°C and 5% $CO_2$ in a humidified environment. Non-adherent cells were eluted from the column at a rate of 1 drop every 2 to 3 sec. If an experiment called for highly purified B-cell depleted T cells, nylon wool-nonadherent spleen cells were passaged through a second nylon wool column. Once- or twice-enriched nylon wool non-adherent

splenocytes were depleted of NK cells by the incubation for 30 min at room temperature with 10 μg/ml anti-asialoGM1 rabbit polyclonal antiserum (Wako Chemicals, Richmond, VA) in PBS. Cells were washed twice and then resuspended in Low-Tox rabbit complement (1/12 dilution in PBS; Cedarlane Laboratories, Hornby, OH) for 45 min at 37°C and 5% $CO_2$ in a humidified environment. Cells were washed in PBS and the debris discarded. The cells were then washed twice more in PBS and counted.

**2.6 T cell activation by anti-CD3 mAb**

T cells were activated by incubation for 40-45 h in the presence of hamster anti-mouse CD3 mAb (hybridoma supernatant from clone 145-2C11) at a dilution of 1:20. Unless stated otherwise, all drugs, antibodies and cytokines were added at the beginning of culture along with anti-CD3 mAb. Antibodies to B7-2 (clone GL1) and B7-1 (clone 16-10A1), in the form of hybridoma supernatants, were used at a final dilution of 1:10 (4 μg/ml for anti-B7-2 and 0.2 μg/ml for anti-B7-1). T cell activation by immobilized anti-CD3 mAb was accomplished by precoating the wells of 24- or 96-well flat bottom plates (Sarstedt). Five μg/ml of anti-CD3 mAb (ascites) in borate buffer (3.02 g boric acid, 29.2 g NaCl, 4.77 g sodium borate (Borex).$10H_2O$; made up to 500 ml with $ddH_2O$; pH 8.35) was added to 2 ml or 200 μl wells overnight. Wells were coated in volumes of 500 μl and 100 μl for 2 ml and 200 μl size wells, respectively. Coated wells were washed 3 times with PBS. T cells were counted and seeded at 8 x $10^6$ cells in 2 ml of cRPMI per well in a 24-well flat bottom tissue culture plate or at the stated numbers in 96-well flat bottom plates for

40-45 h, or as required, at 37°C and 5% $CO_2$ in a humidified atmosphere. At the end of the activation period cell viability was measured by trypan blue exclusion. Cells treated with the various reagents were counted and equal numbers of viable cells were used in $^{51}$-Cr-release assays.

**2.7 B cell isolation and activation by LPS**

Splenocytes were depleted of RBC by osmotic shock and resuspended at $10^7$ cells/ml in cRPMI. Macrophages were removed by adherence to the inner surface of a 50 ml polystyrene culture flask (Sarstedt) for 1 h at 5% $CO_2$/37°C in a humidified atmosphere. The non-adherent cells were removed and pelleted by centrifugation for 10 min at 500 x *g*. The supernatant was discarded, the cells resuspended in 500 µl of PBS and incubated with anti-Thy-1 mAb (New England Nuclear, Boston, MA) at 10 µg/ml for 30 min at room temperature. Cells were centrifuged, washed twice, resuspended in Low Tox Rabbit complement and incubated for 45 min at 5% $CO_2$, 37°C in a humidified atmosphere to deplete T cells. The resulting B cell preparation was washed twice to remove cellular debris. B cells were seeded at 5 x $10^6$ cells/ml in cRPMI and incubated for 3 d in the presence of 5 µg/ml LPS (Sigma). Activated B cells were washed 3 times in cRPMI and used for FACS analysis, or added to resting T cells at a ratio of 1:10.

**2.8 $^{51}$Chromium release assay.**

Anti-CD3-activated T cells were washed 3 times in PBS and seeded in a 96-well V-

bottom microtitre plate at a frequency of 250,000 or 125,000 cells in 100 µl of cRPMI. P815 target cells were labelled with $^{51}Cr$ by incubation with 100 µCi of $Na_2{}^{51}CrO_4$ (ICN Biochemicals Canada) for 1 h. P815 target cells were then washed 3 times in cRPMI and 5000 $^{51}Cr$-labelled target cells were added to each well in a volume of 100 µl. The final effector to target cell ratio in the wells was 50:1 or 25:1. The microtitre plate was incubated at 37°C and 5% $CO_2$ in a humidified atmosphere for 4 h, and then centrifuged at 1500 x *g*. A 100 µl aliquot of supernatant was removed for analysis with a Beckman Gamma 8000 sample counter. The spontaneous release of $^{51}Cr$ from target cells was determined by incubation in cRPMI alone while the maximum $^{51}Cr$ release was obtained by treating target cells with 100 µl of 10% SDS. The following equation was used to quantitate cytotoxicity: (experimental release - spontaneous release)/(maximum release - spontaneous release) x 100 = % lysis.

**2.9 T cell proliferation assay**

After 48 h of stimulation with anti-CD3 mAb alone or in combination with various treatments, a 100 µl volume of T cells was transferred to triplicate wells of a 96-well round-bottom microtitre plate. The cultures were pulsed with 0.5 µCi of [$^3$H]TdR and maintained at 37°C and 5% $CO_2$ in a humidified atmosphere for 6 h. Cultures were harvested onto glass fibre mats (ICN) using a Titer-Tek multiple sample harvester and [$^3$H]TdR incorporation was determined in a Wallac 1410 liquid scintillation counter.

## 2.10 Conjugation Assay

A 1 mg/ml neutral red dye (Sigma) solution was prepared in cRPMI medium and centrifuged at 750 x *g* for 10 min. The supernatant was removed, sterilized using a filter syringe and diluted 1:2 in cRPMI medium. P815 cells were incubated in the neutral red dye for 30 min at 37°C in a humidified environment containing 5% $CO_2$. Next the P815 cells were washed extensively and resuspended to a concentration of 5 x $10^6$ cells/ml in cRPMI medium. Anti-CD3-activated T cells were also resuspended in cRPMI at a concentration of 5 x $10^6$ cells/ml. A 0.1 ml aliquot of T cells was combined with an equal volume of neutral red dye-labeled tumor cells in 12 x 75 mm round-bottom polystyrene tubes (Becton Dickinson, Lincoln Park, NJ). The cells were pelleted and incubated for 30 min at 37°C in a humidified environment containing 5% $CO_2$. Next the cells were put on ice. T cell-tumor cell conjugates were gently resuspended five times with a Pipetman set at 200 µl and the percentage of T cells bound to target cells was calculated by counting the number of T cells which had conjugated to the neutral red-dyed tumor cells. At least 100 cells were counted in each sample.

## 2.11 Flow cytofluorometric analysis

Approximately $10^6$ anti-CD3-activated T cells, LPS-activated B cells, freshly isolated B cells and freshly isolated, highly purified (two consecutive nylon wool column passages) T cells were placed in 12 x 75 mm polystyrene round bottom tubes and washed once with PBS. The supernatant was discarded and cell pellets were resuspended in one of the following: 1 µg/ml rat anti-mouse CD8α, 1/2 dilution of hybridoma supernatant

containing rat anti-mouse CD4 (clone GK1.5), 1/2 dilution of hybridoma supernatant containing rat anti-mouse LFA-1, 1 μg/ml hamster anti-mouse CD28, a 1/2 dilution of hybridoma supernatant containing rat anti-mouse B7-2 (clone GL1), a 1/2 dilution of hybridoma supernatant containing hamster anti-mouse B7-1 (clone 16-10A1), or 1 μg/ml of rat anti-B7-1 mAb (clone RM80) in PBS with 0.2% sodium azide and 2.5% BSA. This concentration of primary antibody was found to be saturating in prior experiments. The cells were then incubated for 30 min on ice and in the dark. Cells were then washed twice with 200 μl of PBS/0.2% sodium azide/2.5% BSA and resuspended in 100 μl of PBS/0.2% sodium azide/2.5% BSA containing either 10 μg/ml FITC-conjugated mouse anti-rat IgG or FITC goat anti-hamster IgG fragment F(ab')2 (Jackson ImmunoResearch), depending on the primary antibody used. This was followed by incubation for 45 min on ice in the dark. It should be noted that anti-CD3-activated T cells stained with FITC-labelled anti-hamster secondary antibody were pre-treated (30 min on ice and in the dark) with 10 μg/ml of unlabelled goat anti-hamster fragment F(ab')2 IgG in 2.5% BSA, 0.2% sodium azide in PBS in order to prevent binding of the secondary antibody to surface bound anti-CD3 mAb. Following incubation with the FITC-labelled secondary antibody, cells were washed once with 200 μl PBS/0.2% sodium azide/1% BSA, washed twice more with 200 μl PBS/1% BSA, and resuspended in 200 μl of storage buffer (1% paraformaldehyde, in PBS). Cells were stored in the dark at 4°C until the percentage of fluorescent cells was determined by analysis of $10^4$ cells with a FACSCAN (Becton Dickinson Canada, Mississauga, ONT).

**2.12 Production of radiolabelled cDNA probes**

The cDNA (1.0 kb) coding for murine Gzm B, a gift from Dr. R. C. Bleackley (University of Alberta, Edmonton, AT), was supplied in pUC19. The β-actin cDNA was supplied by Dr. C. Lazier, Dalhousie University (with permission of Y. Valotaire, Universite de Rennes, Cedex, France). Competent *E. coli* cells were transfected according to previously described procedures (287) and were grown overnight in 50 ml of YT broth at 37°C with constant shaking. The following morning cells were pelleted by centrifugation for 5 min at 6000 x *g*. The supernatant was gently aspirated and 1.0 ml of ice cold solution I [(50 mM glucose, 25 mM Tris-Cl (pH 8) and 10 mM EDTA (pH 8)] was added. The tube was vortexed briefly and 5 ml of freshly prepared solution II (0.2 N NaOH, 1.0% SDS) was added to the tube. After vigorous shaking the tube was incubated for 5 min on ice. A volume of 3.75 ml of ice cold solution III (60 µl 5.0 M potassium acetate, 11.5 ml glacial acetic acid and 28 ml $ddH_2O$) was added to the tube. The suspension was inverted vigorously for 2 min and then placed on ice for 10 min. The solution was then centrifuged at 6000 x *g* for 10 min and the supernatant transferred to a fresh tube. A half volume of phenol (pH buffered to 8.0 with 1 M Tris) and chloroform (24:1 chloroform:isomamyl alcohol) was then added. The phases were mixed by vortexing for 2 min and then placed on ice for 10 min. The mixture was centrifuged for 10 min at 10,000 x *g* and the upper aqueous phase was carefully removed to a fresh tube. An equal volume of ice-cold isopropanol was added to precipitate the nucleic acids, shaken briefly and placed on ice for 30 min, followed by centrifugation for 20 min at 6000 x *g*. The isopropanol was discarded by gentle aspiration and the pellet was allowed to air dry. The pellet was resuspended in

500 µl of $ddH_2O$ in a 1.5 ml microfuge tube and 1.0 µl of a 10 mg/ml solution of RNAse (Sigma) was added to the solution (to eliminate contaminating RNA) which was then placed at 37°C for 1 h. Following this, equal volumes of 250 µl of phenol and chloroform were added to the tube. The phases were combined by vortexing for 2 min and then separated by centrifugation in a microfuge at 10,000 x *g* for 5 min. The resulting aqueous phase was transferred to a new 1.5 ml tube and an equal volume of chloroform was added. These phases were mixed by vortexing for 1 min and then centrifuged for 5 min. The upper aqueous phase (approximately 400 µl) was transferred to a clean tube and 45 µl of 3.0 M sodium acetate and 900 µl of 95% ethanol was added and mixed. This mixture was then placed at -20°C overnight to precipitate the DNA. DNA was pelleted by centrifugation at 10,000 x *g* for 30 min at 4°C and the ethanol decanted. The precipitated DNA was washed in 1 ml of 75% ethanol, centrifuged at 10,000 x *g* at 4°C for 10 min, and the pellet was air-dried. Dried, purified plasmid DNA was then reconstituted in an appropriate volume (usually 100-200 µl) of $ddH_2O$. cDNA inserts were isolated from purified plasmids by restriction digestion with appropriate enzymes cutting the plasmid at sites flanking the insert. Digested DNA was electrophoresed across a 0.8% agarose gel and the insert was isolated using a Gene Clean II kit (Bio/Can Scientific), according to the manufacturers instructions.

Insert cDNA was labelled with $\alpha^{32}$P-ATP by random priming according to the manufacturer's instructions (random primed DNA labelling kit; Boehringer Mannheim Biochemicals, Montreal, PQ). Unincorporated radionucleotides were removed by passage

through a Sephadex NICK column (Pharmacia Biotech, Uppsala, Sweden). Briefly, the column was rinsed with 3.0 ml of equilibriating buffer (10 mM Tris-HCl, 1.0 mM EDTA, pH 7.5) and then 25 µl of the random-primed cDNA was added, followed by 2 x 400 µl of equilibriating buffer. The first 400 µl was discarded, the second volume was kept and the radioactive incorporation estimated by placing 1.0 µl into 1.0 ml of scintillation fluid. The cpm/µl was determined in a Wallac 1400 scintillation counter.

**2.13 RNA isolation**

RNA was isolated from T cells with the TRIzol reagent according to the manufacturer's instructions. Briefly, 5 x $10^6$ T cells were pelleted at 500 x *g* and the supernatant removed by gentle aspiration. The cells were resuspended in 1 ml of TRIzol and vigorously mixed by pipetting with a 1000 µl Pipetman. The TRIzol/cell lysate mixture was then immediately transferred to a 1.5 ml Eppendorf tube and stored at -70°C, if not immediately needed. The TRIzol/cell lysate mixture was allowed to sit for 3 min at room temperature, mixed with 200 µl of chloroform and mixed by inversion for 15 sec. Aqueous and organic phases were separated by a 15 min centrifugation at 12,000 x *g*. Approximately 60% of the aqueous phase was removed and transferred to a new 1.5 ml Eppendorf tube. Five hundred µl of -20°C isopropanol was added to the tube which was mixed by inversion and incubated at room temperature for 10 min. Following this, precipitated RNA was pelleted by centrifugation at 12,000 x *g* for 10 min. The supernatant was gently aspirated from the pellet and discarded. A 1 ml volume of -20°C 75% ethanol

was added. The $ddH_2O$ used to make the 75% ethanol had been previously treated with the RNAse inhibitor DEPC (Sigma Chemical Co.) as previously described (287).The tube containing the 75% ethanol and precipitated RNA was vortexed briefly and then centrifuged at 12,000 x *g* for 10 min. The ethanol was gently aspirated and the RNA pellet was vacuum-dried for 5 min at room temperature. The RNA was resuspended in 10-20 µl of DEPC-treated $ddH_2O$. OD readings were taken at 260 and 280 nm to assess the concentration and purity of the RNA, as previously described (287).

**2.14 RNA electrophoresis**

All equipment and glassware used in this procedure were pre-soaked in bleach or hydrogen peroxide solution to remove RNAses. A 30 ml 1.5% agarose (Sigma) solution (in $ddH_2O$) was prepared by warming in a microwave oven. When the solution had cooled sufficiently, 5 ml of 10 X MOPS/EDTA pH 7.0 (0.4 M monopholinpropanesulphonic acid, 100 mM sodium acetate, 10 mM EDTA) and 8.75 ml of 37% formaldehyde (Fisher Scientific) was added. The solution was poured into a gel tray and allowed to solidify. The gel was submerged in a gel box filled with 1 X MOPS/EDTA pH 7.0. For optimal resolution the gel was pre-run for 15 min at 70 V. Five µg of RNA was mixed with 1.0 µl of 10 X MOPS/EDTA, pH 7.0, 3.5 µl of 37% formaldehyde, 8.5 µl deionized formamide, 2.0 µl of a 1:20 dilution of a 10 mg/ml stock solution of ethidium bromide (Sigma). The total volume was brought up to 20 µl and incubated at 65°C for 10 min and then immediately placed on ice. Prior to loading, 2 µl of RNA loading buffer (322 µl buffer A

[294 µl 10 X MOPS/EDTA pH 7.0, 706 µl $H_2O$ formaldehyde/formamide: 89 µl formaldehyde (37%) and 250 µl deionized formamide], 5.0 mg xylene cyanol [Sigma], 5.0 mg bromocresol green [Fisher Scientific Inc.] and 400 mg sucrose) was mixed with each sample. Samples were loaded into the gel along with 5 µg of RNA ladder (0.24-9.5 kb; Gibco-BRL, Gaithersburg, MD). Samples were electrophoresed at 70 V until the dye front had migrated 75% of the gel length. Pictures were then taken of the gel using a UV illuminator and Polariod camera.

RNA that did not need to be denatured, like that shown in one-step RT-PCR data, was electrophoresed across a 1% agarose gel in a 1 X TAE running buffer. RNA was mixed with TE buffer and normal loading dye and then loaded onto the gel. Samples were run and photographed as described previously.

**2.15 RNA transfer from agarose gel to Nytran membranes**

RNA was transferred from the agarose gel to a Nytran membrane according to manufacturer's instructions. Briefly, Nytran membrane (S&S Maximum Strength Nytran Plus, Schleicher & Schuell, Keene, NH) was pre-soaked in $ddH_2O$, followed by immersion in 20 X SSC (175.3 g NaCl, and 88.2 g sodium citrate in 1.0 L of $ddH_2O$, pH 7.0). Whatmann 3MM filter paper was placed on the porous white plate of the vacuum blotting transfer apparatus (Vector Systems, Toronto, ON) followed by the Nytran membrane. The plastic sealing mask was then layered over the Nytran and the gel placed on top of the sealing mask completely covering the opening in the center. RNA was transferred at a pressure of 80 mbar during sequential 5 min washes with 5 ml of 50 mM NaOH, 10 mM

NaCl and 5 ml of 0.1 M Tris-Cl, pH 7.4. After these washes the gel was covered with 20 X SSC and transferred for 2 to 4 h until the RNA had migrated out of the gel as assessed by UV illumination. The Nytran blot was air dried and the RNA was cross-linked by baking for 3 h at 80°C or UV cross-linking on a UV Stratalinker 1800 (Stratagene, La Jolla, CA).

**2.16 Northern Hybridization**

The RNA blot was prehybridized in a Hybaid rotating hybridization oven (Hybaid, Halifax, NS) for 2 h at 42°C in 5 ml of fresh hybridization solution (50% deionized formamide, 5 X Denhardt's reagent, 0.1% SDS, 100 μg/ml salmon sperm DNA, and 5 X SSPE) in a 50 ml conical tube (Sarstedt). Eppendorf tubes (1.5 ml) containing 1.5 x $10^7$ cpm of $^{32}P$ random prime-labeled cDNA probes for Gzm B or β-actin were boiled for 10 min to denature the cDNA, and then immediately placed on ice for 5 min to preserve the single-stranded state. Pre-hybridization solution was discarded and 5 ml of fresh hybridization solution was added along with the radiolabelled cDNA probe. The blot was hybridized overnight at 42°C with constant rotation. The following morning the hybridization solution was discarded and the blot was then washed twice for 30 min with 50 ml of 6 X SSPE at room temperature followed by a 30 to 60 min wash at 65°C with 1 X SSPE. The blot was removed from the 50 ml conical tube and excess moisture was removed by briefly blotting with filter paper. Finally, the blot was wrapped in saran wrap and exposed to Amersham Hyperfilm for various lengths of time at -70°C. Nytran membranes were stripped as suggested by the manufacturer and reprobed as needed.

**2.17 Semi-quantitative (Two step) RT-PCR**

Total RNA was isolated using TRIzol reagent as previously described and was reverse transcribed (1 μg) in a 0.5 ml microfuge tube in a 20 μl volume containing 200 U of Moloney murine leukemia virus reverse transcriptase, 1 μg of random hexanucleotide primers, 0.5 mM dNTPs, and 5 mCi of $^{32}$P-dCTP. The reaction mixtures were incubated at 37°C for 1 h, then boiled for 10 minutes and stored at -20°C. A 1 μl aliquot was removed and counted on a Wallac 1410 liquid scintillation counter. The final volume was adjusted to 200 μl with pyrogen-free $H_2O$ and a volume containing 50,000 cpm of cDNA was used for subsequent PCR. cDNA in a total volume of 50 μl in a 0.5 ml microfuge tube was amplified in the presence of 1/10 dilution of 10 X PCR buffer (2 M KCl, 1M Tris-HCl pH 8.4, 1 M $MgCl_2$, 1 mg/ml BSA) 2.5 units of Taq DNA polymerase, 0.2 mM dNTPs, and 0.5 μM of each primer. PCR mixtures were contained by overlaying with 100 μl of mineral oil (a kind gift from Dr. D. Mahoney, Dalhousie University, Halifax, NS). The amplification protocol for GAPDH (25 cycles), β-actin (25 cycles), Gzm B (28 cycles) and perforin (30 cycles) were as follows: denaturation 92°C for 30 sec, annealing at 57°C for 30 sec and primer extension at 72°C for 1 min (2 min for Gzm B). Fas-ligand (35 cycles) was amplified as follows: 94°C for 1 min, 57°C for 1 min, and 72°C for 1.5 min. The following primers were used for PCR. The size of the PCR amplicon is given after the reverse primer. 'F' refers to forward primer and 'R' refers to reverse primer.

GAPDH (F) 5'-ACTCACGGCAAATTCAACGGC-3'

GAPDH (R) 5'-ATCACAAACATGGGGGCATCG-3' (product size: 247 bp)

β-Actin (F) 5'-CTGGAGAAGAGCTATGAGC-3'

β-Actin (R) 5'-TTCTGCATCCTGTCAGCAATG-3' (product size: 241 bp)

Perforin (F) 5'-TCAATAACGACTGGCGTGTGG-3'

Perforin (R) 5'-GTGGAGCTGTTAAAGTTGCGG-3' (product size: 252 bp)

Granzyme B (F) 5'-GCCCACAACATCAAAGAACAG-3'

Granzyme B (R1) 5'-AACCAGCCACATAGCACACAT-3' (product size: 216 bp) (26)

Granzyme B (R2) 5'-GAGAACACATCAGCAACTTGGG-3' (product size: 889 bp)

Fas ligand (F) 5'-ATGGTTCTGGTGGCTCTGGT-3'

Fas ligand (R) 5'-GTTTAGGGGCTGGTTGTTGC-3' (product size: 362 bp) (85)

IL-2 (F) 5'-TGATGGACCTACAGGAGCTCCTGAG-3'

IL-2 (R) 5'-GAGTCAAATCCAGAACATGCCGCAG-3' (product size: 170 bp)

One Gzm B reverse primer (R1) was used for the timecourse, RAP/rIL-2, and the ActD half-life studies, while the second Gzm B reverse primer (R2) was used for the anti-B7-2 mAb and IL-12 studies. The β-actin primer sequences were designed by Dr. A. Stadnyk (Dalhousie University, Halifax, NS). All primers were designed to bind intron-bridging exons of the respective gene. If the murine gene exon-intron structure was not known, the position of introns was estimated from the rat gene homologue. PCR products were visualized by electrophoresis across an ethidium bromide-stained 1.5% agarose gel. Relative levels of PCR products were quantified by densitometric analysis of gel photographs.

## 2.18 One step RT-PCR analysis

Total RNA was isolated using TRIzol reagent as previously described and was reverse transcribed (1 μg) and amplified in a one step reaction using RT-PCR beads (Pharmacia). The reaction was carried out in a 0.5 ml microfuge tube in a 50 μl volume containing 1 μg of random hexanucleotide primers, 0.5 μM of each gene-specific PCR primer, and 1 μg RNA. Pyrogen-free water was added to a volume of 50 μl and the solution was overlaid with 100 μl of mineral oil. Synthesis of cDNA was facilitated by incubation at 42°C for 30 min followed by 95°C for 5 min. The same reaction mixture was then subjected to the appropriate number of cycles and temperatures required for the particular pair of primers being used. PCR products were visualized by electrophoresis across an ethidium bromide-stained 1.5% agarose gel. The quantity of RNA employed in the one-step RT-PCR reaction was also electrophoresed as a visual control for equal template loading.

## 2.19 Gzm B activity assay

Gzm B activity in the cytosolic fraction of CTL was measured using the Gzm B-specific synthetic substrate Boc-Ala-Asp thiobenzyl ester purchased from Enzyme Systems Products (Dublin, CA). CTL lysates were prepared by combining $10^6$ CTL with 0.25 ml of lysis buffer (PBS containing 0.5% NP40 and 0.4 mM $Ca^{2+}$) and incubating on ice for 30 min. Lysates were cleared by high speed centrifugation. A 180 μl volume of the reaction mixture (PBS with 0.2 mM Boc-Ala-Asp thiobenzyl ester and 0.1 mM 5,5'-dithiobis(2-

nitrobenzoic acid)) was added to 20 µl of cell lysates in wells of a 96-well flat-bottom microtitre plate (Sarstedt). Wells containing lysis buffer alone served as negative controls. After 90 min at room temperature the absorbance at 405 nm was read using a Titertek plate reader (ICN). An absorbance of 0.01 was arbitrarily defined as 1 unit of esterolytic activity.

**2.20 Detection of granule exocytosis by measurement of tryptase activity**

To measure tryptase activity released during granule exocytosis by anti-CD3-activated CTL, 0.5 x $10^6$ CTL were seeded in 100 µl of cRPMI in triplicate wells of a 96-well V-bottom plate. One hundred ng/ml PMA and 500 ng/ml ionomycin were added to each well and the cells were incubated for 2 h at 37°C and 5% $CO_2$ in a humidified atmosphere. Following this, plates were centrifuged at 500 x *g* and 50 µl of cell-free supernatant was transferred to empty wells of a 96-well flat-bottom microtitre plate. Then 100 µl of 1 mM dithio-bis(2-nitrobenzoic acid) (Boehringer Mannheim, Laval, PQ) and 50 µl of 2 mM Nα-CBZ-L-lysine thiobenzylester (Sigma) (both in PBS) were added to each well. The plate was incubated for 2 h at room temperature and the absorbance was read at 405 nm using a Titertek plate reader. An absorbance of 0.01 was arbitrarily defined as 1 unit of esterolytic activity. The absorbance from unstimulated cells was subtracted from that of stimulated cells.

**2.21 Perforin protein detection by Western blot analysis**

Postnuclear proteins were obtained from 5 x $10^6$ PBS-washed 48 h-activated T cells

by treatment with 200 µl of ice-cold lysis buffer (1% Nonidet P-40, 5 mM EDTA, 1 mM PMSF, 10 µg/ml leupeptin/pepstatin, and 10 µg/ml aprotinin) for 30 min on ice. The cell suspension was vortexed every 5 min and after 30 min the lysates were transferred to 1.5 ml microfuge tubes. Cell lysates were cleared by centrifugation at 14,000 x *g* for 6 min, and the supernatant was immediately stored at -20°C. Samples were diluted 1:1 with 2 X Laemmli sample buffer containing 2-mercaptoethanol, and boiled for 5 min. Protein samples were electrophoresed across a 12% SDS-polyacrylamide gel under reducing conditions at a constant voltage setting of 200 V/60 mA. Briefly, protein samples and pre-stained low range SDS-PAGE standard (Bio-Rad) were heated for 5 min at 95°C. Approximately 15 µl of protein sample and 5 µl of pre-stained standard were loaded into the wells of the polyacrylamide gel with a Hamilton syringe (VWR Scientific, Toronto, ON). After the dye front had travelled to the bottom of the gel, electrophoresis was halted. Separated proteins were transferred onto a 0.45-µm nitrocellulose membrane (Mandel Scientific Co., Guelph, ON) using a Bio-Rad Mini Trans Blot Module for 1 h at 100 V/250 mA under the conditions recommended by the manufacturer. After transfer, the nitrocellulose membrane was allowed to air-dry, blocked with 3% BSA (Boehringer Mannheim, Laval, PQ) in 10 mM Tris (pH 7.5) in 100 mM NaCl and 0.1% Tween-20 (Sigma) for 1 h at room temperature. All blocking steps and washes were performed with gentle agitation. Modified blocking buffer (1% BSA in 10 mM Tris (pH 7.5), 100 mM NaCl, and 0.1% Tween-20) containing 2.5 µg/ml of rat anti-mouse perforin mAb (Kamiya Biomedical Co., Tukwila, WA) was next applied to the membrane which was allowed to sit

overnight at 4°C. The blot was washed with 10 mM Tris (pH 7.5), 100 mM NaCl, and 0.1% Tween-20 for 30 min at room temperature. The wash buffer was changed every 5 min. Following washing, the nitrocellulose membrane was immersed in modified blocking buffer containing 1.6 μg/ml of alkaline phosphatase-conjugated AffiniPure mouse anti-rat IgG (H+L) (Jackson ImmunoResearch Laboratories) for 2 h at room temperature. Following additional washes, perforin protein bands were visualized by the addition of 150 μg/ml of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (BCIP was dissolved in DMSO) and 335 μg/ml of nitro blue tetrazolium (NBT) (NBT was dissolved in dimethyl formamide) in substrate solution (200 mM Tris (pH 9.5), 10 mM $MgCl_2$). Once significant band staining was observed the color reaction was stopped by the addition of distilled water. Nitrocellulose membranes were air-dried and stored in the dark.

**2.22 Cytokine detection by sandwich ELISA**

Flat bottom 96-well microtitre ELISA plates (Costar, Cambridge, MA) were coated with capture anti-cytokine mAb in 0.1 M phosphate buffer (pH 9.0) at 4°C overnight. Capture mAb was used at the following concentrations. Anti-IL-2, anti-IL-4, and anti-IL-6 mAb were used at 1 μg/ml while anti-IL-10, anti-TNFα, and anti-IFN-γ were used at 4 μg/ml. All antibodies were purchased from Pharmingen and used at the suggested concentrations for this assay. After coating, the plates were washed with Tris-buffered saline (TBS) 3 times for 3 min each. Plates were post-coated with 100 μl/well of 1 mg/ml BSA in TBS for 2 h at room temperature. Next, plates were washed 3 times in TBS for 3

min. Supernatants from anti-CD3-activated T cell cultures and standards were added in a volume of 100 µl. Standards were diluted in 1 mg/ml BSA in TBS/Tween. Plates were incubated overnight at 4°C and washed 3 times in TBS for 3 min each. Biotinylated detection anti-cytokine mAb was next added (100 µl per well) at 0.5 µg/ml in 1 mg/ml BSA in TBS/Tween and incubated at room temperature for 2 h. Plates were then washed 4 times in TBS for 3 min each. Extravidin-peroxidase (Sigma) was diluted 1/1000 in 1 mg/ml BSA in TBS/Tween and added in a 100 µl volume per well. This was incubated at room temperature for 30 min. Plates were then washed 4 times in TBS for 3 min each. Following this, 100 µl of 3,3',5,5'-tetramethylbenzidine (TMB) peroxidase substrate solution (Kirkegaard Perry Laboratories, MD) was added and plates were stored in the dark while color developed. The reaction was stopped by the addition of 100 µl of 1M $H_3PO_4$. Absorbance at 450 nm was read with a Titertek plate reader (ICN).

**2.23 Determination of antibody concentration in hybridoma supernatants by ELISA**

The concentration of mAb in the supernatants of anti-B7-1 (clone 16-10A1) and anti-B7-2 (clone GL1) producing hybridomas were determined by ELISA. This was accomplished by first pre-coating flat-bottom 96-well microtitre plates with 50 µl of 10 µg/ml of rabbit anti-rat IgG (ICN) or rabbit anti-hamster IgG (Sigma). Capture antibody was diluted in borate buffer and incubated overnight at 4°C. The following morning wells were washed 3 times with 200 µl PBS. A blocking solution of 1% BSA/$NaN_3$/PBS was added in 200 µl volumes to each well and the plate was incubated at 4°C overnight. Plates

were washed with 3 times with 200 µl PBS per well. Hybridoma supernatant was added in serial dilutions in triplicate alongside known concentrations of isotype standards. The standard used for 16-10A1 supernatant was purified hamster anti-mouse CD28 mAb (Cedarlane) and the standard used for GL1 supernatant was purified rat anti-mouse B7-1 (clone 1G10, rat $IgG_{2a}$, Pharmingen). Standards were diluted in 1% BSA/PBS while hybridoma supernatant was diluted in cRPMI. After 3 h of incubation at room temperature wells were washed 3 times with 200 µl PBS/0.05% Tween 20. Next, 100 µl of rabbit anti-rat IgG-horse radish peroxidase (HRP) or rabbit anti-hamster IgG-HRP secondary antibody (Jackson ImmunoResearch, 1/1000 dilution in PBS/1% BSA) was added to each well. Plates were incubated at room temperature for 1 h followed by 2 washes with 200 µl of PBS/0.05% Tween 20 and one wash with 200 µl PBS. HRP-activity was detected by added 140 µl of substrate solution to each well. Substrate solution consisted of 25 ml $ddH_2O$, 12.1 ml of 0.1 M citric acid, 12.9 ml of 0.2 M $Na_2HPO_4$, 20 mg (or one tablet) o-phenylenediamine (Sigma) and 20 µl $H_2O_2$. The substrate solution was made fresh each time and hydrogen peroxide was added just before use. The reaction was allowed to proceed until sufficient color had developed and then halted by adding 40 µl of 2.5 M $H_2SO_4$. The absorbance was read at 490 nm on a Titertek plate reader (ICN).

### 2.24 Statistical analysis

Statistical comparisons of data were performed using the Instat statistics program (GraphPad Software, Inc., San Diego, CA). Student's *t*-test or one-way ANOVA and the

Bonferroni test were used where appropriate. Values of $p<0.05$ were considered to be statistically significant.

## 3.0 Results

## Section I: Reversible and non-reversible aspects of RAP-mediated inhibition of CTL function

### 3.1 The presence of RAP during CTL activation with anti-CD3 mAb inhibits the development of cytotoxicity

Initial studies on the effect of RAP on T cell function revealed that RAP inhibits T cell activation, as measured by proliferation in response to mitogens (166). Other studies have shown that RAP-treatment during CTL induction results in killer cells with decreased cytotoxicity (213). These studies provide important clues as to effect of RAP on T cells and the immune system, but also cause other questions to be raised. For example, what is the target of inhibition in CTL effector function? In order to answer this question, T cells were isolated from female C57BL/6 spleens and depleted of RBC by osmotic lysis, followed by depletion of the majority of B cells by nylon wool adherence, and NK cells by treatment with anti-asialoGM1 antibodies and complement. These 'purified' T cells were activated for 48 h in the presence of soluble anti-CD3 mAb, which induces the development of strong MHC-unrestricted lytic activity against the P815 mastocytoma cells, as measured by $^{51}$Cr-release (19). In contrast, unactivated murine T cells do not lyse P815 target cells (287a).

In order to study the effect of RAP on CTL activation, a working concentration of the drug needed to be determined. Therefore, RAP was added at 0.1, 1, and 10 ng/ml concurrently with anti-CD3 mAb to T cells. After 48 h of culture, the cells were washed and used as effectors in a $^{51}$Cr-release assay against P815 target cells. While the DMSO vehicle control did not decrease the levels of cytotoxicity exhibited by anti-CD3-activated

CTL, RAP at concentration of 0.1 ng/ml decreased lysis of P815 target cells by approximately 50% (Figure 1). Higher concentrations of RAP (1 and 10 ng/ml) showed a dramatic decrease in P815 lysis. While both 1 and 10 ng/ml RAP resulted in similar levels of cytotoxic inhibition (approximately 90%), 10 ng/ml of RAP proved to be toxic to T cells as assessed by trypan blue dye exclusion and total cell recovery. Thus, 1 ng/ml of RAP was used to treat T cells in all subsequent experiments. It should be noted that RAP did not totally inhibit CTL activation since between 10-25% of control levels of P815 lysis was seen in all experiments following RAP treatment. This suggests that RAP-insensitive cytotoxic activation pathways are also induced by anti-CD3 mAb.

### 3.2 RAP-resistant IL-2R-signaling pathways cannot substitute for RAP-sensitive signaling pathways during CTL activation

The mechanism by which RAP inhibits T cell activation is thought to be, in part, due to a blockade of certain signal transduction pathways emanating from the IL-2R while leaving IL-2 mRNA and protein production unaffected (125,170). Signals transduced from the IL-2R are known to be important in killer cell activation and gene transcription of cytolytic mediators such as Gzm B and perforin (106,108). In order to confirm the inhibitory effect of RAP on IL-2R-signaling, 100 U/ml exogenous recombinant (r)IL-2 was added to T cell cultures at the same time as anti-CD3 mAb, with or without RAP. This concentration of rIL-2 was used for all subsequent experiments. After 48 h of culture, the effector cells were washed and used in a $^{51}$Cr-release assay against P815 target cells. The results of this experiment are shown in Figure 2. As previously shown, RAP-treatment

Figure 1. The addition of RAP to T cells cultured in the presence of anti-CD3 mAb decreases the cytolytic activity of the resulting CTL. Purified T cells were stimulated with anti-CD3 mAb alone, in the presence of 0.2% v/v DMSO (vehicle control), or in the presence of the stated amounts of RAP. Following 48 h of culture, cytolytic activity against P815 target cells at the stated E:T ratios was assayed using a $^{51}$Cr-release assay. Results are expressed as the mean percent lysis of P815 target cells ± the standard deviation of three replicates. The results are from one experiment and are representative of at least three separate experiments. One-way analysis of variance (ANOVA) indicates that the variation among column means is extremely significant. (*) denotes a statistically significant difference in killing activity in comparison to the DMSO control, as determined by the Bonferroni multiple comparison's test.

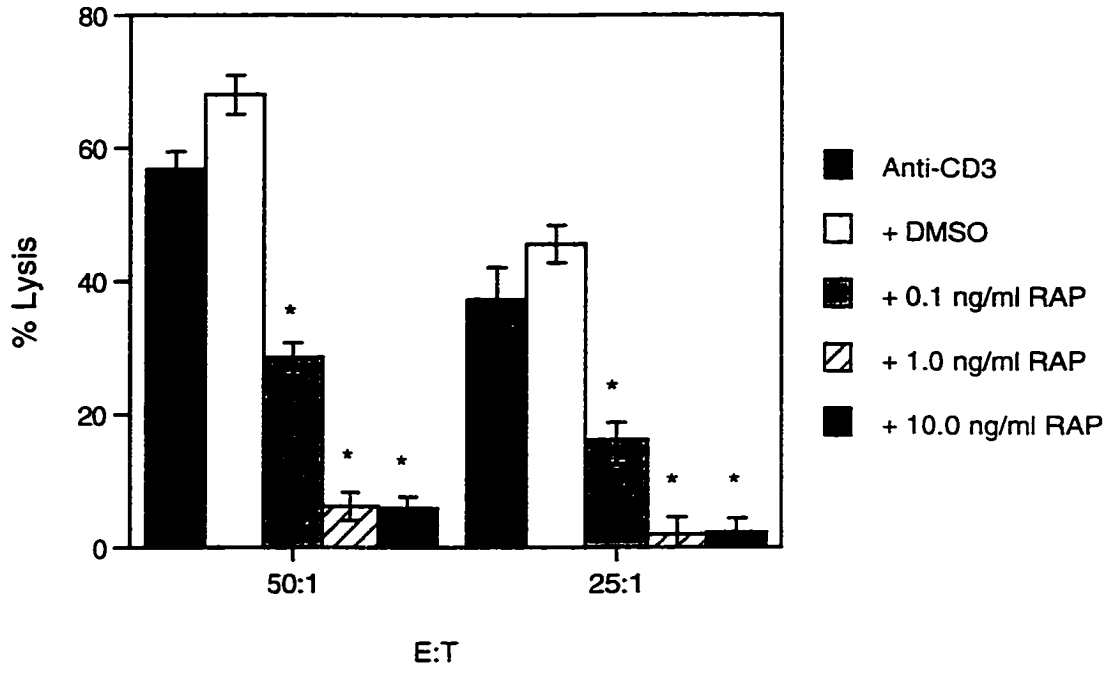

80
60
40
20
0
% Lysis
50:1
25:1
E:T
Anti-CD3
+ DMSO
+ 0.1 ng/ml RAP
+ 1.0 ng/ml RAP
+ 10.0 ng/ml RAP

Figure 1.

Figure 2. Exogenous rIL-2 only partially restores the cytotoxicity of RAP-treated anti-CD3 activated CTL. Purified T cells were stimulated with anti-CD3 mAb alone, or in the presence of 1 ng/ml RAP and 100 U/ml rIL-2, either individually, or in combination. Following 48 h of culture, cytotoxicity against P815 target cells at the stated E:T ratios was determined using $^{51}$Cr-release assay. Results are expressed as mean percent lysis of P815 targets ± the standard deviation of three replicates. The results are from one experiment and are representative of at least three separate experiments. (*) denotes a statistically significant difference in killing activity in comparison to the control, as determined by Student's *t*-test.

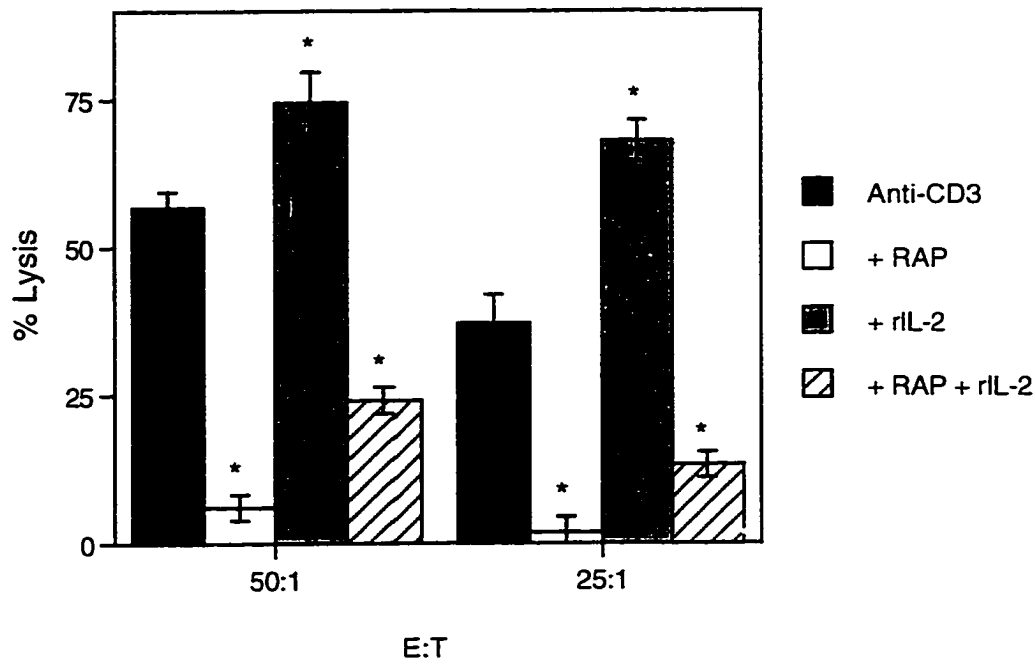



Figure 2.

inhibited the ability of CTL to lyse P815 target cells. The addition of exogenous rIL-2 alone boosted CTL-mediated lysis of P815, as expected. However, in combination with RAP, rIL-2 only slightly reversed the RAP-induced inhibition, resulting in lysis which was still far below control levels. This suggests that the signal transduction pathways associated with the IL-2R that are resistant to RAP cannot completely substitute for the RAP-sensitive signaling pathways also linked to the IL-2R which are involved in the development of cytotoxicity.

### 3.3 Exogenous rIL-2 restores proliferation in RAP-treated T cell cultures

In addition to the MHC-unrestricted cytotoxicity displayed by anti-CD3-activated T cells, progression from $G_0$ into cell cycle is also induced when T cells are exposed to anti-CD3 mAb (16). It is well known that RAP inhibits the proliferation of both B and T cells activated with mitogenic stimuli (166). Therefore, it was of interest to determine whether or not exogenous rIL-2 could restore the proliferation of RAP-treated T cells. T cells were activated with anti-CD3 mAb in the presence or absence of RAP, with or without rIL-2, for 48 h, as described previously. T cell cultures were pulsed with [$^3$H]TdR for the last 6 h of culture. The cultures were then harvested and the amount of [$^3$H]TdR incorporated into DNA was determined by liquid scintillation counting. T cells activated with anti-CD3 mAb alone showed very high levels of [$^3$H]TdR incorporation, whereas proliferation in RAP-treated cultures was almost totally abolished (Figure 3). This is in agreement with prior reports (166). Interestingly, the inhibition of proliferation caused by RAP was always much more profound than the RAP-induced inhibition of cytotoxicity. Exogenous rIL-2

Figure 3. Exogenous rIL-2 completely restores the proliferative capacity of RAP-treated T cells. Purified T cells were activated with anti-CD3 mAb alone, or in the presence of 1 ng/ml RAP, and 100 U/ml rIL-2, individually or in combination. DNA synthesis was measured following 48 h of culture by pulsing with [$^3$H]TdR and measuring [$^3$H]TdR incorporation by liquid scintillation counting. Data are expressed as mean cpm of triplicate cultures ± standard deviation. The results are from one experiment and are representative of at least three separate experiments. (*) denotes a statistically significant difference in DNA synthesis in comparison to the control, as determined by Student's *t*-test.

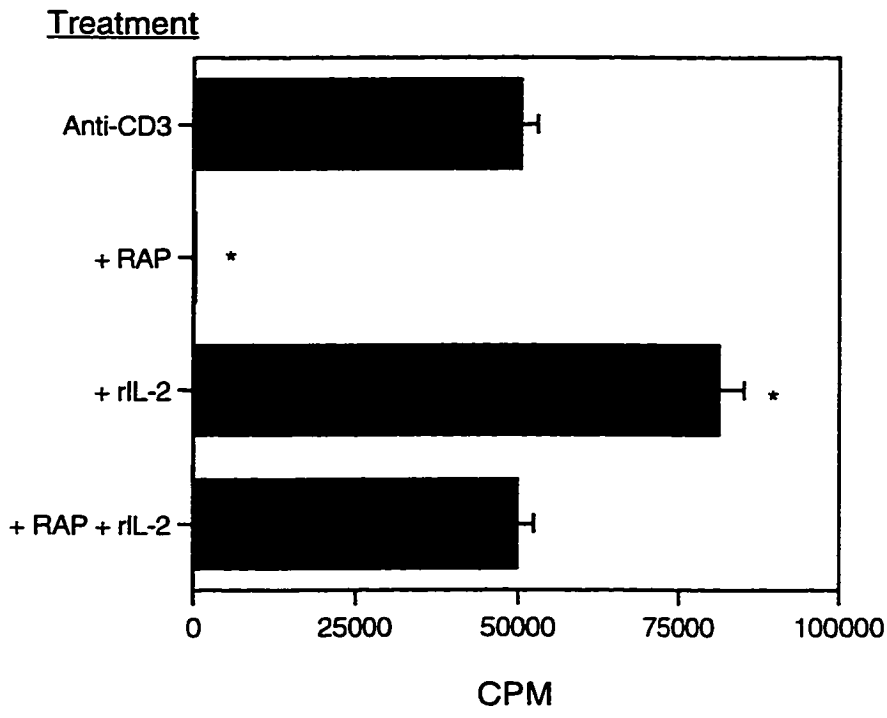

Treatment
Anti-CD3
+ RAP
+ rIL-2
+ RAP + rIL-2
*
*
0
25000
50000
75000
100000
CPM

Figure 3.

increased T cell proliferation above control levels. Surprisingly, the combination treatment of RAP plus rIL-2 restored T cell proliferation to control levels (Figure 3). This suggests that alternative RAP-resistant IL-2R-signaling pathways are able to substitute for RAP-sensitive pathways for the signals needed for T cells to progress into cell cycle.

### 3.4 RAP does not affect the percentage of $CD8^+$ CTL precursors

Most of the anti-CD3-induced cytotoxicity against P815 target cells is mediated by $CD8^+$ CTL (19). In order to rule out the possibility that RAP is decreasing CTL-mediated lysis of target cells by selectively decreasing the $CD8^+$:$CD4^+$ T cell ratios, flow cytometric analysis was performed on CTL activated in the presence or absence of RAP, with or without rIL-2, for 48 h to determine the percentage of $CD4^+$ and $CD8^+$ T cells. As shown in Table 1, none of the treatments notably affected the percentage of $CD4^+$ or $CD8^+$ T cells present following the 48 h incubation period. This suggests that the effects of RAP on $CD8^+$ T cells are more subtle, and that the level of differentiation or activation of these T cells is the most likely target of RAP. It should be noted that the ratio of CD4:CD8-expressing murine spleen-derived T cells falls into the normal ratio of approximately 1:1 (D. Hoskin, personal communication).

### 3.5 Time-course of CTL cytolytic gene expression during 48 h of culture with anti-CD3 mAb

The ability of exogenous rIL-2 to restore T cell proliferation in the presence of RAP suggested that the inhibition mediated by RAP was not a global phenomenon, but was

TABLE 1

The presence of RAP and rIL-2, individually, or in combination during anti-CD3-activation of T cells does not affect the proportion of $CD4^+$ T cells and $CD8^+$ T cells.

| Additions to culture: | Anti-CD3 alone | Anti-CD3 + RAP | Anti-CD3 + rIL-2 | Anti-CD3 + RAP + rIL-2 |
|---|---|---|---|---|
| % $CD4^+$: | 42.5 | 41.9 | 45.9 | 44.9 |
| % $CD8^+$: | 37.7 | 37.8 | 40.1 | 39.9 |

Purified T cells were activated by anti-CD3 mAb in the absence or presence of 1 ng/ml of RAP or 100 U/ml of rIL-2, individually or in combination. Following 48 h of culture, cells were washed and incubated with rat anti-mouse CD4 mAb and rat anti-mouse CD8α mAb. Cells were washed and incubated with FITC-conjugated mouse anti-rat IgG. The percent of $CD4^+$ or $CD8^+$ cells was determined with a FACSCAN.

selective for cytotoxicity. One possibility was that the cytolytic machinery in RAP- and RAP/rIL-2-treated T cell cultures was somehow defective. Since RAP is known to selectively modulate the activation of certain transcription factors (128), cytolytic gene expression seemed a likely target for RAP. In order to test this hypothesis, the mRNA levels of each cytolytic gene needed to be analyzed. The appropriate time-point to harvest RNA from treated-CTL, therefore, needed to be determined. T cells were activated with anti-CD3 mAb for 2, 4, 8, 16, 24, and 48 h. Total RNA was isolated at each time-point and reverse transcribed into cDNA with random hexamers. This cDNA was used in a PCR-reaction with primers specific for β-actin and the cytolytic gene in question. β-Actin expression served as a control for equal cDNA loading. The number of cycles used for each type of PCR was predetermined to provide a product from the log phase of amplicon development. The data are represented as inverted photographs of the original agarose gel used to visualize the PCR products. Densitometric analysis of the relevant amplicon relative to the β-actin amplicon was used to provide an arbitrary unit of mRNA abundance.

**a) Gzm B time-course of expression**

Exon-specific intron-bridging primers specific for the murine Gzm B gene were used in an RT-PCR assay with mRNA isolated from T cells at progressively increasing times following activation with anti-CD3 mAb. Gzm B mRNA is expressed at very low levels in resting splenic T cells (Figure 4), and is barely detectable at 2, 4, and 8 h of culture (Figure 5A). By 16 h of culture Gzm B expression is rising and is very strong by 24 h. Gzm B is expressed at increasingly higher levels up to and including 48 h (Figure 5A). It is known from previous Northern blot analysis that Gzm B mRNA

Figure 4. Comparison of Gzm B and perforin mRNA levels in resting and anti-CD3-activated T cells. T cells were isolated as described in the Methods and Materials. Total cellular RNA was isolated from fresh T cells or following 48 h of culture with anti-CD3 mAb. Using one-step RT-PCR beads (described in the Methods and Materials), single-stranded cDNA was reverse-transcribed from 0.5 μg of RNA with random hexamers followed by PCR reaction with exon-binding, intron-bridging primers specific for Gzm B and perforin. Amplicons were resolved by gel electrophoresis and ethidium bromide staining, scanned and the image inverted. Equal RNA template loading is shown by electrophoresis of the same volume of RNA used in the one-step RT-PCR procedure. Data are from one experiment and are representative of two independent experiments.

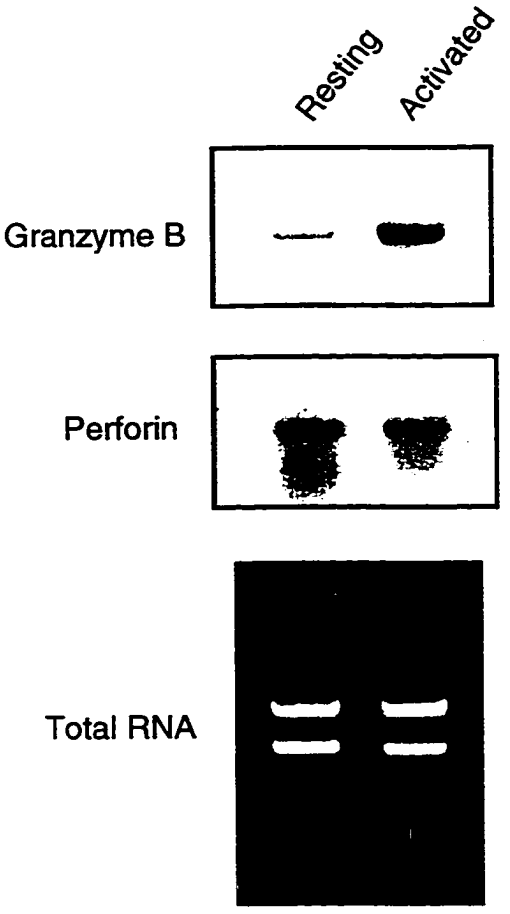



Figure 4.

Figure 5. Time-course of Gzm B, perforin, FasL, and IL-2 mRNA expression by anti-CD3-activated CTL. Purified T cells were incubated with anti-CD3 mAb and total cellular RNA was isolated at the stated time points. Single-stranded cDNA was reverse-transcribed from 0.5 μg of RNA with random hexamers as described in the Materials and Methods. The resulting cDNA template was used in a PCR reaction with exon-binding, intron-bridging primers specific for (A) GzmB, (B) perforin, (C) FasL, and (D) IL-2. β-Actin mRNA levels were also determined by RT-PCR. Amplicons were resolved by gel electrophoresis, and densitometric analysis was performed to quantitate Gzm B, perforin, FasL, and IL-2 expression relative to steady state expression of β-actin. Data are from one experiment and are representative of three independent experiments.

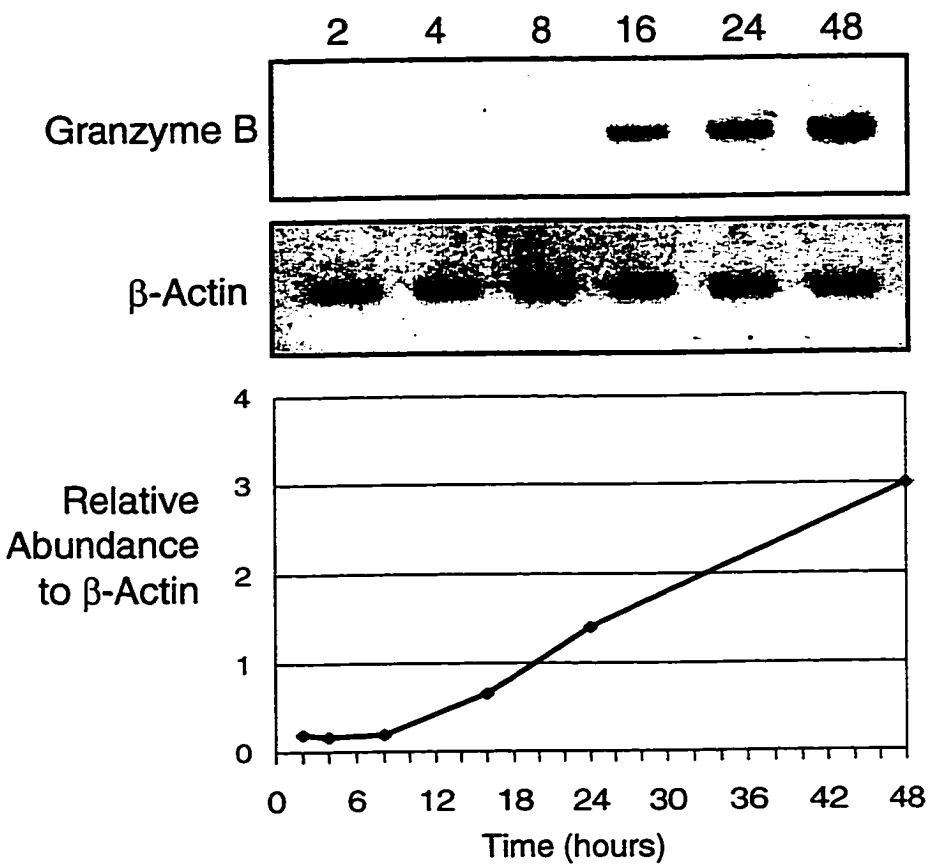

2
4
8
16
24
48
Granzyme B
β-Actin
Relative Abundance to β-Actin
4
3
2
1
0
0
6
12
18
24
30
36
42
48
Time (hours)

Figure 5A.

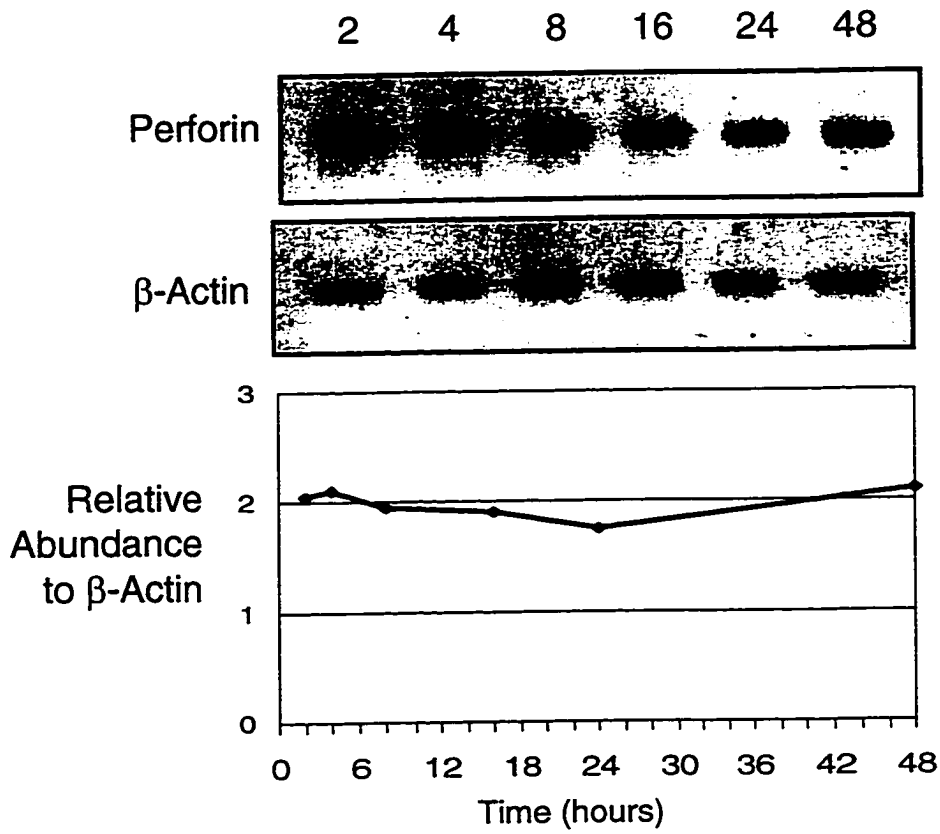

2
4
8
16
24
48
Perforin
β-Actin
Relative Abundance to β-Actin
3
2
1
0
0
6
12
18
24
30
36
42
48
Time (hours)

Figure 5B.

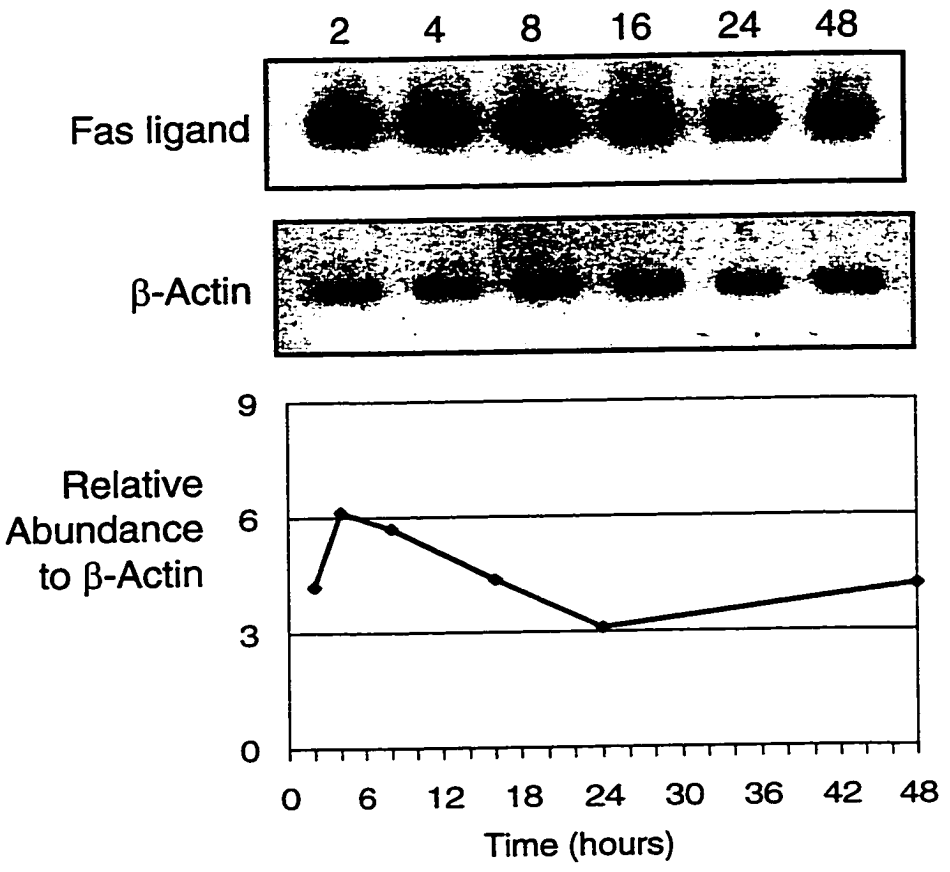

2
4
8
16
24
48
Fas ligand
β-Actin
Relative Abundance to β-Actin
9
6
3
0
0
6
12
18
24
30
36
42
48
Time (hours)

Figure 5C.

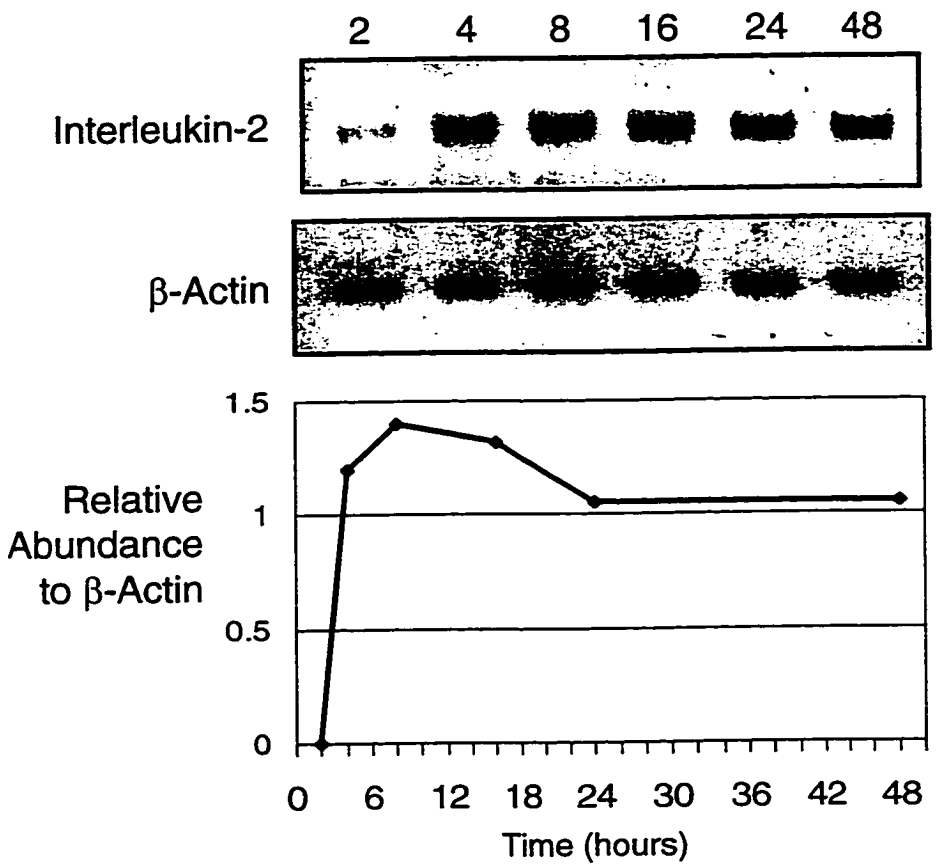

2
4
8
16
24
48
Interleukin-2
β-Actin
1.5
1
0.5
0
Relative Abundance to β-Actin
0
6
12
18
24
30
36
42
48
Time (hours)

Figure 5D.

levels in anti-CD3-activated T cells are maximal at about 48 h (288). Therefore, 48 h was chosen as the time-point to determine maximum Gzm B expression.

**b) Perforin time-course of expression**

Primers specific for the mouse perforin gene were used in a PCR reaction with cDNA from the same T cells used for the Gzm B PCR. Unlike Gzm B, perforin mRNA was present at all time points assayed (Figure 5B). Perforin mRNA in resting T cells was found to be as abundant, if not more, than that of T cells activated for 48 h with anti-CD3 mAb (Figure 4). Since the initial experiments for perforin mRNA detection were done with primers specific for the same exon, it was possible that the amplicon detected was due to equal DNA contamination present at all timepoints assayed. Exon-specific intron-bridging primers were designed for mouse perforin and these experiments were repeated with similar results. This suggests that either murine CTL precursors constitutively express perforin or the regulation for perforin expression is at the translational level, whereas Gzm B expression is transcription-dependent (Figure 4). Since perforin mRNA levels are as high at 48 h as at any other time point, and cytotoxicity at this time is known to be maximal for anti-CD3-activated CTL, it was decided that 48 h was an optimal timepoint to harvest T cell mRNA for the detection of perforin expression.

**c) FasL time-course of expression**

FasL expression over 48 h was more similar to perforin than to Gzm B. Like perforin, FasL expression was present at all timepoints assayed (Figure 5C), including unstimulated T cells. Unlike perforin, FasL mRNA levels peaked between 4 and 6 h post-addition of anti-CD3 mAb. Since cytotoxicity is maximal at 48 h of culture, I chose both 4

and 48 h to look for FasL mRNA.

**d) IL-2 time-course of expression**

The finding that perforin and FasL mRNA levels did not increase appreciably as T cells became cytotoxic was unexpected and cast doubt on the ability of RT-PCR to detect meaningful changes of mRNA levels during CTL differentiation. In order to establish the validity of the data produced by this technique I thought it would be prudent to examine the expression of another gene whose transcriptional regulation is well documented. Therefore, I chose to look at IL-2 mRNA levels which are known to rise very quickly after mitogenic stimulation (224). As shown in Figure 5D, very low levels of IL-2 mRNA were present at 2 h post addition of anti-CD3 mAb. By 4 h IL-2 mRNA levels had increased dramatically and were maintained at high levels for the remainder of the 48 h culture. This agrees with past reports (224) and supports RT-PCR as a valid measurement of relative increases and decreases of mRNA levels in a population of T cells.

## 3.6 Exogenous rIL-2 restores Gzm B and perforin mRNA levels to normal in RAP-treated CTL

**a) RT-PCR determination of Gzm B mRNA levels in RAP-treated CTL**

Gzm B is required for the rapid induction of DNA fragmentation in target cells by CTL (56). Therefore, it was important to determine the effect of RAP on Gzm B mRNA levels in activated CTL. Although it has been reported that RAP treatment decreases Gzm B mRNA levels in allografts, and that this correlates with increased organ survival times, the source of Gzm B in these grafts was not identified (206). T cells were purified as

described and activated with anti-CD3 mAb in the presence or absence of RAP, with or without rIL-2 for 48 h. mRNA was then isolated and RT-PCR analysis was used to determine the levels of Gzm B mRNA for each treatment, relative to β-actin mRNA levels. Gzm B expression in anti-CD3-activated CTL is decreased by approximately 50% with RAP-treatment (Figure 6A), which is in agreement with a prior report (206). The addition of exogenous rIL-2 increased Gzm B levels above control levels while the addition of rIL-2 to RAP-treated cultures restored Gzm B levels to slightly above control values (Figure 6A). This is a surprising finding, given that CTL treated with RAP and rIL-2 killed target cells with far less efficiency than control CTL (Figure 2).

**b) Perforin mRNA levels in RAP-treated CTL**

The importance of perforin in target cell lysis has been shown with perforin knockout mice. CTL and NK cells from such animals are incapable of inducing lysis in Fas-deficient target cells (38). Like RAP plus rIL-2-treated CTL, Gzm B expression is normal in CTL from perforin knockout mice (38). Therefore, it was possible that the decreased cytotoxicity observed with RAP plus rIL-2-treated CTL was due to decreased levels of perforin mRNA expression. However, perforin mRNA levels, as determined by RT-PCR, among the different treatments mirrored the Gzm B mRNA profile. Thus, RAP decreased perforin expression to approximately half control levels, rIL-2 increased perforin expression, and treatment with RAP plus rIL-2 resulted in perforin mRNA levels which were above control values (Figure 6B). This result was unexpected, considering that the vast majority of cytotoxicity induced against P815 target cells by anti-CD3-activated CTL is due to granule exocytosis, as deduced by $Ca^{2+}$ chelation studies (31 ± 3% lysis of P815 in

Figure 6. Exogenous rIL-2 restores Gzm B and perforin mRNA expression to control levels in RAP-treated CTL cultures. Purified T cells were incubated with anti-CD3 mAb alone, with 1 ng/ml RAP, or 100 U/ml rIL-2, individually or in combination. Following 4 and 48 h of culture, total cellular RNA was isolated from T cells. Single-stranded cDNA was reverse-transcribed from 0.5 μg of RNA with random hexamers as described in the Materials and Methods. The resulting cDNA template was used in a PCR reaction with exon-binding, intron-bridging primers specific for Gzm B, perforin, and FasL. β-Actin mRNA levels were also determined by RT-PCR to control for equal cDNA loading. Gzm B (A) and perforin (B) mRNA levels were assayed at 48 h of culture while FasL mRNA levels were assayed at both 48 (C) and 4 h (D) of culture. PCR amplicons were resolved by gel electrophoresis and ethidium bromide staining, and quantified by densitometric analysis. Data are from one experiment and are representative of three independent experiments.

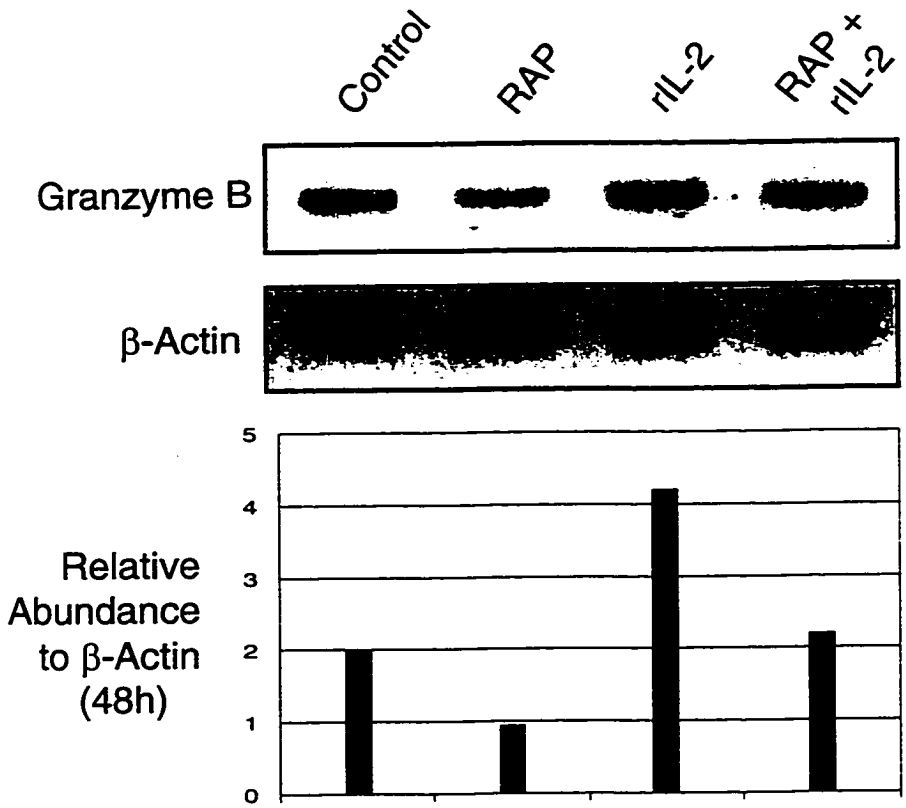

Control
RAP
rIL-2
RAP + rIL-2
Granzyme B
β-Actin
Relative Abundance to β-Actin (48h)
5
4
3
2
1
0

Figure 6A.

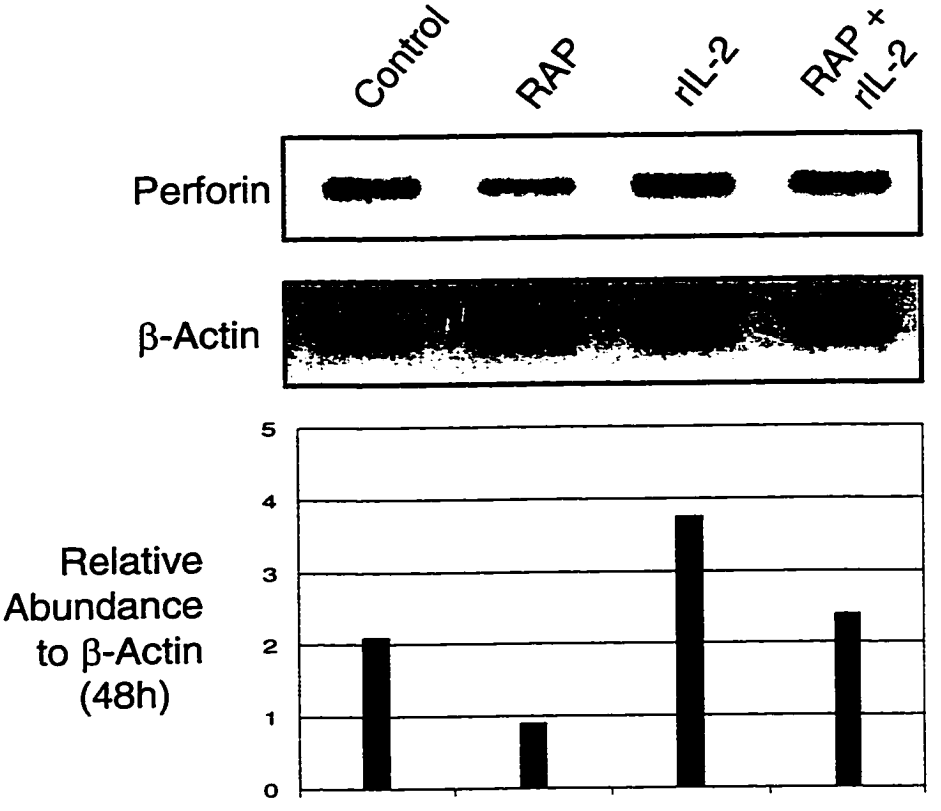

Control
RAP
rIL-2
RAP + rIL-2
Perforin
β-Actin
Relative Abundance to β-Actin (48h)
5
4
3
2
1
0

Figure 6B.

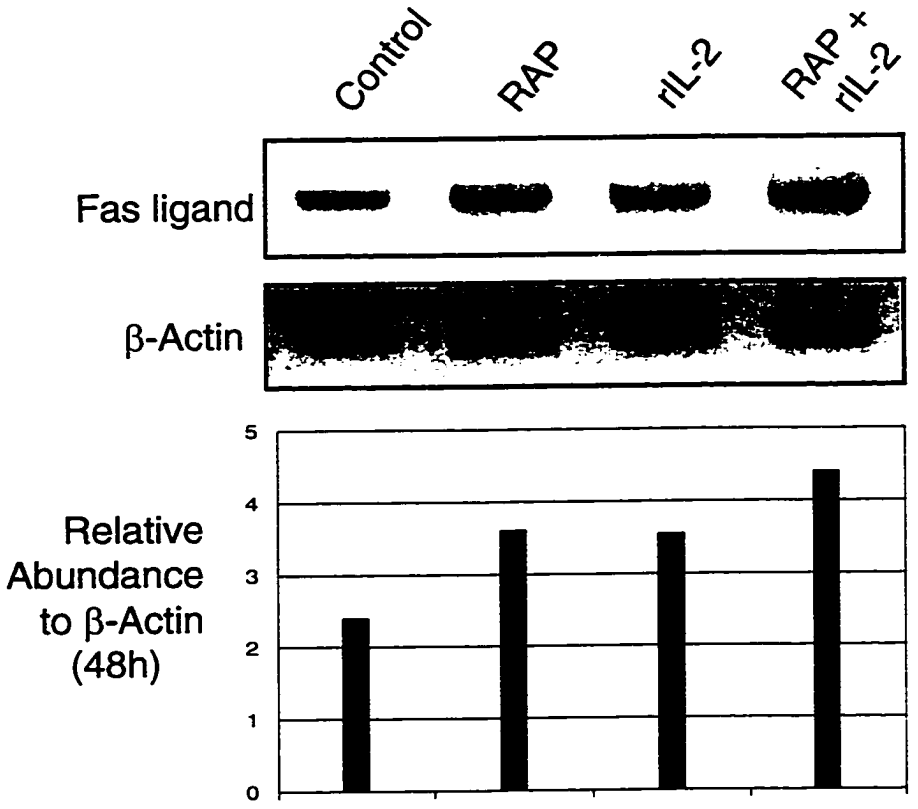

Control
RAP
rIL-2
RAP + rIL-2
Fas ligand
β-Actin
Relative Abundance to β-Actin (48h)
5
4
3
2
1
0

Figure 6C.

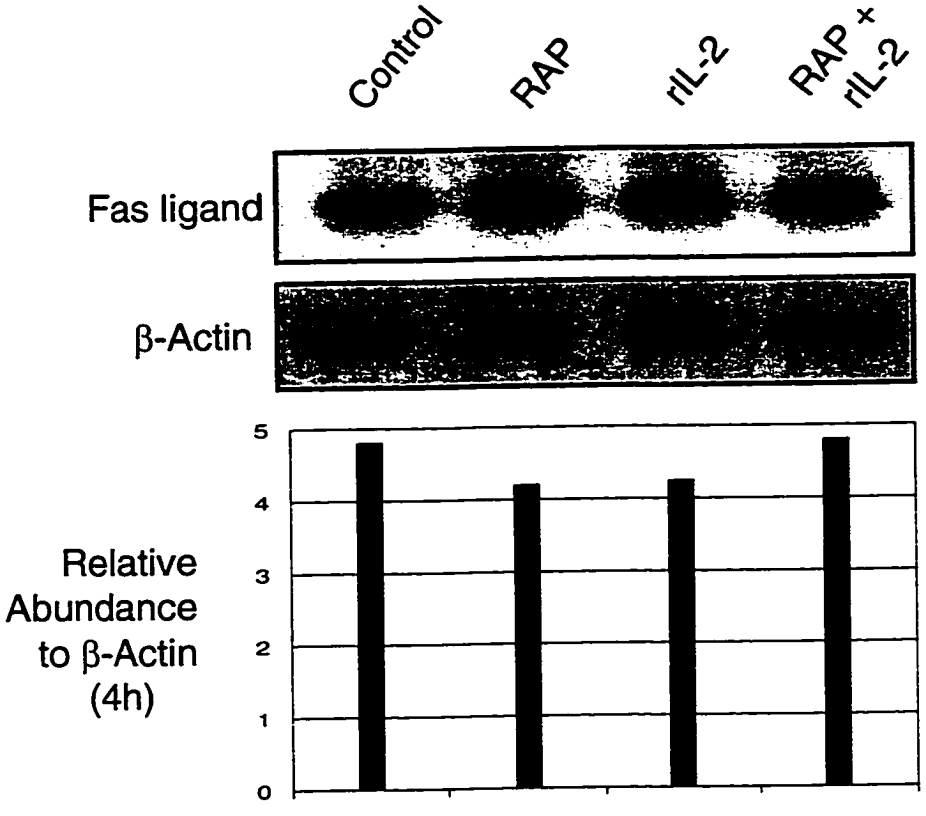

Control
RAP
rIL-2
RAP + rIL-2
Fas ligand
β-Actin
Relative Abundance to β-Actin (4h)
5
4
3
2
1
0

Figure 6D.

normal media vs. 3 ± 1% lysis of P815 in media containing 4 mM EGTA, at an E:T of 50:1). Therefore, the reason for the impaired killing displayed by RAP plus rIL-2-treated CTL, despite normal Gzm B and perforin expression, is unclear.

**c) FasL mRNA levels in RAP-treated CTL**

Because Fas/FasL-induced apoptosis is thought to be the second most important weapon CTL have in their arsenal to kill target cells, it was of interest to determine the effect of RAP on FasL mRNA levels. Unlike perforin and Gzm B, FasL mRNA levels at 48 h were increased when CTL were activated in the presence of RAP (Figure 6C). An increase was also observed following rIL-2-treatment. Furthermore, the combination of RAP plus rIL-2 showed levels of FasL mRNA higher than RAP or rIL-2-treatment alone. A totally different expression profile was seen in CTL activated for 4 h. In this case, the level of FasL mRNA expression was approximately the same for all treatments (Figure 6D).

**d) Northern blot analysis of Gzm B mRNA levels in RAP-treated CTL**

Northern blotting has historically been used to determine changes in mRNA levels, but with the development of PCR, it has become more practical to use semi-quantitative RT-PCR to study gene expression. In order to eliminate any doubt that RT-PCR analysis will supply at least semi-quantitative results, a Northern blot analysis was performed with the RNA isolated at 48 h of culture from CTL induced with anti-CD3 mAb with or without RAP and/or rIL-2 and probed with $^{32}$P-labelled Gzm B and β-actin cDNA. In agreement with RT-PCR results, Northern blot analysis showed that control CTL had abundant Gzm B mRNA while Gzm B mRNA expression was totally abrogated by RAP treatment (Figure 7). Exogenous rIL-2 increased Gzm B mRNA levels above control values. T cells treated

with RAP and rIL-2 exhibited Gzm B mRNA levels which were comparable to control levels (Figure 7). Interestingly, the decrease in Gzm B expression due to RAP-treatment was more dramatic when analyzed by Northern blotting versus RT-PCR. This suggests that the decreases seen using RT-PCR are actually understated and, in reality, may be much higher. This also shows that RT-PCR analysis is a reliable measure of overall changes in the abundance of specific mRNAs.

### 3.7 RAP does not affect the stability of Gzm B, perforin and FasL mRNA transcripts

RAP decreased the levels of perforin and Gzm B mRNA, and increased FasL mRNA expression after 48 h of culture (Figures 6A, B, and C). However, it was not clear whether the changes in mRNA levels were due to changes in the rate of gene transcription or changes in the rate of mRNA degradation. In order to determine whether RAP affected the half-life of these mRNA species, purified T cells were activated for 48 h with anti-CD3 mAb in the presence or absence of RAP. At 48 h of culture, actinomycin D (ActD) was added (time 0) and total RNA was harvested at this and successive timepoints. The RNA was then subjected to RT-PCR analysis and the levels of the relevant transcript were compared to mRNA levels at time 0.

Gzm B mRNA levels from CTL activated in the presence of anti-CD3 mAb steadily declined over the 12 h period following the addition of ActD. Gzm B mRNA transcripts in RAP-treated CTL were also seen to decline over the 12 h period at a similar rate (Figure 8A). This suggests that RAP does not affect the rate at which Gzm B mRNA degrades. Similarly, the rate at which the control-derived perforin and FasL mRNA transcripts

Figure 7. Northern Blot analysis of Gzm B mRNA levels in RAP, rIL-2, or RAP plus rIL-2-treated CTL. Five μg of RNA were isolated from T cells activated with anti-CD3 mAb for 48 h in the absence or presence of 1 ng/ml RAP, 100 U/ml rIL-2, or RAP plus rIL-2. The RNA was electrophoresed, transferred to a Nytran membrane, and probed with $^{32}$P-labelled Gzm B cDNA, stripped and reprobed with $^{32}$P-labelled β-actin cDNA. Northern probe signals were subjected to densitometric analysis to quantitate Gzm B expression relative to steady state expression of β-actin. Data are from one experiment and are representative of three independent experiments.

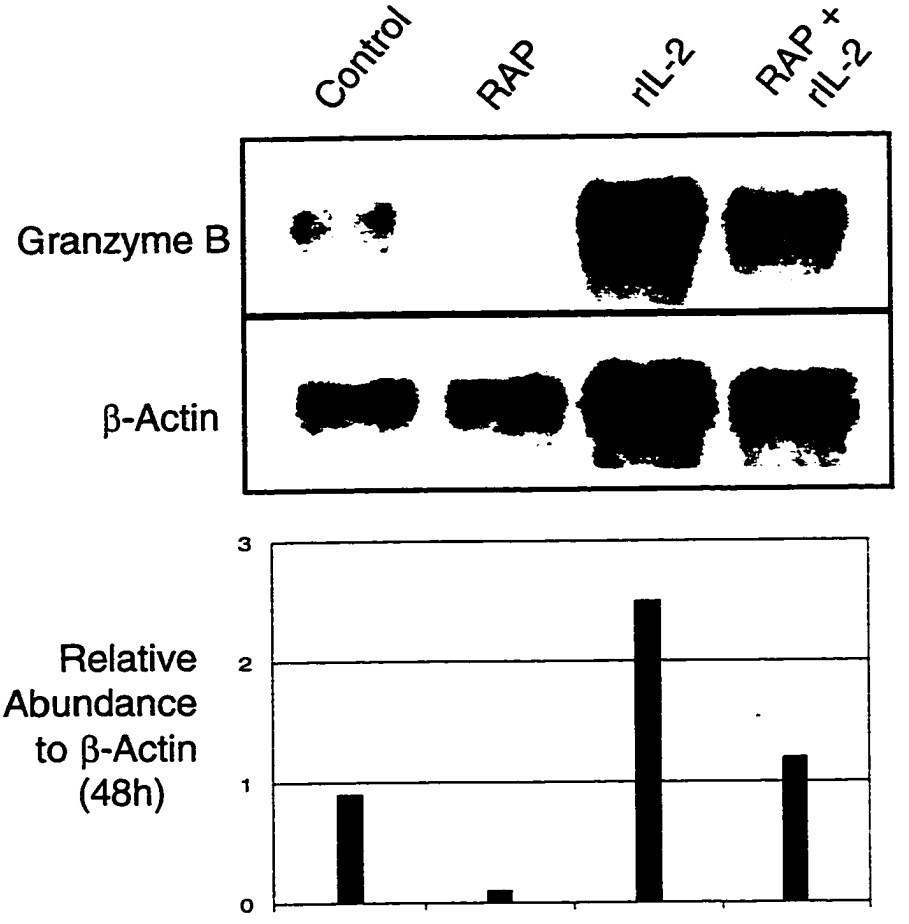
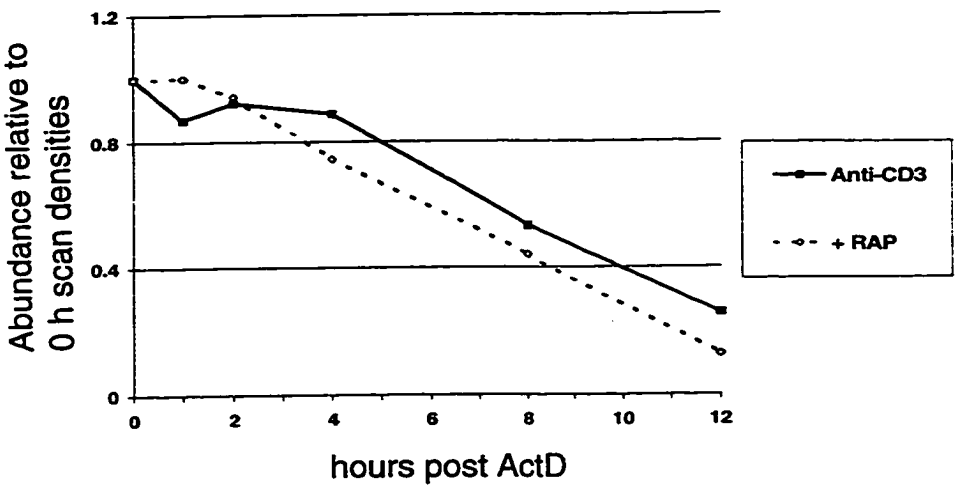

Control
RAP
rIL-2
RAP + rIL-2
Granzyme B
β-Actin
Relative Abundance to β-Actin (48h)
3
2
1
0

Figure 7.

Figure 8. RAP does not affect the stability of Gzm B, perforin, and FasL mRNA transcripts in anti-CD3-activated CTL. Purified T cells were incubated with anti-CD3 mAb alone or with 1 ng/ml RAP. Following 48 h of culture, 5 μg/ml of actinomycin D were added to replicate cultures (0 h) and total cellular RNA was isolated from these cultures at the indicated timepoints. Single-stranded cDNA was reverse-transcribed from 0.5 μg of RNA with random hexamers as described in the Materials and Methods. The resulting cDNA template was used in a PCR reaction with exon-binding, intron-bridging primers specific for (A) GzmB, (B) perforin, (C) FasL, and (D) IL-2. PCR amplicons were resolved by gel electrophoresis and ethidium bromide staining, and quantified by densitometric analysis. Results are expressed as the densitometric abundance relative to 0 h actinomycin D-treated T cell cultures. Data are from one experiment and are representative of two independent experiments.

A. Granzyme B




B. Perforin

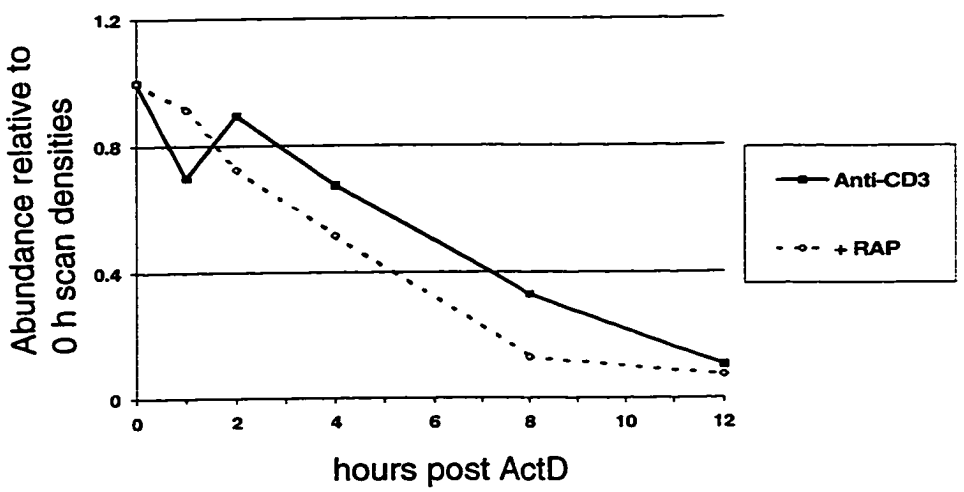
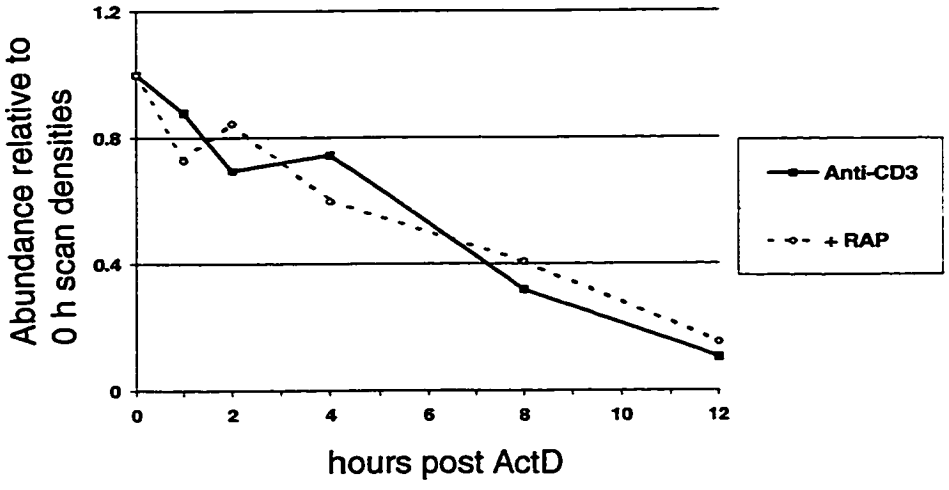



Figure 8.

C. Fas ligand




D. IL-2

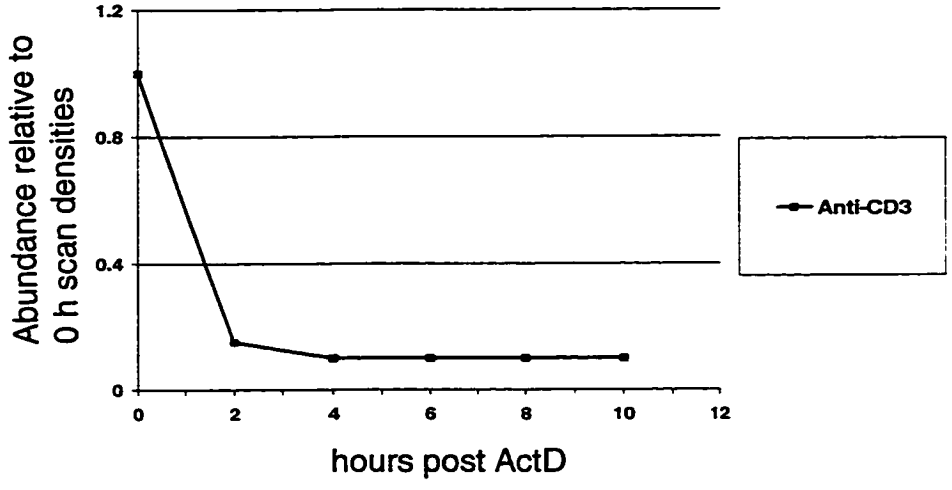



Figure 8.

degraded over 12 h was approximately the same as the degradation rate of mRNA transcripts from RAP-treated cultures (Figures 8B and C, respectively). This suggests that the changes RAP induces in the overall levels of Gzm B, perforin, and FasL mRNA expression are due to different efficiencies of transcription and not effects on mRNA stability.

The rates at which the mRNA transcripts of the genes degraded over time were all very similar. In order to rule out the possibility that the data might be artifactual, I decided to analyze the half-life of IL-2 mRNA, an mRNA species whose rate of decay is well documented. In contrast to the rates of decline exhibited by the other mRNA species, IL-2 had a very short half-life (Figure 8D), which agrees with prior reports (224). After 2 h the relative levels of IL-2 mRNA had declined to a degree that was only achieved after 12 h with Gzm B, perforin or FasL (Figure 8D). This proves that different rates of mRNA decay can be detected in this assay. Furthermore, Gzm B, perforin and FasL mRNAs all have approximately the same half-life, which is much longer than that of IL-2 mRNA.

### 3.8 Gzm B-type protease activity is decreased by RAP and is restored by rIL-2

In order to reconcile the decreased cytotoxic ability of CTL treated with RAP plus rIL-2 with the high levels of Gzm B and perforin mRNA expressed in these cells, I decided to measure the amounts of the actual proteins. Because RAP is known to selectively inhibit the translation of certain mRNAs (168,169), it was possible that RAP might be inhibiting the translation of Gzm B and perforin mRNA. To detect the presence of Gzm B protein, a colorimetric assay was used which measures Gzm B-type protease activity. The cytosolic

fraction of CTL activated with anti-CD3 mAb in the presence or absence of RAP and/or rIL-2 for 48 h was incubated with the synthetic Gzm B substrate Boc-Ala-Ala-Asp thiobenzyl ester. Cleavage of this substrate results in a color change and the absorbance measured at 405 nm gives an indication of the amount of Gzm B-like protease activity present in the cytosol. Interestingly, the amount of Gzm B-like protease activity in each treatment group mirrored the Gzm B mRNA profile (Figure 9). Treatment with RAP alone decreased substrate cleavage levels by at least 50%. On the other hand, treatment with rIL-2 boosted Gzm B activity above control values, while treatment with RAP plus rIL-2 resulted in protease levels which were also above control values (Figure 9). Assuming that Gzm B is the only protease being measured in this assay, this suggests that the low levels of cytotoxicity induced by RAP plus rIL-2 are not due to a translational block in Gzm B expression.

### 3.9 Perforin protein levels in anti-CD3-activated CTL are decreased by RAP and restored by exogenous rIL-2

I also examined the possibility that perforin mRNA in RAP-treated CTL was not being translated. A monoclonal antibody for murine perforin was obtained and used in Western blot analysis of the cytosolic fractions of CTL activated by anti-CD3 mAb for 48 h in the presence or absence of RAP and/or rIL-2. As shown in Figure 10, perforin protein levels mirrored perforin mRNA levels. RAP-treated CTL had approximately half the perforin content of control CTL. In contrast, rIL-2-treated CTL had much more perforin than control CTL. RAP plus rIL-2-treated CTL also had more perforin than untreated CTL.

Figure 9. Exogenous rIL-2 restores Gzm B-like enzymatic activity to control levels in RAP-treated CTL. Purified T cells were stimulated with anti-CD3 mAb in the presence of 1 ng/ml RAP, 100 U/ml rIL-2, or RAP plus rIL-2. Following 48 h of culture, postnuclear lysates were prepared from equal numbers of T cells, and added to a colorimetric reaction mixture containing synthetic Gzm B substrate as described in Materials and Methods. Data are expressed as mean arbitrary units of esterolytic activity ± standard deviation. Data are from one experiment and are representative of three independent experiments. (*) indicates a statistically significant difference compared with control untreated CTL, as determined by Student's *t*-test.

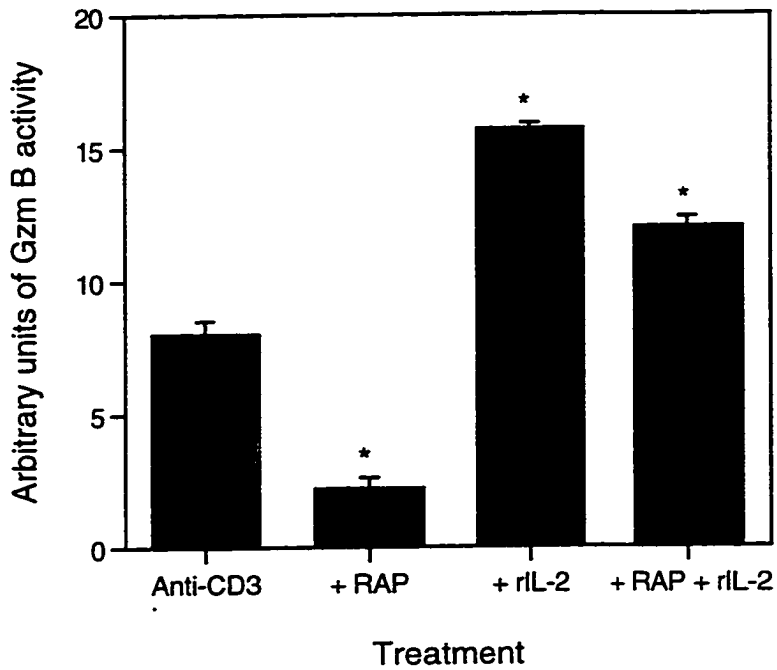

20
15
10
5
0
Arbitrary units of Gzm B activity
*
*
*
Anti-CD3
+ RAP
+ rIL-2
+ RAP + rIL-2
Treatment

Figure 9.

Figure 10. Exogenous rIL-2 restores perforin protein to control levels in RAP-treated CTL. Purified T cells were stimulated with anti-CD3 mAb in the presence of 1 ng/ml RAP, 100 U/ml rIL-2, or RAP plus rIL-2. Following 48 h of culture, postnuclear lysates were prepared from equal numbers of T cells, and perforin expression was analyzed by Western blotting with a rat anti-mouse perforin mAb as described in Materials and Methods. Protein bands were quantified by densitometric analysis and are expressed as the relative abundance compared to perforin expression in untreated CTL. Data are from one experiment and are representative of two independent experiments.

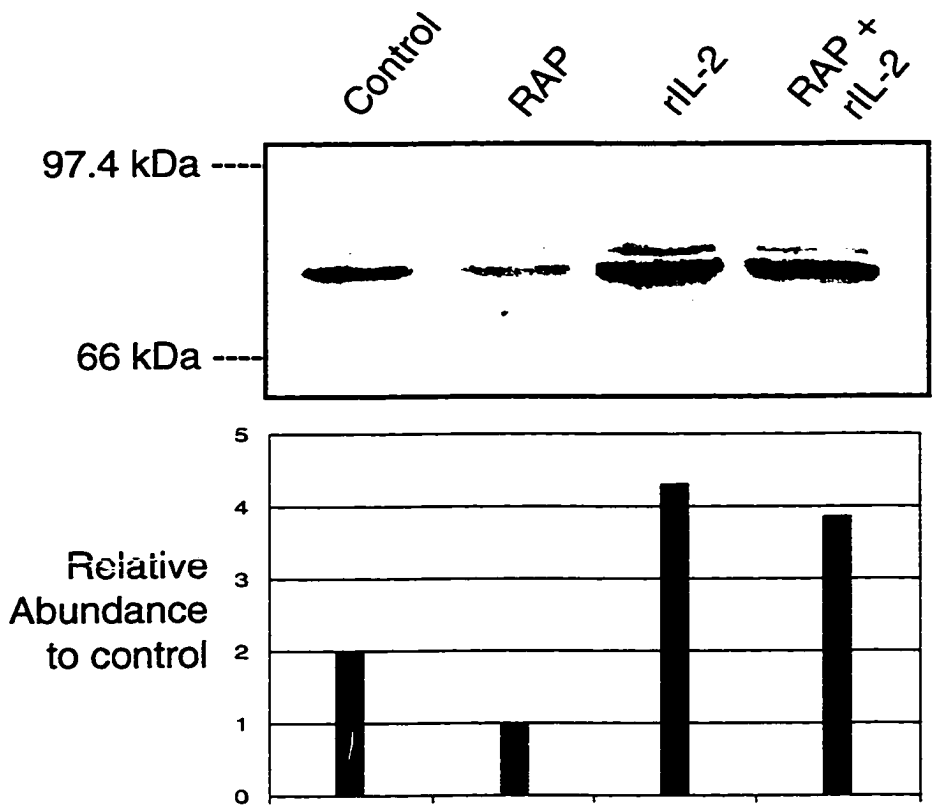

Control
RAP
rIL-2
RAP + rIL-2
97.4 kDa
66 kDa
Relative Abundance to control
5
4
3
2
1
0

Figure 10.

This shows that the block in the killing of RAP plus rIL-2-treated CTL is not due to retarded perforin protein synthesis.

### 3.10 Conjugation to P815 target cells and LFA-1 surface levels are normal in RAP plus rIL-2-treated CTL

The RT-PCR, enzymatic, and Western blot data on Gzm B and perforin expression by CTL treated with RAP plus rIL-2 seems to be at odds with the low levels of cytotoxicity displayed by CTL activated in the presence of RAP plus rIL-2. These CTL express more Gzm B and perforin mRNA and protein than untreated CTL, yet have a greatly diminished ability to kill susceptible target cells. However, RAP could be affecting the expression or function of other molecules involved in the cytolysis of target cells. The ability of CTL to conjugate with target cells was tested. Control and RAP plus rIL-2-treated CTL adhered to P815 with similar efficiency (48 ± 13% conjugation for RAP plus rIL-2-treated CTL vs. 53 ± 5% conjugation for control untreated CTL, in one experiment representative of two independent experiments). In addition, there were no notable changes in the surface levels of LFA-1 in the RAP plus rIL-2-treated CTL in comparison to control CTL, as determined by flow cytometric analysis (Figure 11). LFA-1 is known to be important in P815 target cell binding by anti-CD3-activated CTL (289).

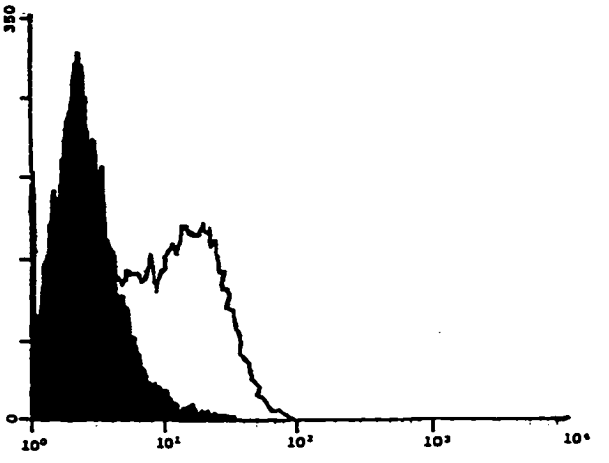
### 3.11 Granule Exocytosis triggered by PMA plus ionomycin treatment is normal in RAP plus rIL-2-treated CTL

The intracellular machinery controlling granule exocytosis was also examined.

Figure 11. LFA-1 surface expression in control vs. RAP plus rIL-2-treated T cells. T cells were activated with anti-CD3 alone or in the presence of 1 ng/ml RAP plus 100 U/ml rIL-2 for 48 h. Cells were then stained with 1 μg/ml rat-anti-mouse LFA-1 followed by a second incubation with the 1 μg/ml FITC-conjugated mouse-anti-rat IgG. Flow cytometric analysis was performed with a FACSCAN. Filled peaks indicate unstained cells while open peaks indicate stained cells. The x-axis represents fluorescence intensity and the y-axis shows the relative cell number.

LFA-1

Control




RAP + rIL-2

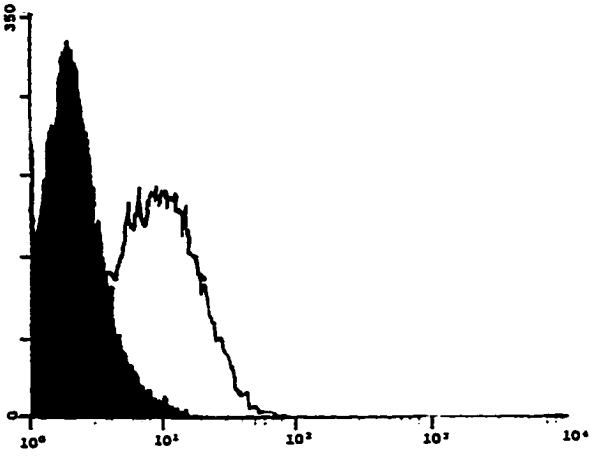



Figure 11.

Granule exocytosis was induced in control CTL and RAP plus rIL-2-treated CTL for 2 h with PMA and ionomycin. Granule release was measured in a tryptase-specific assay similar to that used to measure Gzm B enzymatic activity using the Nα-CBZ-L-lysine thiobenzyl ester substrate (49). It was found that both CTL populations exhibited similar levels of exocytosis after 2 h of stimulation with PMA/ionomycin, suggesting that granule delivery mechanisms are intact in RAP plus rIL-2-treated CTL (Figure 12).

## Section II: The role of B7 in anti-CD3 mAb activation of CTL

### 3.12 B7-2, but not B7-1, is a major contributor to anti-CD3 mAb-induced activation of CTL

Costimulation through CD28 provides the second signal necessary for the activation of T cells in terms of proliferation and cytokine production (7). Although it is generally accepted that CD28 costimulation is required to activate cytotoxicity, there is controversy over which B7 ligand can better mediate activation. CTL precursors can be activated in the absence of Th cells by B7-1$^+$ tumor cells (277), as well as by B7-2$^+$ tumor cells (279). In order to determine what role, if any, the B7 family of proteins play in anti-CD3-mediated activation of CTL, blocking monoclonal antibodies to murine B7-1 and B7-2 were obtained. Anti-B7-1 (clone 16-10A1) and anti-B7-2 (clone GL1) mAbs were both used as hybridoma supernatants at a 1/10 final dilution. ELISA assays showed that the concentration of mAb in the supernatant of clone GL1 was approximately 40 μg/ml while that of clone 16-10A1 was approximately 2 μg/ml. Both blocking mAb were added along

Figure 12. RAP plus rIL-2-treated CTL are capable of normal granule exocytosis. T cells were stimulated with anti-CD3 mAb alone or in the presence of 1 ng/ml RAP plus 100 U/ml rIL-2. Following 48 h of culture, equal numbers of T cells were incubated for 2 h with 100 ng/ml PMA and 500 ng/ml ionomycin, centrifuged and the supernatant used in a colorimetric reaction mixture containing synthetic tryptase-specific substrate as described in Materials and Methods. Data are expressed as mean arbitrary units of tryptase activity ± standard deviation. Data are from one experiment and are representative of three independent experiments.

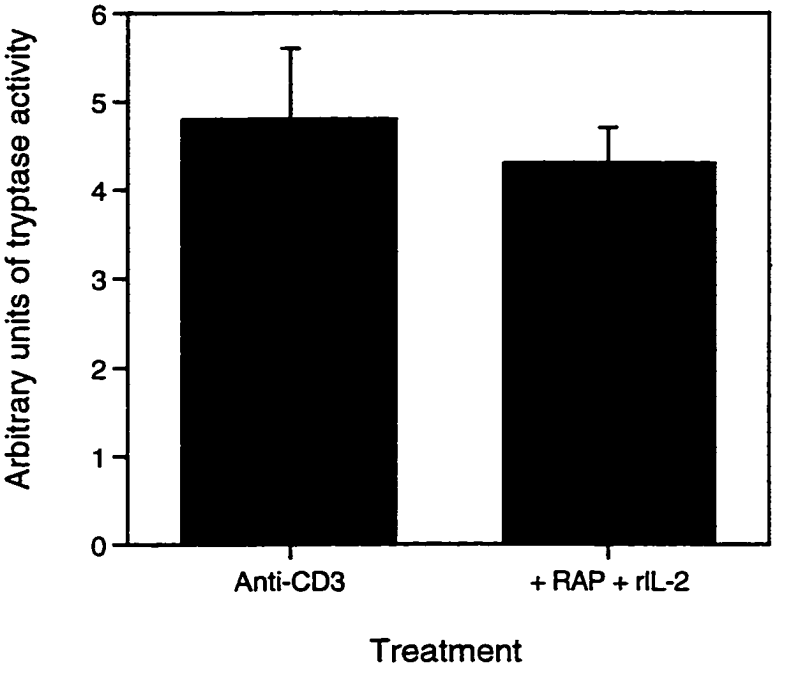

Arbitrary units of tryptase activity
6
5
4
3
2
1
0
Anti-CD3
+ RAP + rIL-2
Treatment

Figure 12.

with anti-CD3 mAb to purified T cells and cultured for 48 h. Cytotoxicity of CTL was then assessed in a $^{51}$Cr-release assay against P815 target cells. As shown in Figure 13, in comparison to CTL activated in the presence of the relevant control immunoglobulin, anti-B7-2- and anti-B7-1 plus anti-B7-2-treated CTL displayed a 70% reduction in their ability to lyse P815 target cells. Anti-B7-1-treated CTL also showed a slight but reproducible decrease in cytotoxicity in comparison to hamster *i*gG isotype control values. This suggests that B7-2 is present in cell culture and binds the majority of CD28 on T cells for their activation. On the other hand, B7-1 does not play a large role in CTL induction under these conditions. These results were confirmed with a different set of blocking antibodies specific for B7-1 and B7-2 (clones RM80 and PO3, respectively). The data are shown in Figure 14. Interestingly, treatment with anti-B7-2 never totally inhibited CTL activation, suggesting that there is a minor pathway of costimulation which is B7-2-independent.

### 3.13 Dose-dependent effect of anti-B7-2 mAb on anti-CD3-induced T cell proliferation

In order to determine the dose-dependent effect of B7-2 blockade on T cell proliferation, T cells were activated with anti-CD3 mAb in the presence of an immunoglobulin control or serial dilutions of anti-B7-2 mAb in the form of hybridoma supernatant (clone GL1). As shown in Figure 15, anti-B7-2-treatment effectively inhibited 90% of T cell proliferation when used at final dilutions of 1/10 to 1/10,000. Proliferation was less affected when anti-B7-2 was used at 1/100,000 dilution. This suggests that anti-B7-2 mAb is at saturating concentrations when used at a final dilution of 1/10. Figure 15

Figure 13. Blocking antibodies to B7-2, but not B7-1, inhibit the induction of cytolytic activity in anti-CD3-activated T cell cultures. Purified T cells were stimulated with anti-CD3 mAb in the presence of blocking antibodies to B7-1 (clone 16-10A1) and B7-2 (clone GL1) (both used as hybridoma supernatants, 0.2 μg/ml and 4 μg/ml final concentration, respectively), either individually, or in combination, or in the presence of 10 μg/ml whole rat IgG. Following 48 h of culture, cytolytic activity against P815 target cells at an E:T of 50:1 was determined using $^{51}$Cr-release assay. Results are expressed as mean percent lysis of P815 targets ± the standard deviation of three replicates. The results are from one experiment and are representative of at least three experiments. (*) denotes a statistically significant difference in killing activity in comparison to the appropriate isotype control, as determined by Student's *t*-test. The anti-B7-1 plus anti-B7-2-treatment group was compared to the rat IgG control.

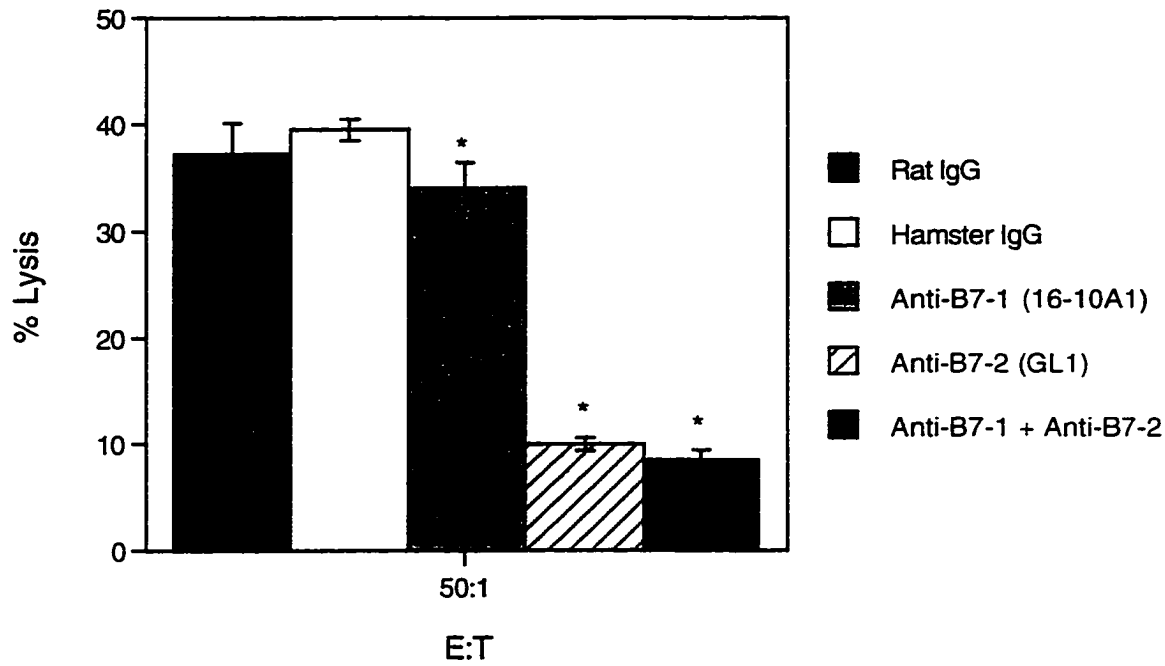

50
40
30
20
10
0
% Lysis
*
*
*
50:1
E:T
Rat IgG
Hamster IgG
Anti-B7-1 (16-10A1)
Anti-B7-2 (GL1)
Anti-B7-1 + Anti-B7-2

Figure 13.

Figure 14. Confirmation of the role of B7-1 and B7-2 during anti-CD3 activation with a different pair of antibodies. Purified T cells were stimulated with anti-CD3 mAb in the presence of 1 μg/ml blocking antibodies to B7-1 (clone RM80) and B7-2 (clone PO3), either individually, or in combination, or in the presence of 10 μg/ml whole rat IgG. Following 48 h of culture, cytolytic activity against P815 target cells at E:T ratios of 50:1 and 25:1 were determined using $^{51}$Cr-release assay. Results are expressed as mean percent lysis of P815 targets ± the standard deviation of three replicates. The results are from one experiment and are representative of at least three experiments. (*) denotes a statistically significant difference in killing activity in comparison to the appropriate isotype control, as determined by Student's *t*-test.

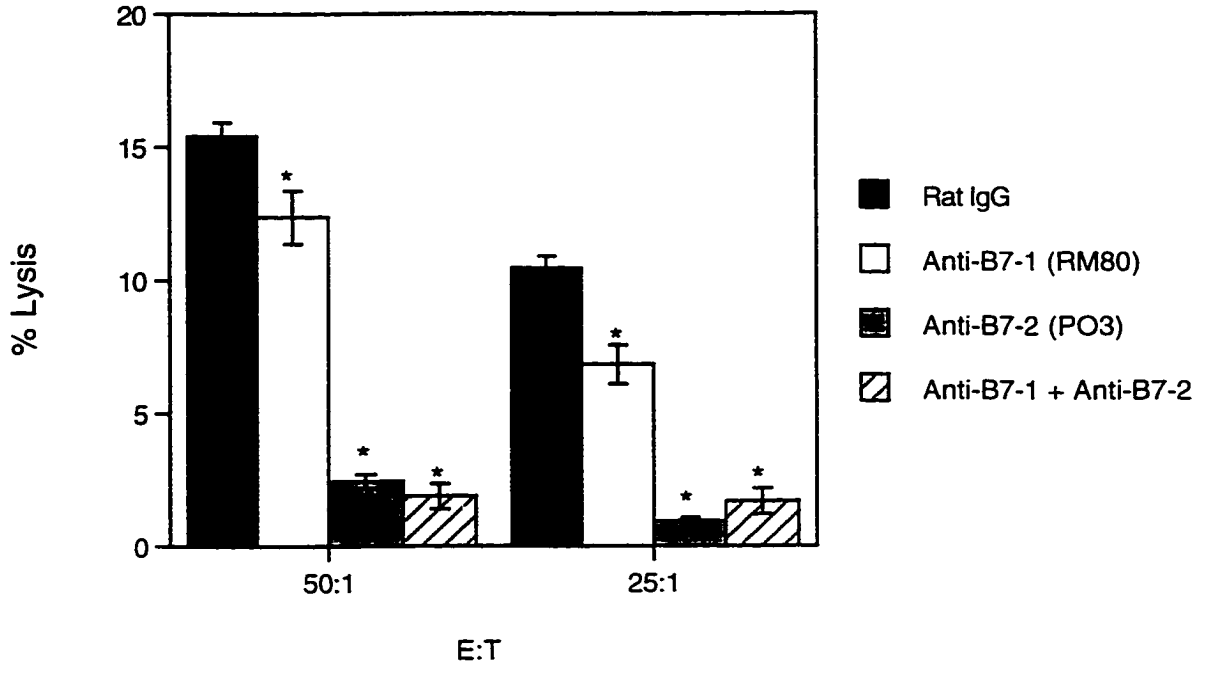

20
15
10
5
0
% Lysis
50:1
25:1
E:T
Rat IgG
Anti-B7-1 (RM80)
Anti-B7-2 (PO3)
Anti-B7-1 + Anti-B7-2

Figure 14.

Figure 15. Anti-B7-2 mAb inhibits proliferation of anti-CD3-activated T cells in a dose-dependent manner. T cells were activated in the presence of 10 μg/ml whole rat IgG or serial dilutions of anti-B7-2 mAb (clone GL1, starting dilution 1/10 or 4 μg/ml). DNA synthesis was measured following 48 h of culture by pulsing with [$^3$H]TdR and measuring [$^3$H]TdR incorporation by liquid scintillation counting. Data are expressed as mean cpm of triplicate cultures ± standard deviation. The results are from one experiment and are representative of at least three separate experiments. (*) denotes a statistically significant difference in DNA synthesis in comparison to the control, as determined by Student's *t*-test.

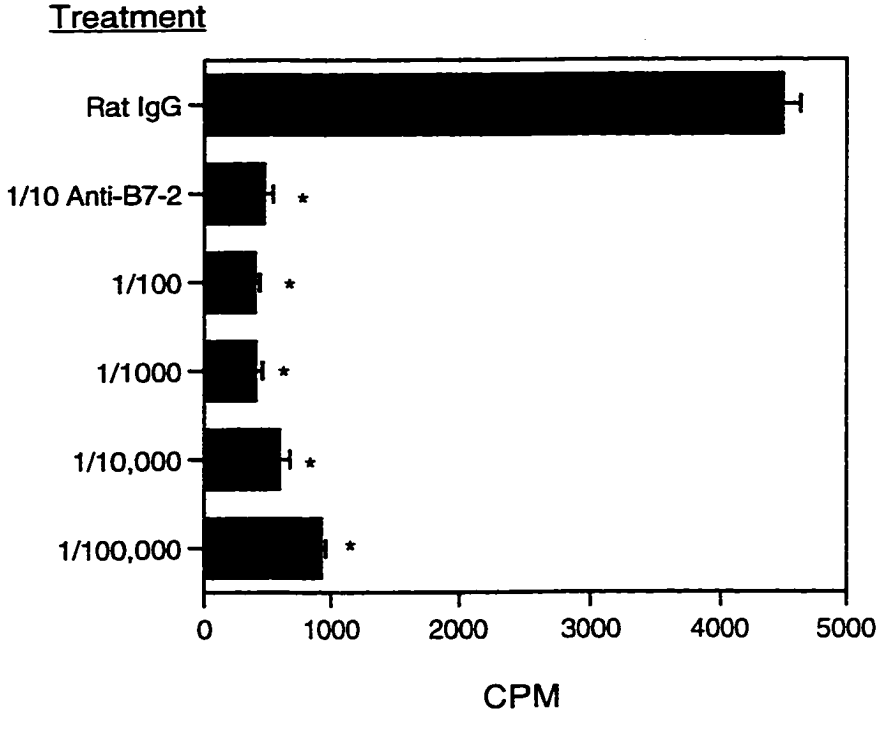

Treatment
Rat IgG
1/10 Anti-B7-2
1/100
1/1000
1/10,000
1/100,000
*
0
1000
2000
3000
4000
5000
CPM

Figure 15.

shows that anti-B7-2 mAb was still saturating at 1/10,000 or about 0.004 μg/ml. When saturating levels of stimulatory anti-CD28 mAb are present during anti-CD3 activation, the resulting cytotoxicity was 2-3 fold higher than that of control CTL suggesting CD28 is mostly unbound (data not shown). The anti-B7-1 mAb was present at 0.2 μg/ml in T cell cultures. As will be discussed later, B7-2 surface protein is expressed at much higher levels than B7-1 in these T cell cultures. Therefore, it is reasonable to assume that anti-B7-1 mAb was used at more than saturating concentrations.

### 3.14 B7-1 and B7-2 molecules are not necessary for effector function of anti-CD3-activated CTL

B7-1- and B7-2-transfected target cells are lysed more efficiently than B7-deficient targets (273). The possibility exists that anti-CD3-activated CTL use B7/CD28 interactions to lyse P815. In order to determine if this was the case, T cells were activated with anti-CD3 mAb and used in a $^{51}$Cr-release assay against P815 target cells. Addition of rat IgG, anti-B7-1, anti-B7-2, or anti-CD28 mAb to the lysis assay did not significantly affect P815 lysis levels (Figure 16). This data suggests that neither B7-1/CD28 nor B7-2/CD28 interactions are required for the lysis of P815 cells by anti-CD3-activated CTL.

### 3.15 Resting murine CD8$^+$ T cells express B7-2 on their surface

It is known that murine CD4$^+$ T cells express both CD28 and B7-2, and that B cells express low levels of B7-2 (240). However, it is unclear if B7-2 is also expressed by murine CD8$^+$ T cells. In order to answer this question, purified CD8$^+$ T cells were obtained

Figure 16. The presence of anti-B7-1 or anti-B7-2 mAb does not inhibit lysis of P815 cells by anti-CD3-activated CTL. T cells were activated with anti-CD3 mAb for 48 h and cytolytic activity against P815 target cells at an E:T ratio of 50:1 was assayed using a $^{51}$Cr-release assay. Effectors and targets were incubated for 4 h in medium alone, 5 μg/ml whole rat IgG, 5 μg/ml anti-B7-1 mAb (clone 1G10), anti-B7-2 mAb (clone GL1 hybridoma supernatant, 4 μg/ml final concentration), or 1 μg/ml anti-CD28 mAb. Results are expressed as the mean percent lysis of P815 target cells ± the standard deviation of three replicates. The results are from one experiment and are representative of at least three separate experiments. (*) denotes a statistically significant difference in killing activity in comparison to the control, as determined by Student's *t*-test.

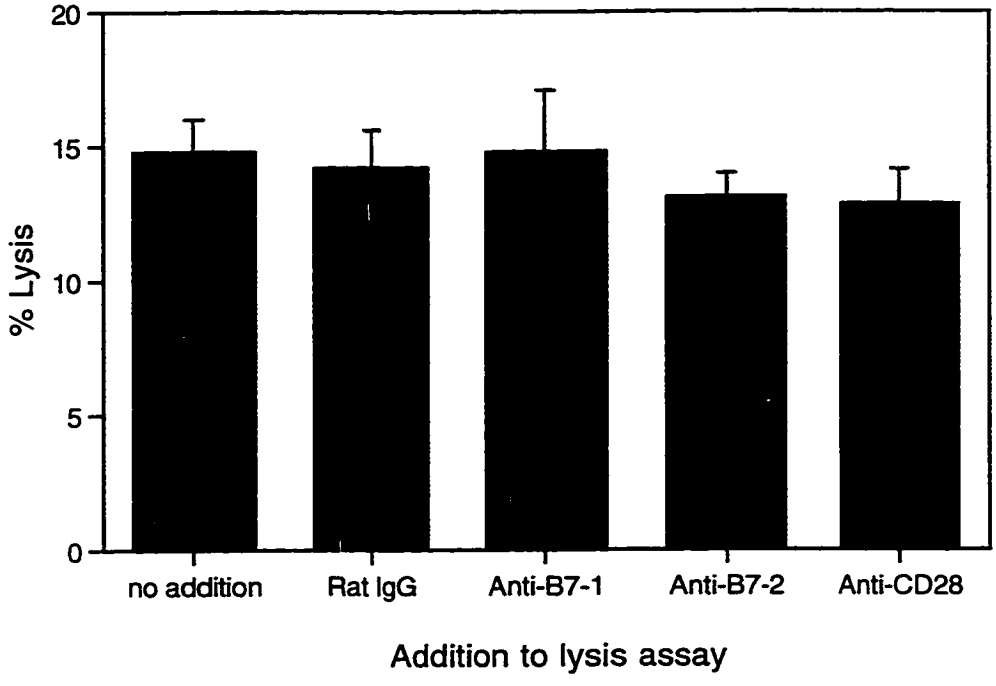

20
15
10
5
0
% Lysis
no addition
Rat IgG
Anti-B7-1
Anti-B7-2
Anti-CD28
Addition to lysis assay

Figure 16.

from $CD4^{-/-}$ mouse splenocytes by double nylon wool passage and treatment with anti-asialoGM1 antibody plus complement to completely remove B cells and NK cells, respectively. The resulting APC-depleted T cell population was subjected to flow cytometric analysis. As shown in Figure 17A, the vast majority of cells expressed high levels of the CD8α chain. Ail T cells were found to express CD28 and B7-2, but at much lower levels than CD8α (Figures 17B and C, respectively). Interestingly, the presence of B7-2 on T cells suggests that some or all of the costimulation during anti-CD3-activation may be supplied by T cells themselves.

### 3.16 B7-2 is directly costimulating $CD8^+$ T cells during anti-CD3-activation

Previous reports have shown that human CTL activity can be activated with immobilized anti-CD3 mAb and either anti-CD28 mAb or B7 costimulation (290). The source of IL-2 for CTL activation in this system is thought to be $CD4^+$ T cells as not all human $CD8^+$ T cells express CD28 (217). However, all murine $CD8^+$ T cells are known to express CD28 (215). In order to determine if B7-2 on APC (or T cells) was directly binding to $CD8^+$ T cells for their activation, purified $CD8^+$ T cells were obtained from $CD4^{-/-}$ mice and activated with anti-CD3 mAb in the presence of rat IgG or anti-B7-2 mAb. Incubation with anti-CD3 mAb activated cytotoxicity in $CD8^+$ T cells in the absence of $CD4^+$ T cells, while treatment with anti-B7-2 mAb again dramatically decreased cytotoxicity (Figure 18). This suggests that B7-2 binds directly to $CD8^+$ T cells for their activation. In addition, T cells depleted of $CD4^+$ T cells by anti-CD4 mAb and C' treatment and then activated with anti-CD3 mAb could be inhibited by the presence of anti-B7-2 mAb (data not shown).

Figure 17. Fresh $CD8^+$ T cells express B7-2. T cells were purified from $CD4^{-/-}$ mice as previously described in Materials and Methods. Cells were then stained with 1 μg/ml rat-anti-mouse CD8α (A), 1 μg/ml hamster anti-mouse CD28 (B), and rat-anti-mouse B7-2 (clone GL1, hybridoma supernatant used 1/2) mAbs (C), followed by a second incubation with the 1 μg/ml FITC-conjugated mouse-anti-rat IgG (A and C), or with 1 μg/ml FITC-conjugated goat F(ab')2-anti-hamster IgG (B). Flow cytometric analysis was performed with a FACSCAN. The x-axis represents fluorescence intensity and the y-axis shows the relative cell number. Percent positive cells are shown in parentheses.

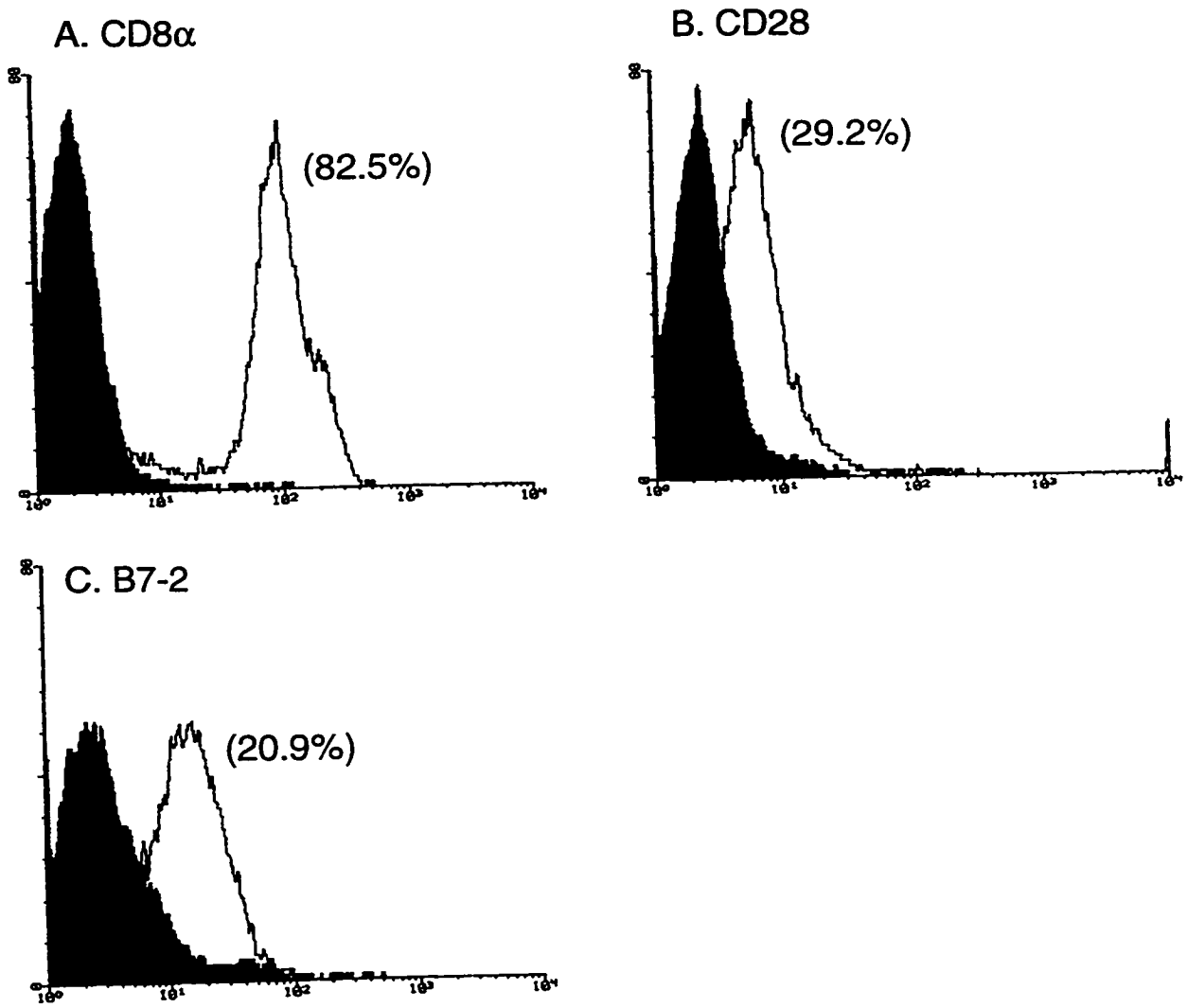



Figure 17.

Figure 18. $CD8^+$ CTL require CD28/B7-2 interactions for activation. Purified T cells from $CD4^{-/-}$ mice were stimulated with anti-CD3 mAb in the presence of blocking antibodies to B7-2 (GL1 hybridoma supernatant, 4 μg/ml final concentration) or in the presence of 10 μg/ml isotype-matched antibody. Following 48 h of culture, cytolytic activity against P815 target cells at an E:T of 50:1 was determined using $^{51}Cr$-release assay. Results are expressed as mean percent lysis of P815 targets ± the standard deviation of three replicates. The results are from one experiment and are representative of at least three experiments. (*) denotes a statistically significant difference in killing activity in comparison to the control, as determined by Student's *t*-test.

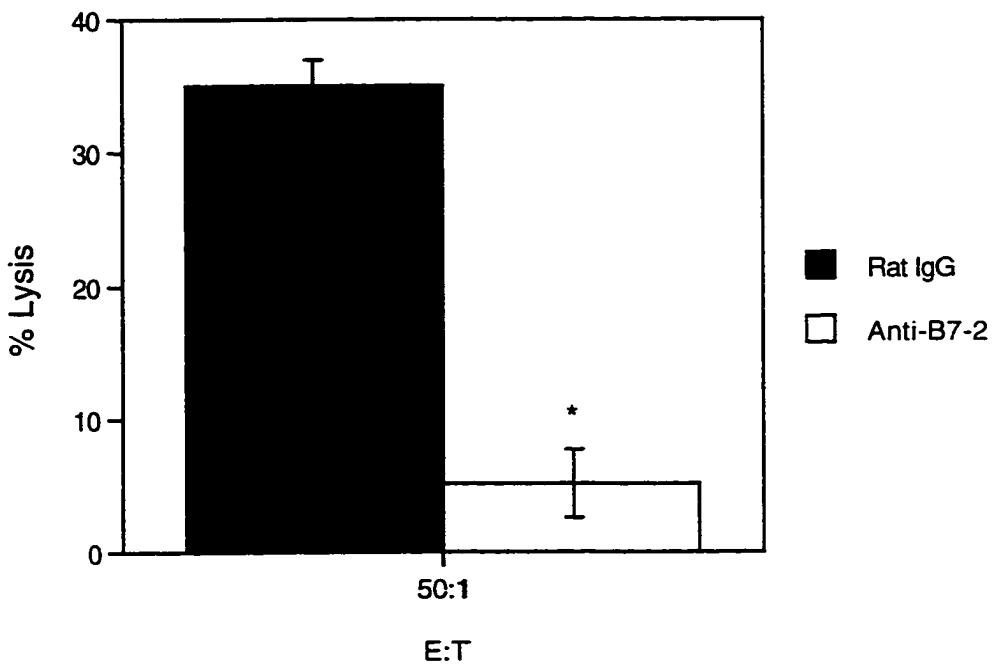



Figure 18.

### 3.17 B7-2 costimulation must be provided within 12 h of anti-CD3 mAb stimulation for optimal CTL induction

In order to determine the time at which B7-2 costimulation is required during the 48 h culture with anti-CD3 mAb for maximum CTL activation, anti-B7-2 mAb was added at different times following the addition of anti-CD3 mAb. After 48 h of culture, cytotoxicity was assessed against P815 target cells. Progressively lesser inhibition was seen the longer anti-B7-2 addition was withheld, with the most inhibition seen at 0, 6, and 12 h timepoints (Figure 19). This suggests that for optimal development of CTL the B7-2 signal is required at the same time as CD3-crosslinking or shortly thereafter. An increase in CTL activation was seen if anti-B7-2 mAb was added at 36 h of culture. However, this effect could not be differentiated from a similar increase in CTL activation caused by the addition of an equal volume of fresh media at this timepoint.

### 3.18 B7-1 expression is not detectable in early T cell cultures, while B7-2 and CD28 expression is constitutive

In order to better understand the kinetics of costimulation during anti-CD3-activation, the levels of B7-1, B7-2 and CD28 expression at different timepoints were determined by flow cytometric analysis. As expected, B7-1 expression was not detectable at 0, 6, and 24 h. However, at 48 h there was a detectable positive shift in anti-B7-1 staining of cells (Figure 20A) agreeing with B7-1 mRNA studies (data not shown). B7-2 expression was present at 0 h and maintained at 6, 24, and 48 h. B7-2 expression is perhaps slightly increased at the latter timepoints (Figure 20B). CD28, like B7-2, was also present

Figure 19. Blockade of B7-2 interaction with CD28 during the first 12 h of culture inhibits the development of anti-CD3-activated CTL. Purified T cells were stimulated with anti-CD3 mAb in the presence of blocking antibodies to B7-2 (GL1 hybridoma supernatant, 4 μg/ml final concentration) added at the indicated times during culture or in the presence of 10 μg/ml isotype-matched antibody. Following 48 h of culture, cytolytic activity against P815 target cells at an E:T of 50:1 was determined using a $^{51}$Cr-release assay. Results are expressed as mean percent lysis of P815 targets ± the standard deviation of three replicates. The results are from one experiment and are representative of at least three experiments. One-way analysis of variance (ANOVA) indicates that the variation among column means is extremely significant. (*) denotes a statistically significant difference in killing activity in comparison to the rat IgG-treatment, as determined by the Bonferroni multiple comparison's test.

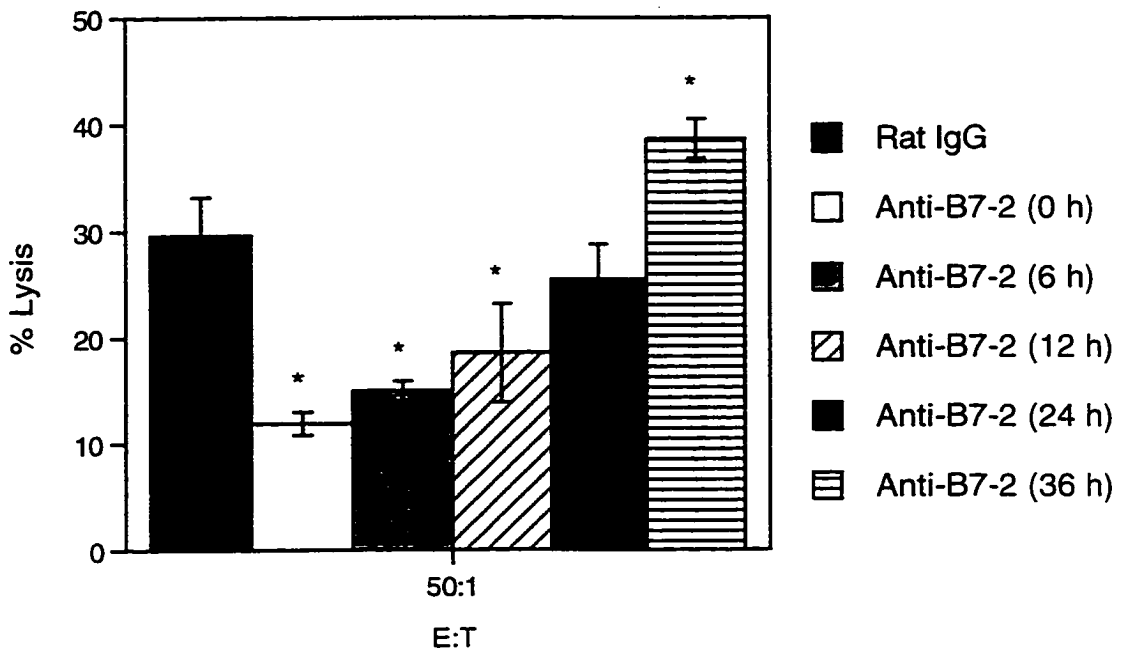

50
40
30
20
10
0
% Lysis
*
*
*
*
50:1
E:T
Rat IgG
Anti-B7-2 (0 h)
Anti-B7-2 (6 h)
Anti-B7-2 (12 h)
Anti-B7-2 (24 h)
Anti-B7-2 (36 h)

Figure 19.

Figure 20. B7-2 and CD28, but not B7-1, are expressed before and after anti-CD3 activation of nylon wool non-adherent, asialoGM1-depleted splenocytes. T cells were purified as previously described in Materials and Methods and activated with anti-CD3 mAb for 48 h. Aliquots of cells were removed from culture at the indicated timepoints and washed. In order to avoid detection of anti-CD3 mAb (hamster IgG isotype), cells to be stained for (A) B7-1 and (C) CD28 expression were incubated sequentially with 1 μg/ml unlabelled goat F(ab')2-anti-hamster IgG, hamster anti-mouse B7-1 (clone 16-10A1, final dilution 1/2) or 1 μg/ml hamster anti-mouse CD28, and 1 μg/ml FITC-conjugated goat F(ab')2-anti-hamster IgG. Cells stained for (B) B7-2 were incubated sequentially with rat-anti-mouse B7-2 (clone GL1, final dilution 1/2) and 1 μg/ml FITC-conjugated mouse anti-rat IgG. Filled peaks indicate non-specific binding of the FITC-conjugated antibody, while open peaks indicate specific staining with the primary antibody. Flow cytometric analysis was performed with a FACSCAN. The x-axis represents fluorescence intensity and the y-axis shows the relative cell number. The mean channel fluorescence is depicted in the square brackets.

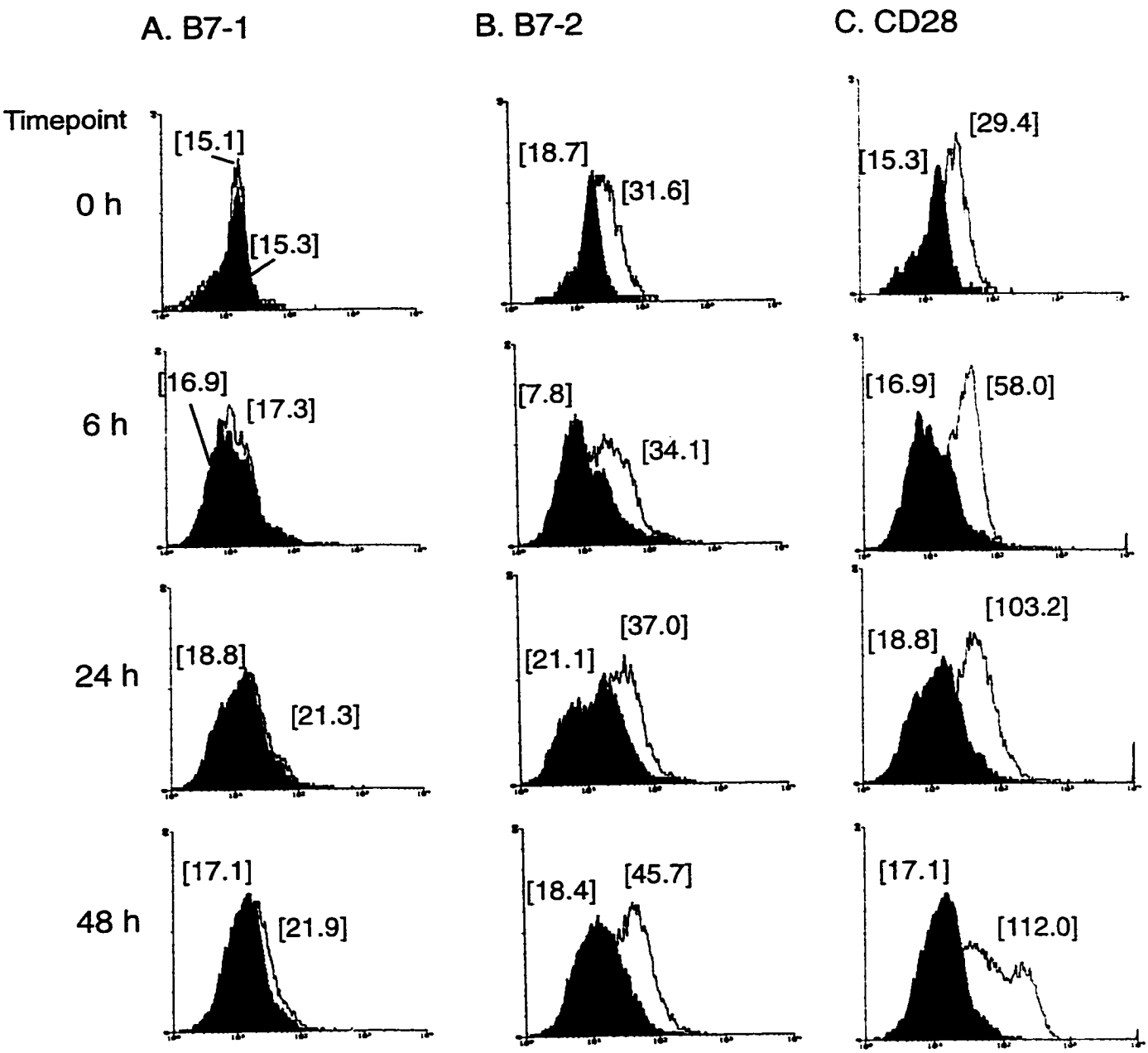

A. B7-1
B. B7-2
C. CD28
Timepoint
0 h
6 h
24 h
48 h
[15.1]
[15.3]
[18.7]
[31.6]
[29.4]
[15.3]
[16.9]
[17.3]
[7.8]
[34.1]
[16.9]
[58.0]
[18.8]
[21.3]
[37.0]
[21.1]
[103.2]
[18.8]
[17.1]
[21.9]
[18.4]
[45.7]
[17.1]
[112.0]

Figure 20.

at all timepoints. However, at 48 h there appeared to be an increase in CD28 expression (Figure 20C).

### 3.19 B7-1 can contribute to CTL activation only in the absence of both anti-B7-1 and anti-B7-2 mAb

The data from the flow cytometric studies described in 3.18 suggested that anti-B7-1 mAb does not significantly affect anti-CD3-activation of CTL because B7-1 expression is not detectable during the early stages of T cell culture (Figure 20A). However, transfection studies have shown that B7-1 is a potent costimulator of CTL activation (277). To determine if B7-1 can costimulate anti-CD3-induced CTL activation, purified T cells were activated with anti-CD3 mAb in the presence of 3 d-LPS-activated B cells, with or without blocking antibodies to B7-1 and/or B7-2. LPS-activated B cells are known to express high levels of B7-1 (242). To confirm this, B7-1 expression in LPS-activated B cells was analyzed by flow cytometric analysis (Figure 21). In comparison to freshly isolated unactivated, B cells, LPS-activated B cells expressed much higher levels of B7-1 and B7-2 (Figure 21). Following 48 h of culture with anti-CD3 mAb, effector cells were washed and used in a $^{51}$Cr-release assay with P815 target cells. Data are shown in Figure 22. In comparison to T cells activated with anti-CD3 in the presence of LPS-B cells alone, the presence of anti-B7-1 mAb slightly, but significantly, reduced lysis levels, while anti-B7-2 mAb or the combination of anti-B7-1 plus anti-B7-2 mAb drastically inhibited cytotoxicity. These data suggest that B7-1 cannot effectively substitute for B7-2 during CTL costimulation. However, in the presence of B7-2 costimulation B7-1 also contributes in

Figure 21. LPS-activation induces B7-1 expression and increases B7-2 expression on B cells. B cells were purified from splenocytes by plastic adherence and treatment with anti-Thy-1 mAb plus C'. The resulting B cells were activated by culture in the presence of 5 μg/ml LPS for three days. Fresh and 72 h LPS-activated B cells were incubated with rat anti-mouse B7-1 (clone RM80, 1 μg/ml) or with rat anti-mouse B7-2 (clone GL1, 4 μg/ml final concentration), followed by incubation with 1 μg/ml FITC-conjugated mouse anti-rat IgG. Flow cytometric analysis was carried out with a FACSCAN. The x-axis represents fluorescence intensity and the y-axis shows the relative cell number. Percent positive cells are shown in parentheses and mean channel fluorescence is shown in square brackets.

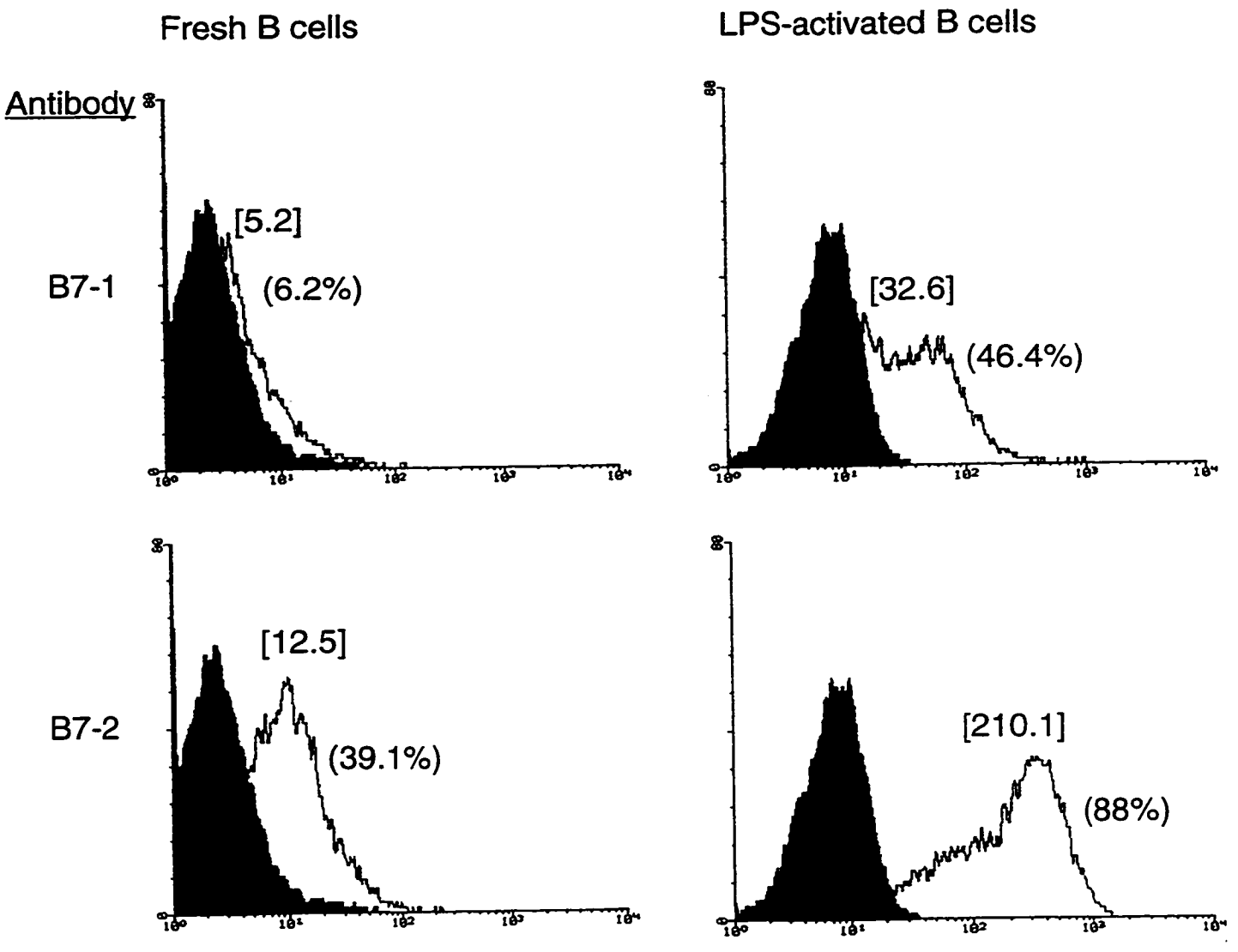

Fresh B cells
LPS-activated B cells
Antibody
B7-1
B7-2
[5.2]
(6.2%)
[32.6]
(46.4%)
[12.5]
(39.1%)
[210.1]
(88%)

Figure 21.

Figure 22. B7-1 can contribute to anti-CD3-activated CTL costimulation, but cannot substitute for B7-2. Purified T cells were stimulated with anti-CD3 mAb and LPS-activated B cells (10% of total cells) in the presence of blocking antibodies to B7-1 (clone 16-10A1) and B7-2 (clone GL1) (both used as hybridoma supernatants, 0.2 μg/ml and 4 μg/ml final concentrations, respectively), either individually, or in combination. Following 48 h of culture, cytolytic activity against P815 target cells at the indicated E:T ratios was determined using $^{51}$Cr-release assay. Results are expressed as mean percent lysis of P815 targets ± the standard deviation of three replicates. (*) denotes a statistically significant difference in killing activity in comparison to the appropriate isotype control, as determined by Student's *t*-test.

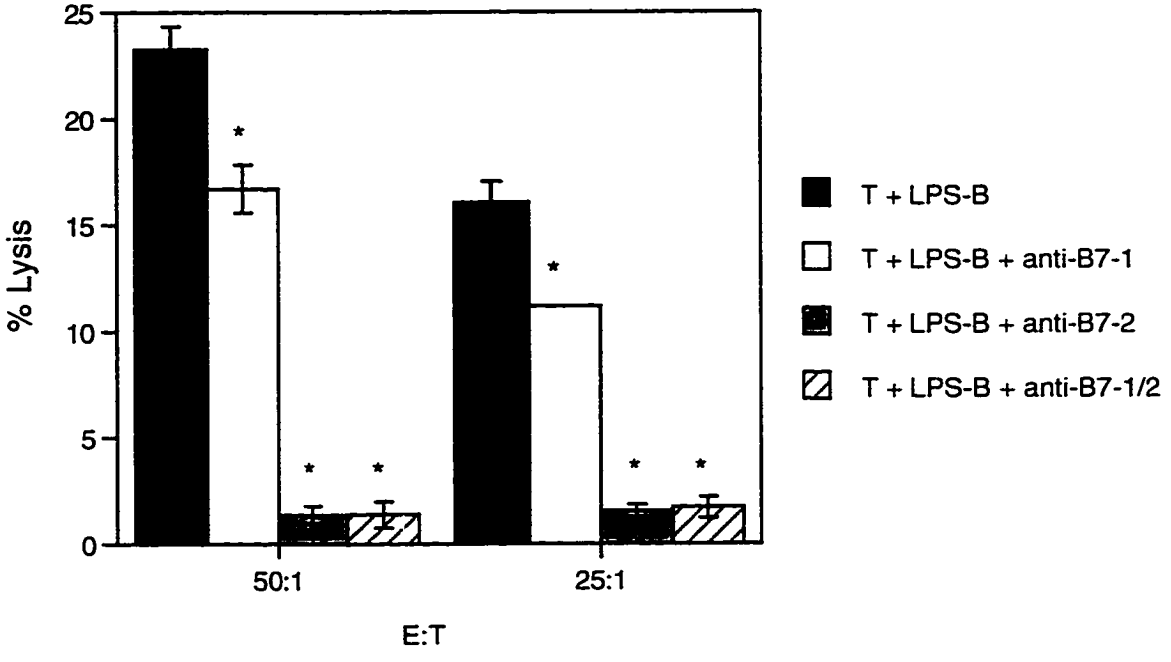

25
20
15
10
5
0
% Lysis
*
*
*
*
*
*
50:1
25:1
E:T
T + LPS-B
T + LPS-B + anti-B7-1
T + LPS-B + anti-B7-2
T + LPS-B + anti-B7-1/2

Figure 22.

minor ways to the development of cytotoxicity.

### 3.20 Highly purified, APC-free T cells can provide their own costimulation for proliferation

$CD8^+$ T cells express B7-2 (Figure 17C) and prior reports have proven that $CD4^+$ T cells also express B7-2 (243). In order to address the possibility that T cells may be able to provide their own costimulation during anti-CD3-activation, an assay was devised where T cells could be activated in the absence of contaminating APC. T cells from normal mice were twice-passaged through nylon wool and then treated with anti-asialoGM1 antibody plus complement to remove NK cells. Since one nylon wool passage removes approximately 90% of the non-T cells in a spleen cell population, it is reasonable to assume good T cell purity following two nylon wool column passages (291). The original article describing the ability of nylon wool to bind non-T cells showed that repeated column passages resulted in much more pure T cell populations (292). These T cells were then added to the wells of 96-well flat bottom plates that had been pre-coated with anti-CD3 mAb. The plastic-immobilization of anti-CD3 mAb mimics the necessary binding of the Fc segment of anti-CD3 mAb to the FcR on APC (293). FcR-mediated binding of anti-CD3 mAb is vital for T cell activation (294). The cultures were incubated for 48 h, pulsed during the last 6 h with [$^3$H]TdR and harvested. Pure T cells activated with immobilized anti-CD3 mAb displayed a significant amount of proliferation (Figure 23). This suggests that the costimulation required for T cell proliferation in this system of activation is supplied by the T cells themselves. Costimulation by contaminating APC was ruled out in a companion

Figure 23. Highly purified, APC-depleted T cells can supply their own costimulatory signal. Twice nylon wool-passaged T cells were seeded at $10^6$ cells/well in a flat-bottom 96 well plate that had been pre-coated with 5 μg/ml anti-CD3 mAb or added to untreated wells containing 5 μg/ml of soluble anti-CD3 mAb. Included in both panels, represented by once nylon wool-passaged T cells, are controls for the effects of APC-mediated anti-CD3 mAb-crosslinking and costimulation. During the last 6 h of the 48 h culture period the wells were pulsed with [$^3$H]TdR, harvested and counted with a liquid scintillation counter. The results are from one experiment and are representative of two independent experiments.

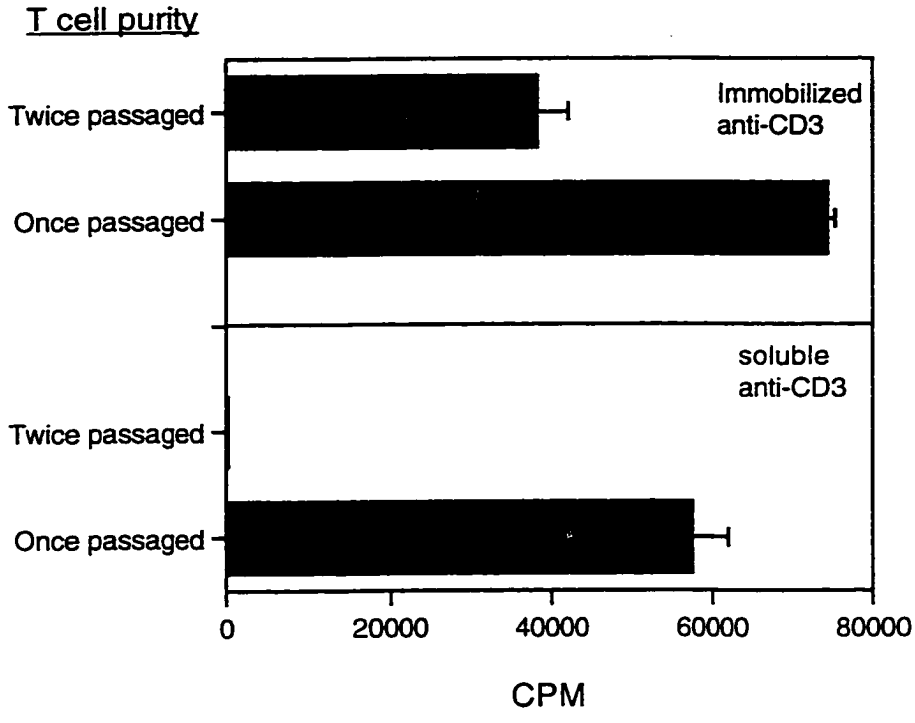

T cell purity
Twice passaged
Once passaged
Immobilized anti-CD3
soluble anti-CD3
Twice passaged
Once passaged
0
20000
40000
60000
80000
CPM

Figure 23.

culture plate where purified T cells were cultured for 48 h with soluble anti-CD3 mAb. As shown in previous experiments, this treatment results in the activation of single nylon wool column-passaged T cells. However, double-passaged T cells did not proliferate in the presence of soluble anti-CD3 mAb (Figure 23). This suggests that there were no $FcR^+$ accessory cells present to cross-link the anti-CD3 mAb, ruling out possible APC contamination can be ruled out. Thus, any costimulation was provided by the T cells themselves.

### 3.21 B7-2 on T cells does not contribute to T-T cell costimulatory interactions

$CD8^+$ T cells isolated from the mouse spleen express B7-2 (Figure 17C) and purified, APC-free T cells proliferate when anti-CD3 mAb is artificially cross-linked via plastic immobilization (Figure 23). If costimulation is required for T cell activation in this system, then B7-2 on neighbouring T cells may be involved in activating proliferation. Blockade of B7-2 was previously shown to inhibit T cell proliferation (Figure 15). To determine if B7-2 on T cells is playing a role in costimulating immobilized anti-CD3-induced T cell proliferation, blocking anti-B7-2 mAb was added to purified, APC-depleted T cells on anti-CD3 mAb-coated plates. As shown in Figure 24, the presence of anti-B7-1 or anti-B7-2 mAb, either individually or in combination, during the 48 h activation period did not affect the proliferative capacity of T cells. The absence of $FcR^+$ APC is shown by the lack of proliferation of T cells cultured in the presence of soluble anti-CD3 mAb. The costimulation provided by B7-2 during anti-CD3-activation of T cells obtained by a single passage through a nylon wool column is therefore most likely due to resting B cells and not

Figure 24. B7-2 on T cells cannot costimulate other T cells to proliferate. Twice nylon wool-passaged T cells were seeded at 600,000 cells per well in a 96 well flat bottom plate precoated with anti-CD3 mAb. Cells were cultured for 48 h in the presence or absence of anti-B7-1 mAb (clone 16-10A1, 0.2 μg/ml final concentration) and/or anti-B7-2 mAb (clone GL1, 4 μg/ml final concentration). A control well for detection of APC contamination contained only soluble anti-CD3 mAb. During the last 6 h cultures were pulsed with [$^3$H]TdR, harvested, and counted with a liquid scintillation counter. The results are from one experiment and are representative of two independent experiments.

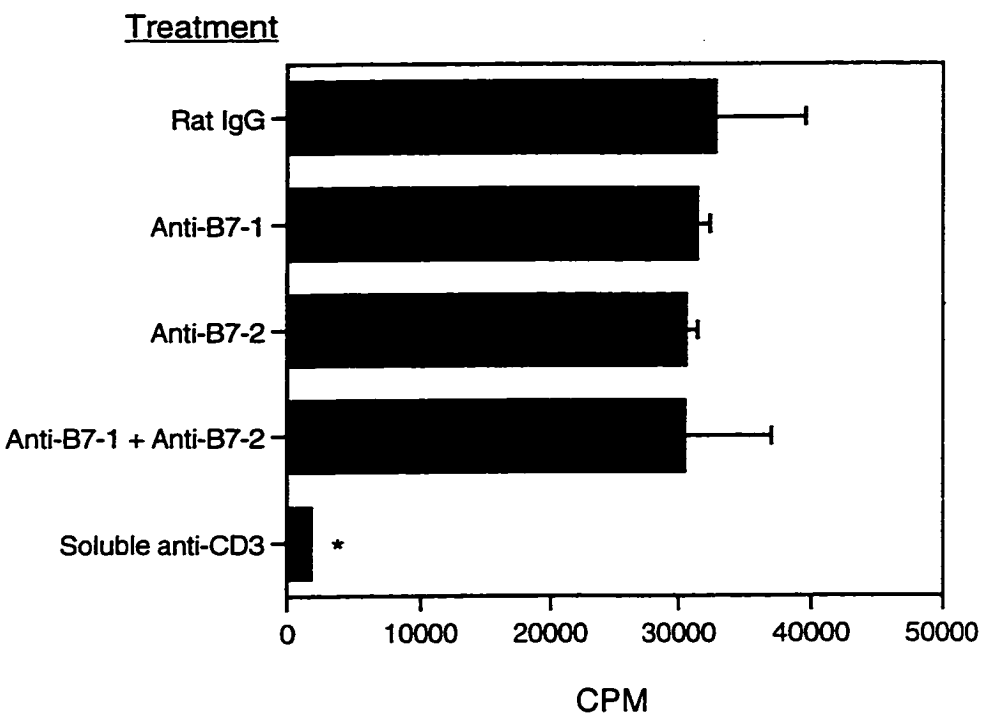

Treatment
Rat IgG
Anti-B7-1
Anti-B7-2
Anti-B7-1 + Anti-B7-2
Soluble anti-CD3
*
0
10000
20000
30000
40000
50000
CPM

Figure 24.

T cells.

### 3.22 Blockade of B7-2/CD28 interactions during activation by anti-CD3 mAb suppresses the production of various cytokines

B7-2 costimulation is known to result in higher production of IL-2 (252). IL-2 is required for anti-CD3-mediated activation of T cells (288). In addition to IL-2, IL-6 (291), IFN-γ (295), and TNF-α (296) have also been shown to contribute to anti-CD3 activation of T cells. To determine the effect of B7-2 blockade on the production of other cytokines needed for CTL development following anti-CD3-activation, T cells were activated with anti-CD3 mAb for 48 h in the presence of control immunoglobulin or anti-B7-2 mAb. At the end of culture T cells were separated from the culture medium by centrifugation and the culture supernatant was collected. Cytokine levels in the supernatants were determined by cytokine-specific sandwich ELISA assays. As shown in Table 2, the most abundant cytokines are IFN-γ and IL-6, while IL-2, IL-4, IL-10, and TNF-α were less prevalent. Cultures treated with anti-B7-2 mAb had decreased levels of all cytokines except TNF-α. The most severely affected were IL-6 and IFN-γ.

### 3.23 Exogenous IL-2 or IL-12 can substitute for B7-2 costimulation for CTL induction

Sandwich ELISA analysis showed that supernatants from control anti-CD3-activated T cell cultures had higher levels of IFN-γ, IL-2, IL-6, IL-4, and IL-10 than anti-B7-2-treated cultures (Table 2). To determine whether the addition of exogenous cytokines

TABLE 2

The effect of anti-B7-2-treatment on cytokine production in anti-CD3-activated T cell cultures.

| Treatment | Concentration (pg/ml) | | | | | |
|---|---|---|---|---|---|---|
| | IL-2 | IL-4 | IL-6 | IL-10 | TNF-α | IFN-γ |
| Rat IgG | 24 ± 1 | 7 ± 1 | 743 ± 130 | 48 ± 1 | 32 ± 1 | 1400 ± 280 |
| Anti-B7-2 | 11 ± 1 | 3 ± 1 | 186 ± 4 | 35 ± 1 | 38 ± 2 | 22 ± 2 |

Purified T cells were activated by anti-CD3 mAb in the presence of 5 μg/ml rat IgG or anti-B7-2 mAb (clone GL1, used as hybridoma supernatant, 1/10 final dilution). Following 48 h of culture, cell cultures were centrifuged and the supernatants used in various cytokine-specific sandwich ELISA assays. These data are from one experiment and are representative of three separate experiments.

might substitute for B7-2 costimulation, a panel of cytokines was individually added to T cell cultures at initiation of culture with or without anti-B7-2 mAb. Cytotoxicity was assayed at 48 h. In comparison to cytotoxicity elicited from control cultures, anti-B7-2-inhibition was overcome only by IL-2 or IL-12 (Figure 25). In contrast, IFN-γ, IL-6, and TNF-α did not restore cytotoxicity in anti-B7-2-treated cultures. This suggests that IL-2R and IL-12R provide a signal similar to CD28 for CTL activation, which is different from signals induced by IFN-γ, IL-6 and TNF-α binding to their respective receptors.

### 3.24 IL-12 substitutes for B7-2 costimulation in an IFN-γ-independent manner

Because IL-2 production is known to be a result of CD28 binding B7-2 (252), the successful restoration of CTL activation by the addition of exogenous IL-2 during B7-2 blockade was not surprising. IL-12 is also known to be important for CTL and NK cell activation (106,145). However, the mechanism by which IL-12 might be acting to substitute for B7-2/CD28 interactions was not clear. One possibility was that IL-12 was directly substituting for CD28-ligation by inducing the production of a cytokine. IL-12 is known to induce IFN-γ production in NK cells (148), and IFN-γ is known to be important in during anti-CD3-mediated activation of CTL (291). IL-12 might therefore substitute for CD28-signaling by enhanced IFN-γ production. To investigate this possibility, neutralizing antibodies to IFN-γ and blocking antibodies to IFN-γR were added with anti-B7-2 mAb plus IL-12 to anti-CD3-treated T cell cultures and incubated for 48 h. Following culture, CTL were used in $^{51}$Cr-release assay against P815 target cells. As shown in Figure 26, CTL

Figure 25. IL-2 and IL-12, but not IFN-γ, IL-6, or TNF-α can substitute for B7-2 costimulation during anti-CD3-activation of CTL. T cells were stimulated with anti-CD3 mAb in the presence of 5 μg/ml rat IgG or anti-B7-2 mAb (clone GL1 hybridoma supernatant, 4 μg/ml final concentration) with or without 100 U/ml IL-2, 100 U/ml IFN-γ, 20 ng/ml IL-6, 100 U/ml TNF-α, or 10 ng/ml IL-12. Following 48 h of culture, cytolytic activity against P815 target cells at an E:T of 50:1 was determined using $^{51}$Cr-release assay. Results are expressed as mean percentage of the cytotoxicity of the isotype control, as determined from at least three independent experiments for each cytokine addition.

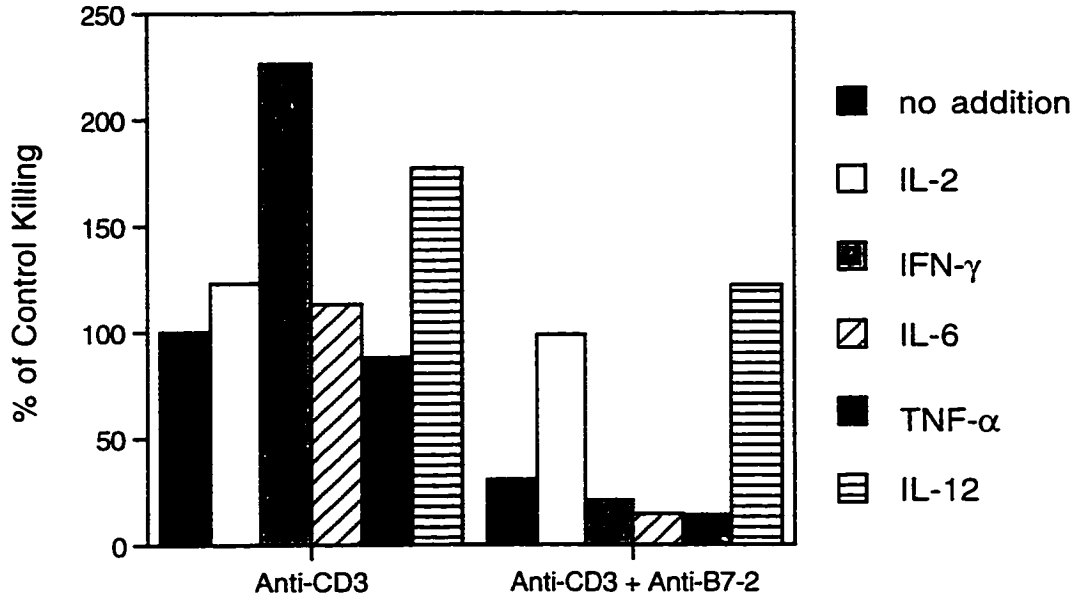

250
200
150
100
50
0
% of Control Killing
Anti-CD3
Anti-CD3 + Anti-B7-2
no addition
IL-2
IFN-γ
IL-6
TNF-α
IL-12

Figure 25.

Figure 26. IL-12 substitutes for B7-2 costimulation through an IFN-γ-independent mechanism. Purified T cells were stimulated with anti-CD3 mAb in the presence of blocking antibodies to B7-2 (GL1 hybridoma supernatant, 4 μg/ml final concentration), 10 μg/ml whole rat IgG, either individually or in combination with 10 ng/ml IL-12, or 10 μg/ml anti-IFN-γ mAb and 10 μg/ml anti-IFN-γR mAb. Following 48 h of culture, cytolytic activity against P815 target cells at an E:T of 50:1 was determined using $^{51}$Cr-release assay. Results are expressed as mean percent lysis of P815 targets ± the standard deviation of three replicates. (*) denotes a statistically significant difference in killing activity in comparison to the control, as determined by Student's *t*-test.

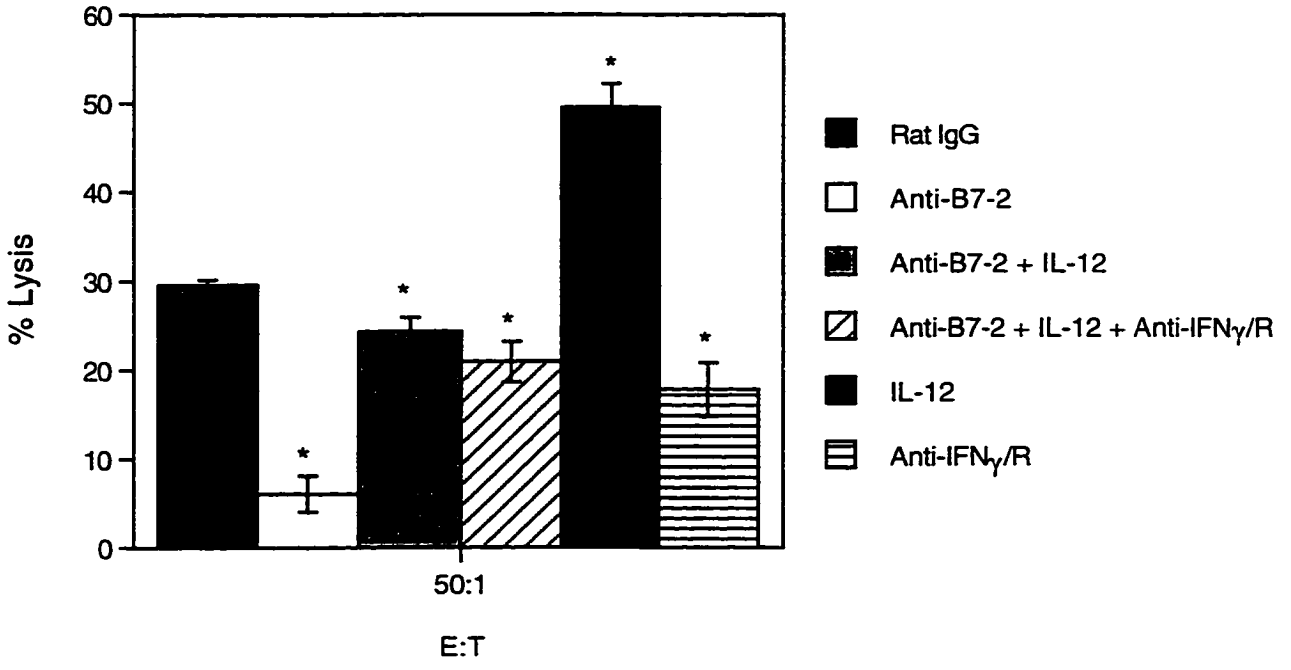

60
50
40
30
20
10
0
% Lysis
50:1
E:T
Rat IgG
Anti-B7-2
Anti-B7-2 + IL-12
Anti-B7-2 + IL-12 + Anti-IFNγ/R
IL-12
Anti-IFNγ/R

Figure 26.

activation in cultures containing IL-12 plus anti-B7-2 was only slightly inhibited by antibodies to IFN-γ and IFN-γR. This suggests that IL-12 is not acting primarily through inducing IFN-γ production to restore CTL activation. This conclusion is supported by the previous cytokine substitution experiment where the addition of exogenous IFN-γ could not restore cytotoxicity in anti-B7-2-treated CTL (Figure 25).

### 3.25 IL-12 substitutes for B7-2 costimulation in an IL-2-dependent manner

One possible explanation for the ability of IL-12 to restore cytotoxicity in anti-B7-2-treated CTL was that IL-12 induced IL-2 secretion. In order to test this hypothesis, T cells were activated with anti-CD3 mAb in the presence of anti-B7-2 mAb and IL-12, with or without neutralizing antibodies to IL-2 and blocking antibodies to the IL-2R. As shown previously, anti-B7-2 mAb inhibited CTL activation and exogenous IL-12 by itself or in combination with anti-B7-2 mAb restored lysis levels above control values (Figure 27). In addition, neutralization of IL-2 and blockade of IL-2R totally inhibited the ability of IL-12 to restore cytotoxicity in anti-B7-2-treated cultures. This suggests that IL-12 is inducing expression of IL-2 in the absence of CD28 stimulation.

### 3.26 Treatment with anti-B7-2 mAb decreases Gzm B gene transcription

The reason for the deficiency in cytotoxicity exhibited by anti-B7-2-treated CTL is not known. However, IL-2 is known to upregulate Gzm B, perforin and FasL expression in CTL (83,106,107) and anti-B7-2-treated T cell cultures have reduced levels of IL-2 (Table 2). Moreover, exogenous IL-2 or IL-12-induced IL-2 production can restore cytotoxicity in

Figure 27. IL-12 substitutes for B7-2 costimulation through an IL-2-dependent mechanism. Purified T cells were stimulated with anti-CD3 mAb in the presence of blocking antibodies to B7-2 (GL1 hybridoma supernatant, 4 μg/ml final concentration), 10 μg/ml whole rat IgG, either individually or in combination with 10 ng/ml IL-12, or 10 μg/ml anti-IL-2 mAb and 10 μg/ml anti-IL-2R mAb. Following 48 h of culture, cytolytic activity against P815 target cells at an E:T of 50:1 was determined using $^{51}$Cr-release assay. Results are expressed as mean percent lysis of P815 targets ± the standard deviation of three replicates. The results are from one experiment and are representative of at least three separate experiments. (*) denotes a statistically significant difference in killing activity in comparison to the control, as determined by Student's *t*-test.

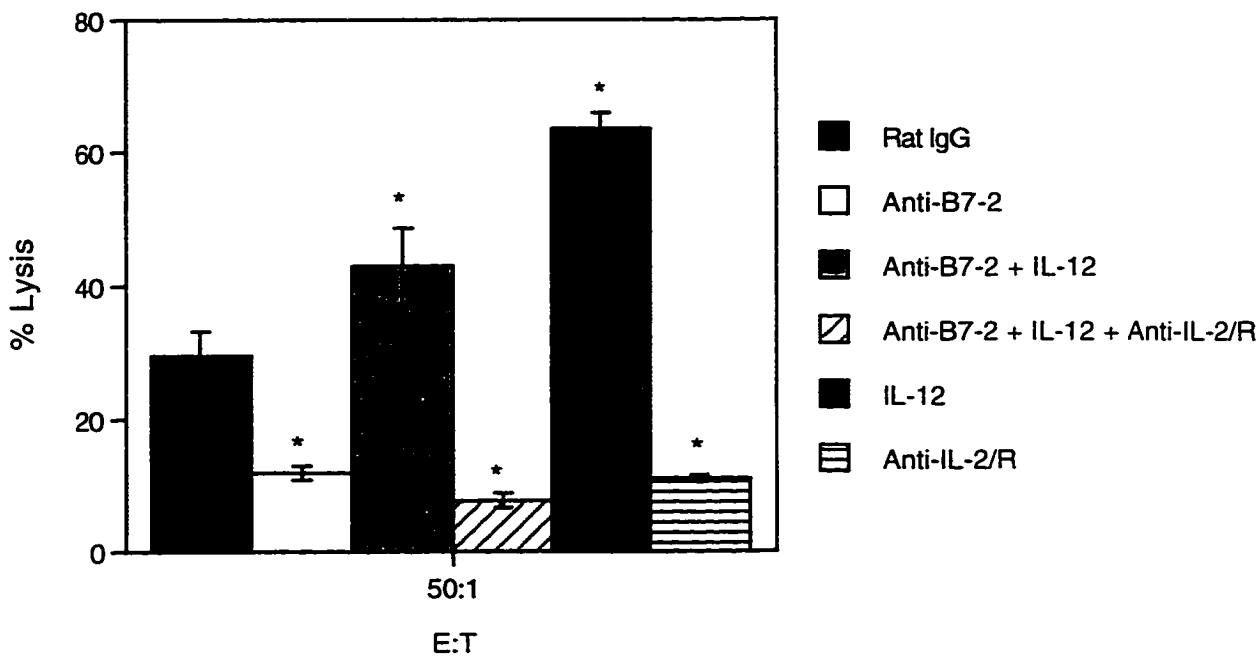

80
60
40
20
0
% Lysis
*
*
*
*
*
50:1
E:T
Rat IgG
Anti-B7-2
Anti-B7-2 + IL-12
Anti-B7-2 + IL-12 + Anti-IL-2/R
IL-12
Anti-IL-2/R

Figure 27.

B7-2-deprived T cell cultures (Figure 25). Taken together, these findings suggest that the lack of IL-2 in anti-B7-2-treated cultures results in decreased levels of cytolytic protein and, therefore, cytotoxicity. In order to test this idea, T cells were activated with anti-CD3 mAb in the presence of control immunoglobulin or anti-B7-2 mAb for 48 h. Total RNA was then harvested and subjected to RT-PCR analysis with exon-specific, intron-bridging primers for Gzm B, perforin and FasL. In comparison to CTL induced in the presence of isotypic control antibody, anti-B7-2-treated CTL showed a 50% decrease in Gzm B mRNA levels (Figure 28A). Equal levels of GAPDH show that the difference is not a result of unequal cDNA content in the PCR reaction. In contrast, perforin mRNA levels at 48 h were slightly increased (Figure 28A). However, this increase was not always reproducible. FasL mRNA levels at 4 h were unaffected by anti-B7-2-treatment (Figure 28B).

The decrease in Gzm B mRNA levels in anti-B7-2-treated CTL could be due to a variety of factors. In order to determine whether decreased mRNA stability is the cause of lower Gzm B levels in anti-B7-2-treated CTL, half-life studies with ActD were carried out. T cells were activated with anti-CD3 mAb in the presence of a control immunoglobulin or anti-B7-2 mAb for 48 h. At this time ActD was added. Total RNA was harvested at different times following ActD addition and subjected to RT-PCR analysis with Gzm B or IL-2 specific primers. With either treatment, Gzm B mRNA levels declined at a similar rate over time (Figure 29). The degradation rate of IL-2 mRNA in rat IgG-treated T cells was included as a positive control. As expected, IL-2 transcripts were found to degrade more rapidly than Gzm B mRNA. These data suggest that the decrease in Gzm B mRNA levels in anti-B7-2-treated cultures is due to a slower rate of transcription.

Figure 28. Blockade of B7-2 interactions results in decreased Gzm B but not perforin or FasL mRNA levels in anti-CD3-activated CTL. Purified T cells were incubated with anti-CD3 mAb in the presence of 10 μg/ml rat IgG or anti-B7-2 mAb (clone GL1, 4 μg/ml final concentration). Total cellular RNA was isolated following 4 and 48 h of culture. Single-stranded cDNA was reverse-transcribed from 0.5 μg of RNA with random hexamers as described in the Materials and Methods. The resulting cDNA template was used in a PCR reaction with exon-binding, intron-bridging primers specific for (A) GzmB, perforin, and (B) FasL. GAPDH mRNA levels were also determined by RT-PCR at 4 and 48 h of culture. Amplicons were resolved by gel electrophoresis and ethidium bromide staining. scanned, image inverted, and densitometric analysis was performed to quantitate Gzm B, perforin, and FasL expression relative to steady state expression of GAPDH. Data are from one experiment and are representative of three independent experiments.

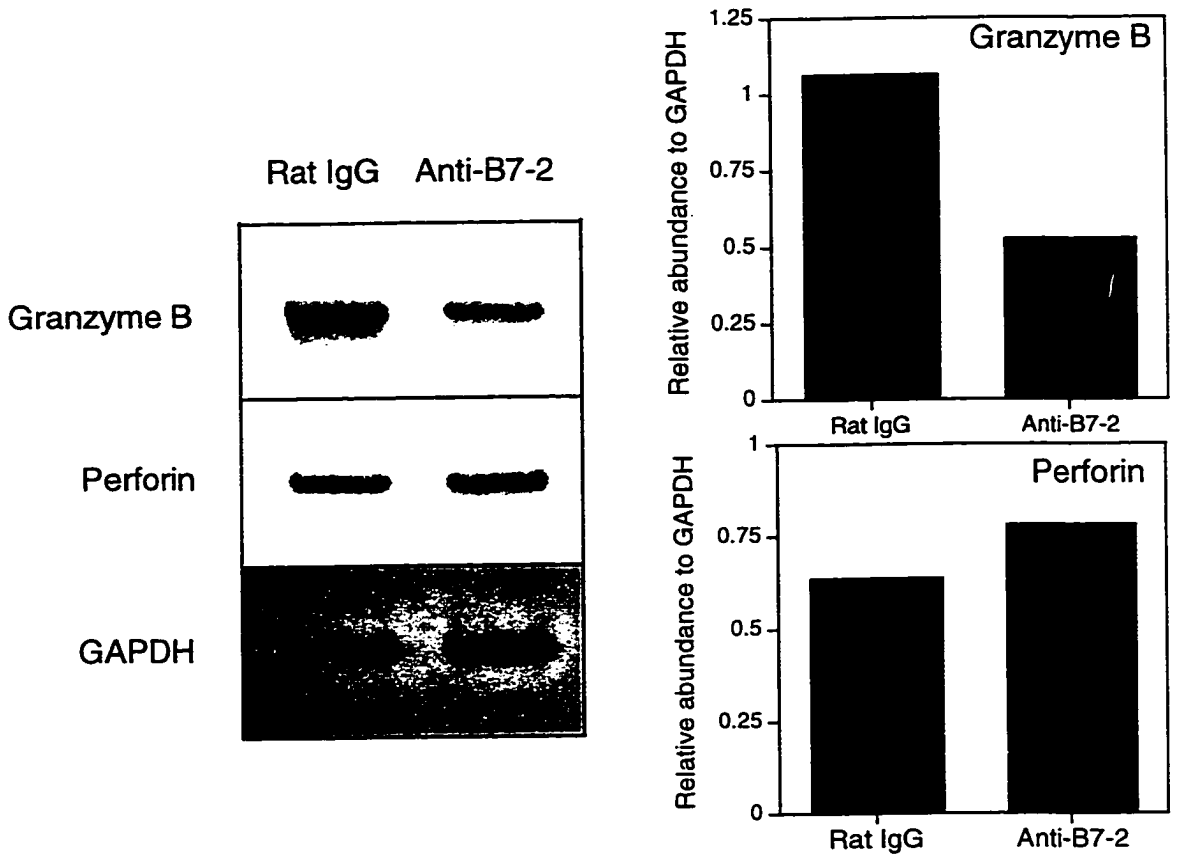

Rat IgG
Anti-B7-2
Granzyme B
Perforin
GAPDH
Granzyme B
Relative abundance to GAPDH
1.25
1
0.75
0.5
0.25
0
Rat IgG
Anti-B7-2
Perforin
Relative abundance to GAPDH
1
0.75
0.5
0.25
0
Rat IgG
Anti-B7-2

Figure 28A.

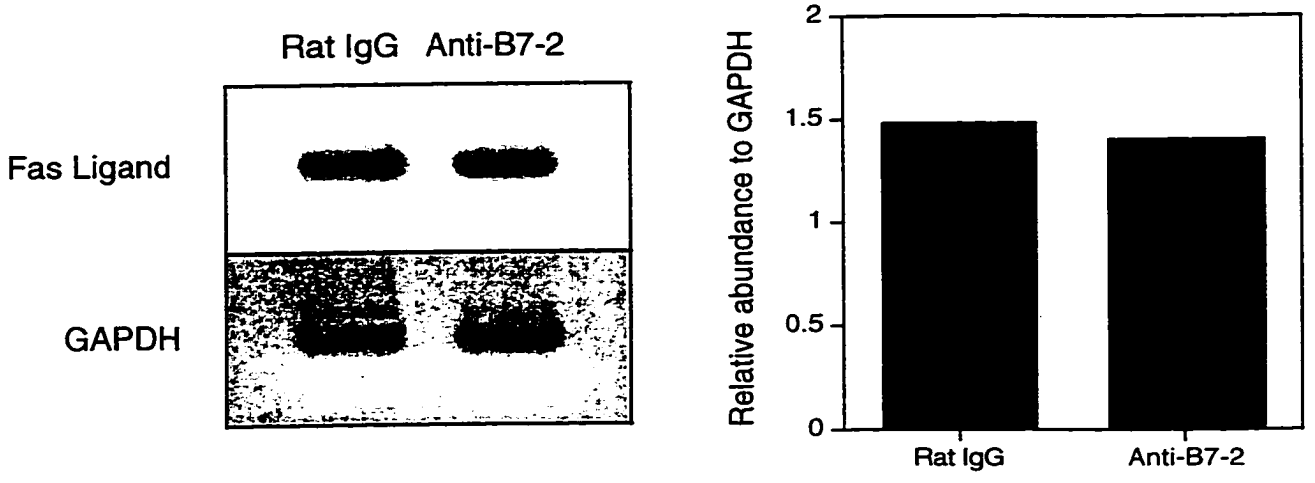

Rat IgG
Anti-B7-2
Fas Ligand
GAPDH
Relative abundance to GAPDH
2
1.5
1
0.5
0
Rat IgG
Anti-B7-2

Figure 28B.

Figure 29. Gzm B mRNA from control and anti-B7-2-treated CTL decay at similar rates. T cells were incubated with anti-CD3 mAb alone or with anti-B7-2 mAb (clone GL1 hybridoma supernatant, 4 μg/ml final concentration). Following 48 h of culture, 5 μg/ml of actinomycin D were added to replicate cultures (0 h) and total cellular RNA was isolated from these cultures at the indicated timepoints. Single-stranded cDNA was reverse-transcribed from 0.5 μg of RNA with random hexamers as described in the Materials and Methods. The resulting cDNA template was used in a PCR reaction with exon-binding, intron-bridging primers specific for Gzm B and IL-2. PCR amplicons were resolved by gel electrophoresis and ethidium bromide staining, and quantified by densitometric analysis. Results are expressed as the densitometric abundance relative to 0 h actinomycin D-treated T cell cultures. Data are from one experiment and are representative of at least three independent experiments.

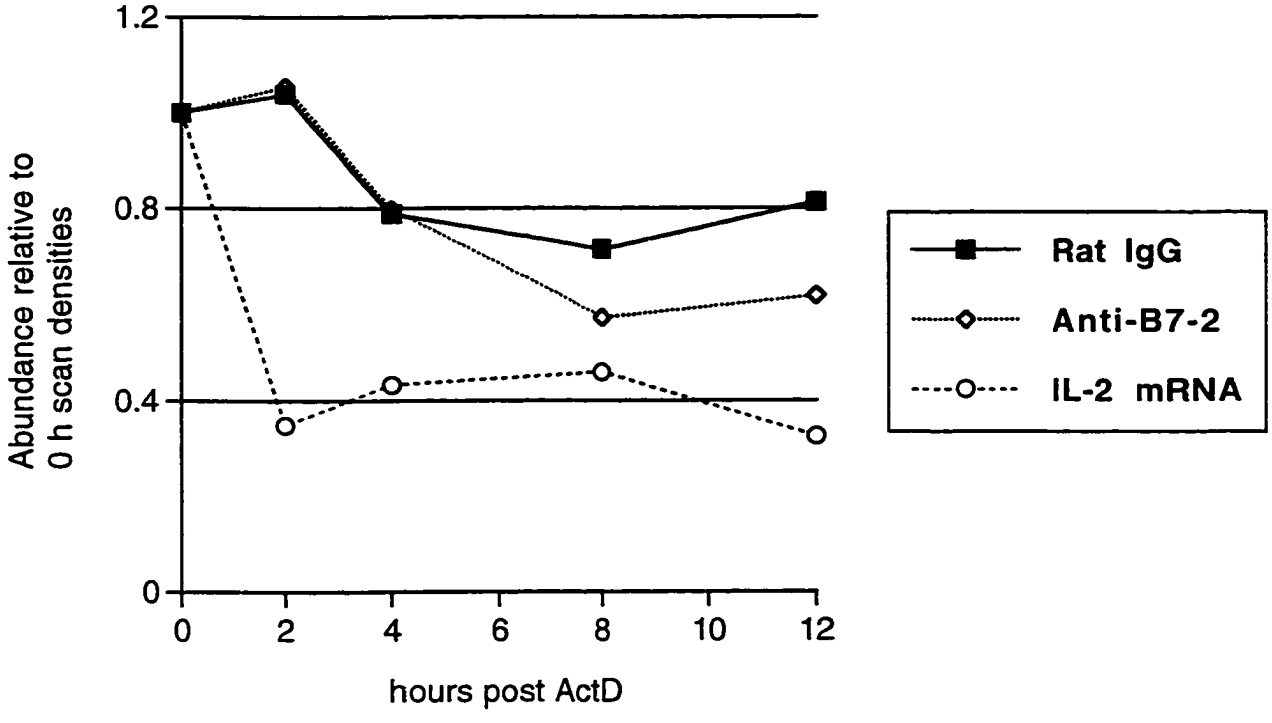

1.2
0.8
0.4
0
Abundance relative to
0 h scan densities
0
2
4
6
8
10
12
hours post ActD
Rat IgG
Anti-B7-2
IL-2 mRNA

Figure 29.

### 3.27 IL-12 restores Gzm B mRNA levels in anti-B7-2-treated CTL

Addition of exogenous IL-12 restored cytotoxicity in anti-B7-2-treated CTL (Figure 25). The reason for this restoration of cytotoxicity is not immediately clear. There have been reports that IL-12 in combination with IL-2 can increase the transcription of Gzm B and perforin genes in NK cells (297). Therefore, to determine if IL-12 was reversing the inhibitory effect of B7-2 blockade on Gzm B mRNA expression, T cells were activated with anti-CD3 mAb in the presence of a control immunoglobulin, anti-B7-2, IL-12, or the combination of anti-B7-2 and IL-12. After 48 h of culture total RNA was isolated and subjected to one-step RT-PCR analysis with Gzm B-specific primers. The results of this experiment are shown in Figure 30. Anti-B7-2-treatment totally abrogated Gzm B expression in comparison to control CTL. Addition of exogenous IL-12 increased expression of Gzm B above control levels. Treatment with anti-B7-2 mAb in combination with IL-12 restored Gzm B mRNA to control levels (Figure 30). As with Northern blot analysis, total RNA is shown as a loading control. These data suggest that IL-12 is able to reverse the inhibitory effect of B7-2 blockade on CTL induction by upregulating Gzm B gene expression.

Figure 30. IL-12 restores Gzm B mRNA expression in anti-B7-treated CTL. Purified T cells were incubated with anti-CD3 mAb in the presence of 10 μg/ml rat IgG, anti-B7-2 mAb (clone GL1, 4 μg/ml final concentration), 10 ng/ml IL-12, or 4 μg/ml anti-B7-2 mAb plus 10 ng/ml IL-12. Total cellular RNA was isolated following 48 h of culture. Using one-step RT-PCR beads as described in the Methods and Materials, single-stranded cDNA was reverse-transcribed from 0.5 μg of RNA with random hexamers followed by PCR reaction with exon-binding, intron-bridging primers specific for GzmB. Amplicons were resolved by gel electrophoresis and ethidium bromide staining, scanned and the image inverted. Equal RNA template loading is shown by electrophoresis of the same volume of RNA used in the one-step RT-PCR procedure. Data are from one experiment and are representative of two independent experiments.

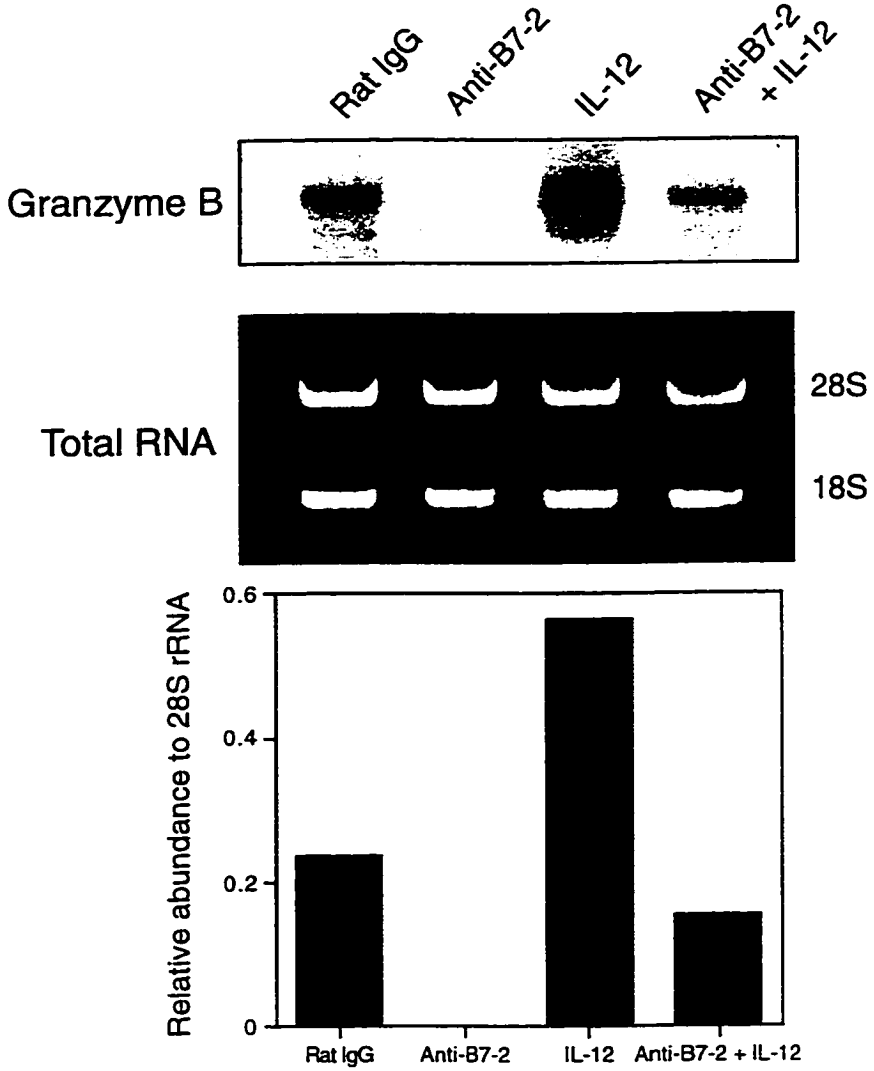

Rat IgG
Anti-B7-2
IL-12
Anti-B7-2 + IL-12
Granzyme B
Total RNA
28S
18S
Relative abundance to 28S rRNA
0.6
0.4
0.2
0
Rat IgG
Anti-B7-2
IL-12
Anti-B7-2 + IL-12

Figure 30.

## 4.0 Discussion

The immunosuppressive effect which RAP displays in models of allograft transplantation agrees with the early initial reports that RAP inhibits T cell proliferation in response to mitogenic stimuli. However, apart from a report showing that CTL activity is diminished in transplant recipients receiving RAP, there have been no detailed studies of the effects of RAP on CTL activation. Furthermore, the effect of RAP on cytolytic molecule expression is unknown, although one report has shown that Gzm B mRNA levels in allografts of animals receiving RAP are decreased compared to allografts from untreated animals (206). *In vitro* activation of murine CTL by incubation with anti-CD3 mAb provides a convenient system to study the effects of a particular agent during the differentiation of CTL. However, polyclonal activation of CTL normally having a wide range of antigen specificities has both advantages and disadvantages. Allospecific CTL will normally be restricted to a small range of antigens and so the MHC-unrestricted cytotoxicity displayed by anti-CD3-activated CTL is not a precise model of *in vivo* target cell recognition. On the other hand, the simplicity of this activation system allows for easier dissection of the signaling pathways inhibited by RAP. Moreover, the variety of cytokines, costimulatory ligand/receptor interactions, and APC which allow CTL to become activated would complicate efforts to delineate the specific effects of RAP in an *in vivo* model system.

In order to determine the effect of RAP on CTL gene expression, a working concentration of the drug needed to be determined. It was found that at concentrations of 10 ng/ml and higher, RAP was toxic to T cells, as assessed by trypan blue dye exclusion

and total cell recovery. Thus, 1 ng/ml was chosen as the best concentration to study the inhibitory effects of RAP on live cells. The toxicity of RAP seen at concentrations above 1 ng/ml could be the result of many factors. RAP is known to suppress the production of new ribosomes by specifically inhibiting the translation of those mRNAs and rRNAs coding for ribosomal subunits and elongation/translation factors (169). At saturating concentrations of RAP (10 ng/ml), all ribosome production may be shutdown. No new proteins are produced which are required for constant cellular maintenance, even at the resting stage, and thus, the cell dies. When used at 1 ng/ml, RAP may not affect ribosome numbers to such a great extent, allowing T cells to maintain housekeeping gene expression.

RT-PCR was adopted as the assay of choice to evaluate mRNA levels for Gzm B, perforin and FasL. While Gzm B was readily detected in Northern blots of total RNA (Figure 7), similar attempts to detect perforin and FasL mRNA met with failure. This was most likely due to the use of a perforin probe from a disparate species (rat) and the fact that both perforin and FasL mRNA are much less abundant than Gzm B mRNA. In fact, Gzm B mRNA is almost as prevalent as β-actin and GAPDH in activated CTL. In order to increase the sensitivity of mRNA detection, oligonucleotide primers were designed for the various cytolytic genes and used in semi-quantitative RT-PCR analysis. While Northern blotting is unquestionably a more reliable method of quantitating mRNA levels, RT-PCR can also be used to compare relative levels of specific mRNAs if the proper controls are performed (298). These include determination of non-saturating thermocycling number and a secondary RT-PCR for a gene whose expression is more constant than the one in question. In order to validate RT-PCR as a reliable measurement of mRNA levels, I decided to look

at the expression of IL-2 mRNA during T cell activation by anti-CD3 mAb. Small amounts of IL-2 mRNA could be detected as little as 2 h-post anti-CD3 addition and by 4 h there was a sharp increase in IL-2 mRNA levels (Figure 5D). This high level of IL-2 mRNA expression was maintained throughout the 48 h period of culture. This data agrees with past studies where IL-2 mRNA timecourse expression was assayed in murine T cells by Northern blot analysis after stimulation by anti-CD3 mAb (22). An almost exact match for the profile of IL-2 mRNA timecourse expression is seen in TCR-transgenic T cells responding to specific antigen (240). Thus, I am confident that the IL-2 timecourse mRNA profile is a trustworthy control for the accuracy of semi-quantitative RT-PCR.

If the number of cycles used during PCR in order to generate a visible amplicon is an indication of the amount of mRNA present, then at 48 h in order of decreasing mRNA quantity: β-actin = GAPDH > Gzm B > perforin > FasL. Initial RT-PCR time-course experiments showed that Gzm B expression was almost undetectable early during anti-CD3-activation, but started to appear by 16 h, and became increasingly greater at 24 and 48 h (Figure 5A). This is in agreement with prior reports that Gzm B mRNA increases during the first 48 h of CTL activation and peaks before 72 h of activation with anti-CD3 mAb (288). Interestingly, the peak of Gzm B gene expression coincides with the peak of MHC-unrestricted cytotoxicity induced by anti-CD3 mAb (19). This suggests that Gzm B expression is a marker of CTL activation.

In contrast, perforin mRNA was present at approximately equal levels throughout the 48 h activation period (Figure 5B). Perforin mRNA was also found in unstimulated T cells at levels comparable to anti-CD3-activated T cells (Figure 4), implying constitutive

expression of the gene. Initially, this was thought to be an artefact due to genomic DNA amplification because the perforin primers did not bridge an intron. Therefore, I designed new primers which spanned the second perforin gene intron. When used for RT-PCR analysis, perforin mRNA was again found to be constitutively expressed by T cells. These results are supported by a previous study showing that low levels of perforin mRNA and mRNA from a reporter gene under the control of the perforin promoter were found in resting T cells and thymocytes (35). This may suggest that control of perforin expression is regulated at the level of translation, as well as transcription. Interestingly, a subpopulation of $CD8^+$ T cells (20% of $Ly2^+$ splenocytes of 5 week old C57BL/6 mice) in naive mouse spleens contain perforin protein (299). This indicates that the perforin mRNA detected in freshly isolated T cells may be from pre-activated CTL present in the spleens of these mice. Since the mice used in my experiment were not kept under germ-free conditions, it is also possible that they may by expressing perforin mRNA in response to infection by a pathogen. However, if this was the case then Gzm B mRNA levels would also be high in these cells. Since Gzm B mRNA is almost undetectable in resting T cells (Figure 4), it is unlikely that the mice used in my studies were infected. Furthermore, activated CTL express asialoGM1 and so would have been depleted during complement incubation after anti-asialoGM1 treatment (299a). It is also possible that the perforin mRNA detected in fresh splenocyte cultures may be from non-T cells, perhaps from macrophages which have been shown to produce perforin (34), although this is unlikely considering that macrophages are a very minor component following the isolation procedure. NK cells, which are another potential source of perforin mRNA (31), are not present after the

isolation procedure, as shown by the inability of anti-asialoGM1 plus complement-treated spleen cells to lyse YAC tumor targets (300). Whatever the source of perforin mRNA in these cells, it is clear that regulation of perforin expression is different from that of Gzm B.

FasL mRNA, like perforin, was also found throughout the 48 h culture period at relatively equal levels. However, FasL expression was slightly increased between 4 and 8 h of activation and dropped off at 24 h (Figure 5C). By 48 h FasL mRNA levels had again increased. The initial 4 h 'spike' can be attributed to rapid transcription of the FasL gene caused by direct TCR/CD3-signal transduction (83). The rise of FasL expression near 48 h of culture is most likely due to the effect of the increasing levels of cytokines in the activated cultures since high concentrations of IL-2 have both been shown to increase FasL mRNA levels in mouse T cells (83). FasL-mediated killing is sensitive to protein synthesis inhibitors, suggesting that FasL mRNA must be rapidly translated and expressed on the surface of killer cells in order to be effective (301). This, along with the rapid upregulation of FasL mRNA levels upon TCR/CD3 stimulation, suggests that while FasL mRNA may be constitutively expressed, FasL surface protein is only found on recently activated T cells. The intracellular pool of FasL mRNA in resting T cells may allow for rapid protein production. This suggests that, like perforin, FasL expression may be controlled at the level of translation. However, definite proof for this theory needs to be provided by a time-course analysis of FasL surface protein levels by flow cytometry.

The previously demonstrated abilities of RAP to inhibit the proliferation of mitogen-activated T lymphocytes (167), and to reduce expression of Gzm B transcripts in rejecting allografts (206) have been correlated in this study with a direct inhibitory effect of

RAP on anti-CD3-induced activation of murine CTL. In addition to dramatically reducing cytotoxic and proliferative capacities of anti-CD3-activated T cells (Figures 1 and 3), RAP inhibits the accumulation of Gzm B (Figure 6A) and perforin (Figure 6B) mRNA and protein (Figures 9 and 10). While Gzm B, perforin and FasL were found to have relatively long half-lives (Figure 8D), RAP-treatment did not affect the stability of Gzm B, perforin and FasL mRNA transcripts (Figure 8). The stability of IL-2Rα or GAPDH mRNA is also not altered by RAP (302). In contrast, RAP enhances the degradation of both IL-2 and granulocyte-macrophage-colony stimulating factor (GM-CSF) mRNA (302). The half-life of β-actin mRNA, like that of GAPDH mRNA, was not affected by RAP-treatment (data not shown). My results suggest that RAP is mediating its effect on CTL gene expression at the transcriptional level. Due to the well documented blocking effects of RAP on IL-2R signal transduction mediated through $p70^{s6k}$, it is likely that this signal transduction pathway is responsible for initiating proliferation, cytotoxicity, and lytic molecule expression in CTL.

However, other signal transduction pathways also emanate from the IL-2R. These include the mitogen-activated protein kinase (MAPK) pathway and the JAK/STAT pathways (129,303). It is known that these pathways contribute to various aspects of CTL activation. For example, MAPK activation eventually leads to transcription factor AP-1 activation (205). This DNA-binding protein is known to be involved in cell cycle progression (205). The sequence to which it binds is found in the 5' regulatory region of many genes, including the Gzm B, perforin, and IL-2 genes (304-306). Activation of STAT binding proteins is also critical for T lymphocyte development, as shown with JAK3

knockout mice which have drastically decreased numbers of T cells (135). Stat5 has been shown to bind to the enhancer region of the human IL-2Rα gene (134). CTL activation may, therefore, be a net effect of several redundant signaling pathways stemming from the IL-2R. If this is true, it should be possible to reproduce a similar level of CTL activation if one of these pathways is blocked and the others are overstimulated.

The RAP-sensitive $p70^{s6k}$ pathway is known modulate the activation of a subfamily of the CREB DNA binding factors (128). For example, CREB binding activity induced by IL-2 is inhibited by RAP (194). Interestingly, both murine Gzm B and perforin genes contain CRE and CRE-like binding motifs in their 5'-flanking sequence (305,307), suggesting a possible mechanism by which RAP could reduce Gzm B and perforin gene transcription. This is in line with the finding that mutations in the CRE-binding motif in the human Gzm B gene upstream region result in reduced promoter activity (304). However, a recent report indicates that CREB proteins are also activated via the MAPK signaling pathway (301). Since activation of MAPK is not affected by RAP (204), IL-2-induced activation of CRE binding proteins by MAPK may account for residual Gzm B and perforin mRNA expression by CTL induced in the presence of RAP. Overstimulated MAPK-signaling also probably contributes to the enhanced Gzm B and perforin expression in RAP plus rIL-2-treated T cells.

To test the hypothesis that overstimulation of the IL-2R would allow RAP-resistant signaling pathways to substitute for RAP-sensitive pathways, a large quantity of exogenous rIL-2 (100 U/ml) was added with RAP at the beginning of T cell culture in the presence of anti-CD3 mAb. Initial results were very interesting. Although RAP plus rIL-2-induced

cytotoxicity of T cells was still far below control levels (Figure 2), proliferation of RAP plus rIL-2-treated T cells was restored to control levels (Figure 3). This is in line with recent evidence that RAP-resistant signaling pathways linked to the IL-2R are sufficient to drive cellular proliferation (124). This also suggested that the RAP-resistant signaling pathways of the IL-2R can substitute for the RAP-sensitive signaling pathways during the induction of proliferation. In contrast, RAP-sensitive pathways are critical for the acquisition of cytotoxicity and cannot be substituted for by RAP-resistant IL-2R signaling processes. The data further suggests that the RAP-induced blockade in ribosome production, as well as upregulation of cell cycle progression inhibitor $p27^{kip1}$ can be overcome by RAP-resistant IL-2R signaling pathways. This indicates that $p70^{s6k}$ may not be necessary for entry of T cells into cell cycle. The same conclusion is suggested by the recent finding that $p70^{s6k}$-deficient cell lines still proliferate, albeit more slowly than wild-type cells, and that RAP is able to inhibit this residual proliferation (308). My studies also suggest that the characteristics of proliferation and cytotoxicity in activated CTL are not necessarily coupled because RAP-treated T cells did not proliferate but could kill at low levels while RAP plus rIL-2-treated T cells proliferated normally but killed in a suboptimal fashion (Figures 2 and 3).

Unlike proliferation, cytotoxicity in RAP or RAP plus rIL-2-treated cultures was never totally inhibited. This could be the result of several factors. Residual Gzm B enzymatic activity and perforin protein was detected in RAP-treated CTL (Figures 9 and 10). Also, FasL mRNA levels were not decreased in response to RAP-treatment and were, in fact, increased (Figure 6C). If it is assumed that FasL protein levels are also unaffected

by RAP-treatment, then FasL may be mediating the residual killing. However, this seems unlikely as normal P815 are poorly susceptible to anti-Fas mAb-mediated killing (309). In addition, I have shown that the vast majority of P815 killing during the 4 h $^{51}$Cr-release assay is sensitive to EGTA, a chelator of calcium and inhibitor of perforin monomer assembly in target cell membranes. Taken together, the results of the anti-Fas mAb and EGTA experiments suggest that anti-CD3-activated CTL use the granule exocytosis pathway to lyse P815 target cells. Therefore, the low cytotoxicity seen in RAP-treated CTL is probably due to the presence of residual Gzm B and perforin protein.

The ability of other cytokines to restore cytotoxicity in RAP-treated anti-CD3-activated T cell cultures was also tested. Neither IFN-γ nor TNF-α could restore killing levels to control values (A. Makrigiannis, unpublished data). This suggests that the RAP-sensitive IL-2R-signaling pathways necessary for CTL activation cannot be replaced by signals induced by the limited array of cytokines tested. This agrees with the observation that mice which are deficient for the IL-2Rγ subunit also have a profound defect in T cell development and virtually no detectable CTL (310). In contrast, IFN-γR and TNF-αR1 knockout mice have normal *in vivo* CTL development during viral infection (163,311), which further suggests that signals emanating from these cytokine receptors, unlike the IL-2R, are redundant with respect to CTL development.

The restoration of Gzm B and perforin gene expression in RAP plus rIL-2-treated CTL suggested the presence of a RAP-resistant, IL-2-induced transcription factor. The STAT proteins are a possible candidate since their activation is unaffected by RAP (129). IL-2 activates STAT5 (129) with DNA binding activity which is restricted to a 9-bp

palindrome sequence (TTCNNNGAA) (129). A review of the 5' regulatory regions of the murine Gzm B and perforin genes yielded one potential STAT5 binding site in the Gzm B gene and two sites in the perforin gene. This supports the notion that RAP-resistant Gzm B and perforin transcription is perhaps mediated by STAT5. Because the STAT5 binding sequence is a fairly short, the likelihood of it appearing randomly in any sequence is high. In order to confirm the presence of a STAT5 binding sequence, the mouse and human perforin 5' regulatory regions were aligned and the regions of homology compared. Surprisingly, there is very high homology in the 5' region of both genes to a degree which one would only expect in the coding region. The second downstream STAT5 binding sequence was found to be conserved between species with only a single base pair discrepancy within the internal non-restricted 3 bp (human -1089 -> -1081: TTC CGA GAA, mouse -799 -> -791: TTC TGA GAA). STAT5 has recently been found to enhance human perforin gene expression when bound to the perforin 5' regulatory region (P. Gosselin, personal communication). In addition to STAT5, the activating protein-1 complex (AP-1, c-fos/c-jun) is also induced by the IL-2R (122). Like STAT5, AP-1 activation is unaffected by RAP and can bind to the 5' regulatory regions of both the mouse perforin and Gzm B genes (304,305). In humans, AP-1 has been shown to be important in activating Gzm B gene transcription (304). Both of these transcription factors plus the MAPK-induced CREB protein activation may be responsible for the residual transcription of the Gzm B gene in RAP-treated CTL. While perforin expression may be affected to some extent by one or all of these transcription factors, resting murine T cells still transcribe perforin mRNA (Figure 4) when these factors are not activated.

The IL-2-induced restoration of Gzm B and perforin mRNA levels in RAP-treated CTL suggests that transcriptional induction of these genes does not rely on any one signaling pathway. Furthermore, a separate study found that RAP did not inhibit IL-2-induced IL-2Rα mRNA accumulation in anti-CD3-activated T cells (180). These data suggest that the action(s) of a particular transcription factor can be substituted for by increasing the presence or activation of other transcription factors. Perhaps this is an indication of the versatility of CTL which must be able to respond to different types of target cells, that may express different types and levels of costimulatory ligands. The types and levels of particular cytokines which CTL encounter may also differ in different situations.

The normal levels of Gzm B and perforin mRNA found in RAP plus rIL-2-treated CTL seems to be in direct conflict with the poor lysis seen with RAP plus rIL-2-treated CTL (Figure 2). However, it is known that RAP is able to block the translation of certain species of mRNA (168). Thus, it was possible that RAP was affecting Gzm B and perforin expression, but at the translational level. To test this hypothesis, the Gzm B and perforin protein levels in RAP plus rIL-2-treated CTL were compared with control CTL. The aspase activity of Gzm B was used as a read-out for the presence of the protein. It was found that Gzm B-like enzymatic activity was restored to control levels in RAP plus rIL-2-treated CTL (Figure 9). However, it is possible that other granzymes/enzymes are mediating the aspase activity in this assay. The primary amino acid sequence of the other members of the mouse Gzm B family (C to G) are highly conserved and may also cleave after aspartic acid (46). The estimation of perforin protein levels by Western blotting using a murine perforin-

specific monoclonal antibody was more conclusive. Perforin protein bands in lanes containing lysates from RAP plus rIL-2-treated CTL were more intense than those found in control CTL lysates (Figure 10), indicating that the translation of perforin mRNA was not reduced by RAP plus rIL-2 treatment.

A seeming paradox arose from the finding that RAP plus rIL-2-treated CTL have a severe defect in lytic capability, but have normal, if not augmented, Gzm B and perforin protein levels. However, it is possible that RAP is affecting other parameters of CTL function. Although RAP plus rIL-2-treated CTL have sufficient intracellular lytic proteins, deficiencies in the machinery of granule exocytosis might still explain the lack of cytotoxicity. To test this hypothesis, granule exocytosis was triggered by PMA and ionomycin treatment. When tryptase-specific activity of the resulting supernatants was assayed, no difference in granule exocytosis was found between RAP plus rIL-2-treated and control CTL (Figure 12). This suggests that granule exocytosis functions normally in RAP plus rIL-2-treated CTL. This experiment also indicates that total tryptase levels in CTL are unaffected during RAP plus rIL-2-treatment. Because prior studies with anti-CD3 activated murine T cells did not detect any Gzm A mRNA by Northern blot analysis (312), the tryptase activity detected must be of some other origin. In this regard, Gzm K is also a tryptase (41), and is found in activated CTL (47).

The ability of RAP plus rIL-2-treated CTL to form conjugates with P815 target cells was also found to be normal. Furthermore, CTL expression of LFA-1 adhesion molecules was unaffected by RAP-treatment (Figure 11). Thus, the identity of the deficient component of cytotoxicity in RAP plus rIL-2-treated CTL remains a mystery. I believe the

best candidate is the, as yet, unidentified tumor target cell receptor for anti-CD3-activated CTL. The MHC-unrestricted target cell range of anti-CD3-activated CTL (16) suggests that anti-CD3-activated CTL express NK-like tumor receptors. If this 'tumor receptor' is downregulated by both RAP and RAP plus rIL-2 treatment, then the identity of this gene could be established by the process of subtractive hybridization. Subtractive hybridization has been recently utilized to identify a RAP-sensitive gene in human B cells required for proteasome activity (173). However, it is likely that RAP inhibits the expression of many different genes. Isolating one gene in particular may therefore be difficult. It may be easier to simply obtain blocking antibodies to the different newly described murine NK cell tumor receptors, such as those belonging to the Ly-49 family, and perform flow cytometric analyses on RAP plus rIL-2-treated CTL. These antibodies could also be used to block P815 lysis, if added directly to the lysis assay.

There was no change in FasL mRNA levels caused by RAP or rIL-2-treatments at 4 h of culture (Figure 6D). This agrees with IL-2 and RAP affecting IL-2R signaling, which is normally considered to be a later event in T cell activation (11). Conversely, at 4 h the only direct effect on transcription is signaling through the TCR/CD3 complex. The unexpected increase in FasL mRNA levels at 48 h in RAP-treated cultures (Figure 6C) can be explained in one of two ways. The first is a delayed shift in the time-course of expression, such that maximal signals for FasL expression in RAP-treated culture are not present until 48 h of culture. The second is that RAP is somehow inducing a higher level of FasL gene transcription. I believe the second hypothesis is supported by both my data and past reports. First, RAP-treated and control T cell cultures display equal FasL mRNA

levels at 4 h, the time point for peak expression of this gene (Figure 6D). If the delayed shift hypothesis were correct then RAP-treated CTL should have lower levels of FasL mRNA at 4 h. Secondly, RAP-treatment has been reported to increase the expression of certain genes such as the proliferation inhibitor $p27^{kip1}$ (172). Thirdly, it has been shown that other proliferation inhibitors, specifically cisplatin and etoposide, increase expression of Fas by murine P815 mastocytoma (309). Logically, both Fas and FasL expression should be controlled in a co-ordinated manner since the upregulation of one protein is useless in the absence of the other. Finally, I have shown that, unlike Gzm B and perforin, FasL mRNA levels in CTL are increased by a wide variety of inhibitory stimuli, including neutralizing antibodies to IL-6 (295) and pentoxyfilline, a drug known to inhibit the production of Th1 cytokines (313). It is therefore conceivable that FasL, and probably Fas, are directly upregulated when T cells receive abherant or inhibitory stimuli. Perhaps this is a suicide mechanism to dispose of unwanted T cells. This hypothesis is supported by the strange symptoms of Fas and FasL-deficient mice which harbour huge numbers of T cells with deleterious consequences. Clonal expansion of T cells in these mice is not accompanied by clonal death (75,89). Perhaps clonal death is induced by Fas/FasL upregulation in the absence of antigenic or growth factor/cytokine stimulation, as would occur following treatment with RAP.

The absence, or decreased levels of Gzm B and perforin in transplant patients who receive RAP should lead to decreased injury of the transplanted tissue. Decreased levels of Gzm B and perforin have been correlated in the past with increased survival times of allografts (95). In contrast, prior reports have found that fully disparate cardiac allografts

are rejected in perforin-deficient mice just as quickly as in normal mice (101). This suggests that perforin is not involved in acute rejection. However, in the same study, class I MHC disparate, but class II MHC compatible transplants survived much longer in perforin knockout mice than in wild-type mice (101). Fully disparate tissues cause activation of both $CD4^+$ and $CD8^+$ T cells, while class I MHC disparate tissues would only activate CTL. Because of its effectiveness in inhibiting CTL activation, RAP may be more suited to modulating immune responses which are dependent on $CD8^+$ T cell activation. The lack of an inhibitory effect with respect to FasL mRNA levels in RAP-treated cultures suggests that acute rejection mediated by Fas/FasL interactions may be unaffected by RAP treatment. Cardiac tissue is known to express Fas mRNA (69). However, when hearts from Fas-deficient mice are transplanted into normal or perforin knockout mice rejection occurs within a similar timeframe (101). It is noteworthy that this study was done with hearts from *lpr* mice which have leaky Fas expression (314). Since Fas may not have been completely absent from the allografts it is difficult to assess the contribution of Fas to rejection in this study. However, a similar study showed that normal hearts transplanted into *gld* mice were also rejected quite quickly (100). These reports suggest that the inability of RAP to inhibit FasL mRNA levels would have little bearing on the outcome of cardiac transplant survival in patients on a RAP-regimen.

Studies with knockout mice have shown that the absence of perforin and, to a lesser extent, Gzm B indicate a severe defect in CTL-mediated cytotoxicity as assessed by $^{51}Cr$-release assay (36,56). On the other hand, my experiments have shown that the presence of Gzm B and perforin are not always indicative of high lytic potential in CTL. Thus,

cytolytic gene expression and proliferation are not irrevocably linked to cytotoxicity in CTL. This suggests that CTL-mediated cytotoxicity is regulated on several levels, only one of which depends on cytolytic gene expression. Furthermore, by itself cytolytic gene expression is not a predisposition for cytotoxic function. RAP has also been found to differentially affect activation signals in other cell types. Treatment of the J2E erythroid cell line with erythropoietin results in increased proliferation and differentiation into mature-type erythrocytes. Treatment of J2E cells with RAP plus erythropoietin inhibited proliferation but did not affect differentiation (315). I observed that RAP plus rIL-2-treatment of CTL inhibited their differentiation but not proliferation. The effect of RAP plus rIL-2 on CTL is opposite to the effect of RAP plus erythropoietin on erythroid cell lines. This difference may be the result of using an immortal cell line in the former report and normal lymphocytes in the present study. There is also the possibility that the erythropoietin receptor may not have access to the redundant signaling pathways leading to proliferation that the IL-2R has at its disposal. With respect to the opposite effects of the drug/cytokine treatment on differentiation, the only aspect of CTL maturation which was affected by RAP plus rIL-2-treatment was cytotoxicity. All other CTL characteristics studied were normal in RAP plus rIL-2-treated CTL. In contrast, the RAP/erythropoietin study used a much more limited criteria for determining cell differentiation. Therefore, with respect to differentiation, RAP plus rIL-2 and RAP plus erythropoietin had very similar effects on CTL and J2E cells, respectively.

RAP has been shown to be an effective and specific inhibitor of CTL activation. While the inhibition mediated by RAP with respect to proliferation and cytolytic molecule

expression could be restored with exogenous rIL-2, cytotoxicity was still impaired. Transplant rejection mediated through a CTL-dependent mechanism would be expected to be retarded but not totally inhibited as RAP-treated CTL still displayed residual cytotoxicity. As discussed earlier, the residual Gzm B and perforin expression in RAP-treated cells is most likely due to TCR/CD3-signaling. Therefore, in conjunction with a TCR-signaling inhibitor such as CsA, RAP may totally block allospecific CTL function. Furthermore, treatment with RAP alone may not be an appropriate therapy for all T cell-mediated pathologies. For example, individuals suffering from pathologies which are T cell-mediated and FasL-dependent, such as graft versus host disease-associated lymphoid hypoplasia and B cell dysfunction (316), would not benefit from the administration of RAP since RAP does not inhibit FasL expression. The effects of RAP on CTL function reported here provide a greater insight into the mechanism by which RAP prolongs graft survival in transplant patients administered this drug.

B7-1 and B7-2 molecules are both effective at costimulating the activation of T cells (273). Maximal activation of CTL by anti-CD3 mAb is also known to be dependent on the presence of many cytokines including IL-2, IFN-$\gamma$, IL-6, and TNF-$\alpha$ (288,291,295). However, the ligand/receptor interactions between T cells and APC in this system are less well characterized. The identity of the ligand which binds CD28 during anti-CD3-activation of CTL is unknown. Furthermore, the cell on which this ligand is expressed is assumed to be a B cell, but this has not been formally proven. It is also not known whether anti-CD3-activated CTL use their own CD28 surface receptors during anti-CD3 activation,

or if they rely on the cytokines produced by CD28-costimulated Th cells. Finally, the effect of blocking B7/CD28 interactions during anti-CD3 activation on cytolytic molecule expression has not been studied.

Since CD28 is considered to be the second most important ligand/receptor signaling molecule after the TCR/CD3 complex, I decided to examine the role, if any, of the B7 family of proteins in CTL activation. Blocking antibodies to B7-1 and B7-2 (known ligands of CD28/CTLA4) were obtained and added at the beginning of T cell culture with anti-CD3 mAb. It was found that B7-2 was required for the majority of CTL activation in this model system, while B7-1 did not make a significant contribution to CTL development (Figure 13). The effect of anti-B7-2, as well as anti-B7-1 mAb was confirmed with a separate pair of anti-B7-1 and anti-B7-2 antibodies (clones RM80 and PO3, respectively). The lack of costimulation by B7-1 was later found to correlate with the absence of B7-1 expression by freshly isolated nylon wool-non-adherent, asialoGM1-depleted splenocytes (Figure 20A). However, B7-1 was found to increase CTL activation when B7-1$^+$ LPS-activated B cells were introduced into anti-CD3 activated T cell cultures (Figure 22). This contribution of B7-1 to the development of cytotoxicity was only seen when B7-2 interactions were not blocked. There is no evidence that B7-2/CD28 interactions are regulating B7-1 function. Instead, because B7-2 is expressed at much higher levels on LPS-activated B cells than B7-1 (Figure 21), it is possible that antibody-coating of B7-2 molecules is providing a steric hindrance to B7-1-CD28 binding. Regardless, the data agrees with prior reports that both B7-1 and B7-2 can contribute to CTL activation (277,279). Similar results have been reported for the Con A-induced activation of lymph

node cells. Con A responses could be blocked by anti-B7-2 mAb, as well as the combination of anti-B7-1 and anti-B7-2 Fab fragments, while anti-B7-1 mAb had no effect (317). Like the fresh and LPS-activated B cells used in my study, $B220^+$ lymph node cells expressed B7-2 earlier and at higher levels than B7-1. The kinetics of expression of both B7 molecules may also contribute to the dominant effects of B7-2 seen in these studies. In order to determine which molecule is the most efficient at costimulating anti-CD3-induced CTL, accessory cells expressing equal levels of B7-1 or B7-2 at the beginning of culture would have to be employed. However, the data presented here suffice to show that both B7 molecules can contribute to anti-CD3-activation of CTL, albeit it to differing extents.

The ability of both B7-1 and B7-2 to activate CTL with B7-2 playing a more dominant role (Figure 22) completely contradicts the conclusions of a B7-transfection study which found that only B7-1 can activate CTL (277). In this study, P815 mastocytoma cells double-transfected for both B7-1 and B7-2 were irradiated and used as stimulator cells for CD4-depleted (i.e., $CD8^+$) C57BL/6 splenocytes in the presence of blocking antibodies to B7-1 or B7-2. Although the presence of anti-B7-2 mAb did not affect alloantigen-induced $CD8^+$ T cell proliferation, the presence of anti-B7-1 mAb decreased proliferation by half. This suggests that B7-2 cannot costimulate CTL. The difference in the costimulatory ability of these molecules cannot be attributed to transfection efficiencies as both were expressed at comparable levels, as assessed by flow cytometry. Furthermore, the blocking antibodies used by these researchers were the same as those used in my experiments (Figure 22). The reason for the contradiction between my data and this study may be in the nature of the antigenic stimulus. Perhaps P815 cells lack a necessary cofactor for B7-2 signaling

such as CD40. However, if this were true, the implication is that B7-1 and B7-2 cause CD28 to signal in different ways. One possibility is that B7-1 supplies a more 'complete' signal, while B7-2 signaling must be supplemented. However, cytokine production elicited by APC from B7-1 or B7-2 knockout mice (253), or from transfected APC (273), intracellular protein phosphorylation patterns induced by B7-1 or B7-2 molecules (263), and the ability of either B7-1 or B7-2 to similarly activate specific components of the CD28 signaling cascade (264) all lead to the conclusion that B7-1 and B7-2 bind and activate CD28 in an identical fashion. Alternatively, it is possible that the B7-2 cDNA used in the P815 transfection study mentioned above did not contain all the proper genomic elements. It is known that B7-2 has many different isoforms and that some of these do not bind CD28 (282,284). The kind of B7-2 isoform transcribed is dependent on upstream, downstream and perhaps intronic elements. Most likely, all of these elements are not present in a cDNA.

The presence of anti-B7-2 mAb during activation of T cells with anti-CD3 mAb never totally inhibited CTL induction (Figure 13), even though saturating levels of anti-B7-2 mAb were added to culture (Figure 15). In fact, anti-B7-2 mAb, in the form of hybridoma supernatant, could be diluted up to 100,000-fold before the inhibitory effect on T cell activation started to diminish (Figure 15). The concentration of anti-B7-2 mAb in the hybridoma supernatant was estimated to be at about 40 μg/ml by ELISA. The final dilution of hybridoma supernatant was 1/10 during culture or about 4 μg/ml of anti-B7-2 mAb. The ability of very low concentrations of anti-B7-2 mAb to inhibit T cell activation indicates that B7-2 is being expressed on accessory cells at low levels. This suggests that CD28 on T cells, for the most part, is unbound during anti-CD3-activation. This theory is supported by

the finding that when saturating levels of stimulatory anti-CD28 mAb are present during anti-CD3 activation, the resulting cytotoxicity was 2-3 fold higher than that of control CTL activated only with anti-CD3 mAb (A. Makrigiannis, unpublished data).

The amount of residual activation in anti-B7-2-treated T cell cultures varied from experiment to experiment. When the lytic activity of control CTL was very high, anti-B7-2 mediated inhibition was not as pronounced as when CTL were less well activated. Residual activation is most likely due to other signaling ligands and cytokines provided by B cells. For example, CD2 ligation contributes to CTL activation by anti-CD3 mAb (296). Interestingly, simultaneous use of anti-CD2 and anti-B7-2 blocking mAbs totally inhibited CTL activation, as measured by $^{51}$Cr-release assay (B. Musgrave, personal communication). This is in agreement with earlier reports showing that B7-1 and CD2 can synergize to activate CTL (318).

The B7-2-independent costimulation of anti-CD3 activated CTL is not supplied by B7-1. This was shown by the results of combination treatment of anti-B7-1 and anti-B7-2 mAb versus anti-B7-2 mAb alone (Figure 13). Both treatments resulted in the same amount of residual cytotoxicity. Barring the existence of a third CD28-binding protein, the residual activation can be said to be CD28-independent. This agrees with the finding that CTL from CD28 knockout mice can still be activated and function at near normal levels (225). A possible explanation for the strong inhibition of CTL induction seen with anti-B7-2 mAb in this study, and the near normal levels of CTL activity seen in CD28 knockout mice, is that other costimulatory ligands such as CD2 and CD40L, if given enough time, can substitute for CD28-signaling and fully activate CTL. However, CD28-signaling may result in faster

CTL induction, in which case assaying at 48 h may be too soon for the full effects of CD2 and other ligands to be seen.

Highly purified (double nylon wool-passaged) T cells from $CD4^{-/-}$ mice were used to show that B7-2 binds directly to CD28 on $CD8^+$ T cells during their activation (Figure 18). As an independent source of confirmation, isolated T cells were depleted of $CD4^+$ T cells by anti-CD4 mAb and C' treatment and then activated with anti-CD3 mAb. These $CD8^+$ T cells became activated as normal and could be inhibited by the presence of anti-B7-2 mAb (data not shown). However, not all $CD4^+$ T cells could be depleted by antibody plus complement treatment so the CD4-knockout approach was thought to be a more rigorous system. My experiments confirm past reports that CTL can be activated by $B7^+$ target cells in the absence of Th cells (277). B7-binding is not important during the killing phase as addition of anti-B7-1 and/or anti-B7-2 mAb did not affect the cytotoxic activity of anti-CD3-activated CTL (Figure 16). Interestingly, T cells from $CD4^{-/-}$ mice were also found to express low levels of B7-2 (Figure 17C). Although it has been previously reported that whole T cell preparations and purified $CD4^+$ T cells express B7-2 (238,243), I am the first to show that this is also true of $CD8^+$ T cells. The expression of B7-2 by CTL precursors and mature CTL could have several possible roles. In the absence of Th cells, a single CTL binding to a B7-deficient target cell would not become activated due to a lack of IL-2. However, if several CTL are clustered around a target cell one could envisage that CTL-CTL contact could lead to bystander costimulation from B7-2 on adjacent CTL and subsequent production of IL-2 by CTL. Under these conditions CTL might, therefore, promote their own differentiation.

In order to test the possibility that B7-2 on T cells is providing costimulation to neighbouring T cells, highly purified, APC-depleted T cells were seeded onto anti-CD3 mAb-coated plates. The immobilization of anti-CD3 on plastic simulates APC-FcR-mediated cross-linking of anti-CD3 mAb bound to T cells. Under these conditions, any T cell activation detected would be a direct result of T-T cell costimulation. Costimulation due to APC contamination was ruled out because of the lack of proliferation in highly purified T cells incubated with soluble anti-CD3 mAb (Figure 23), which indicates an absence of FcR-bearing accessory cells. Although T cells incubated on immobilized anti-CD3 mAb proliferated at high levels, the addition of blocking anti-B7-2 mAb did not affect proliferation (Figure 24). This suggests that B7-2 on T cells does not productively bind CD28 and confirms recent reports that T cell-expressed B7-2 is unable to stimulate CD28 due to altered glycosylation (283). Nevertheless, at least one study shows that B7-2 on memory $CD4^+$ T cells costimulates naive T cells, along with immobilized anti-CD3 mAb, to produce IL-2 (243). The reason for the discrepancy among the different reports may be due to different numbers of memory T cells present in the mice used in each study. Young mice would be expected to have fewer memory T cells than older mice, so if T cells are isolated from young mice not enough memory cells would be present to show a costimulatory effect for B7-2. Another possibility is that resting and memory T cells express different isoforms of B7-2. This is not unheard of since the CD45 family of proteins, for example, is made up of many different members which are differentially expressed depending on the differentiation state of the T cell.

Another possible explanation for the lack of inhibition by anti-B7-2 mAb in T cells

activated by plastic immobilized anti-CD3 mAb is that this system provides a stimulus for proliferation that is costimulation-independent. However, this hypothesis is not consistent with the finding that the addition of anti-CD28 mAb to immobilized anti-CD3 mAb results in a doubling of T cell proliferation (215). This suggests that T cells activated by immobilized anti-CD3 are responsive to CD28 binding. However, if CD28 signaling is required for T cell activation under these conditions, anti-B7-2 mAb would be expected to at least slightly alter activation levels. This is because the T cells are very crowded in the wells and so B7-2/CD28 interactions must be readily available. The results of the anti-CD28 mAb addition experiment suggest that CD28 is, for the most part, unbound. This, along with the fact that B7-2$^+$ T cells are in close proximity, leads one to conclude that B7-2 expressed by T cells does not bind CD28. Alternatively, other ligand/receptor pairs such as CD2/LFA-3 and CD40/CD40L may be operating in this system. These experiments support the notion that B7-2 costimulation during anti-CD3 mAb stimulation is being provided by B cells and/or macrophages. The finding that freshly isolated B cells were found to express B7-2 agrees with this conclusion (Figure 21). Because B cells and macrophages would be the only other B7-2$^+$ cell populations in these nylon-wool passaged, asialoGM1-depleted cells, B7-2 costimulation must therefore be supplied by these cells.

The presence of B7-2 on both CD4$^+$ and CD8$^+$ T cells suggests that B7-2 must perform some important role. The B7-2 expressed on T cells is known to have different glycosylation patterns than that expressed by APC (283). Furthermore, murine T cells express a unique B7-2 mRNA splice variant (284). These findings suggest a specialized function for the B7-2 molecule in these cells. While T cell B7-2 does not productively bind

CD28, it does retain binding to CTLA4 (282). CTLA4 is expressed late in activation (231). At this time, B7-1 also starts appearing on T cells and T cell expression of B7-2 is upregulated (231,240). This may be an indication that both B7-1 and B7-2 on T cells helps to downregulate the immune response. T cells lacking the CTLA4 gene proliferate wildly, leading to early death of the mutant animal (233). One can envisage the accumulation of activated T cells in the lymph nodes or at a site of infection eventually leading to very crowded conditions. This may allow CTLA4 on T cells to bind B7-1 and/or B7-2 on adjacent T cells in a non-antigen specific manner, resulting in the inactivation of T cells. A similar theory has been contemplated regarding the double expression of Fas and FasL on activated T cells. Mice lacking Fas or FasL display a similar phenotype to CTLA4$^{-/-}$ mice (75,89). While B7-2 single and B7-1/2 double knockout mice have been engineered and found to have retarded humoral responses with respect to antibody class switching and germinal center formation like CD28$^{-/-}$ mice (225,319), the induction of CTL in these animals, either *in vivo* or *in vitro*, has not been studied. In addition, CTL need to be able to kill T cell, as well as non T cell targets. Perhaps B7-2 on activated CTL helps these CTL to bind CD28 on virally infected or transformed T cells.

CD28 was found to be expressed at low levels by a large proportion CD8$^{+}$ T cells (Figure 17B). This agrees largely with prior reports showing that all murine T cells express CD28 (215). Although the levels of CD28 in these T cell cultures increased only slightly during the first 24 h of activation, there was a dramatic increase in CD28 expression by 48 h of activation. At this time-point the CD28 staining pattern (Figure 20C) suggests that CD28 is expressed by T cells at both high and low levels. This indicates CD28$^{hi}$ and CD28$^{low}$ T

cells may arise following anti-CD3 activation.

B7-1 was not found to be expressed in T cell cultures until 48 h post-activation. Even at this time, B7-1 expression was only slightly above background levels (Figure 20A). In contrast, freshly isolated B cells expressed low but detectable levels of B7-1 (Figure 21). Since B cells only comprise about 5-10% of the total cell number of nylon wool non-adherent asialoGM1-depleted splenocytes (D. Hoskin, personal communication), the low level of B7-1 expression on these relatively few B cells in T cell preparations would be difficult to detect. On the other hand, the B7-1 detected at 48 h of culture is most likely a result of B7-1 expression by activated T cells since by this time most residual B cells have been destroyed by the developing CTL, most likely through Fas/FasL interactions. RT-PCR analysis with murine B7-1 gene specific primers of anti-CD3-activated T cell mRNA over a timecourse showed that the most intense amplicon product appeared at 48 h of culture (data not shown). Due to the delay between transcription and protein expression, this suggests that B7-1 surface protein may be expressed at even higher levels one or two days later in the culture. The late expression of B7-1 by activated T cells agrees with results obtained by others (238). Whether B7-2 becomes up- or down-regulated on T cells during activation has a been a point of controversy (238,240). My data indicates that B7-2 levels increase slightly following T cell activation by anti-CD3 mAb.

Addition of anti-B7-2 mAb at different times during the 48 h activation period showed that CTL development was only significantly inhibited when B7-2 interactions were blocked at 0, 6 and 12 h after the addition of anti-CD3 mAb (Figure 19). At 24 and 36 h of culture, addition of anti-B7-2 mAb did not decrease T cell activation. In fact, the 36 h

timepoint addition of anti-B7-2 actually increased cytotoxicity. However, this effect was reproduced by simply adding the same volume of fresh medium, suggesting that the effect was due to replenishment of depleted nutrients (data not shown). The finding that B7-2 signaling is only required early in CTL activation suggests that other mechanisms are able to keep CTL activated once the initial CD28 stimulus is given. These non-B7-2 mechanisms are probably of a cytokine nature. By 24 h of culture, IL-2 levels are starting to rapidly rise in anti-CD3 activated T cell cultures (320). This hypothesis is supported by data showing that exogenous rIL-2 can substitute for B7-2 costimulation in anti-CD3-activated T cell cultures (Figure 25). An alternative explanation is that B7-2/CD28 costimulation is required throughout the 48 h activation period but by 12 h of culture all available B7-2 molecules are already bound by CD28. Therefore, late addition of anti-B7-2 mAb would have no apparent effect on CTL activation.

CD28 stimulation leads to the production of IL-2 (7). Thus, blockade of CD28 signaling results in reduced IL-2 synthesis by T cells (240). In order to determine the effect of B7-2 blockade on the production of other cytokines, cytokine levels in supernatants of control and anti-B7-2-treated anti-CD3-activated T cell cultures were assayed by ELISA. Production of IL-2, IFN-$\gamma$, IL-6, IL-4, and IL-10 were all decreased in anti-B7-2-treated cultures, while TNF-$\alpha$ levels were not affected (Table 2). The ELISA results agree with previous reports that blockade by anti-B7-2 mAb decreased IL-2, IL-4, and IFN-$\gamma$ production by murine T cells (240,252). These results indicate that CD28-signaling is required for the induction of IFN-$\gamma$, IL-6, IL-4 and IL-10, as well as IL-2 gene expression. Alternatively, IL-2 elicited by B7-2-CD28 interactions may in turn elicit the production of

these other cytokines through IL-2R signal transduction. Since CD28, like the IL-2R, signals activates ubiquitous transcription factors such as CREB through $p70^{s6k}$ activation (262), and AP-1 (258), both surface complexes contribute to cytokine gene transcription. However, Northern blot analysis of cytokine mRNA levels in murine T cells after stimulation by immobilized anti-CD3 mAb show that IL-2, TNF-α, TNF-β, IFN-γ, and GM-CSF gene transcription occur by 6 h of culture (22). Furthermore, the presence of co-immobilized anti-CD28 increased the expression of all these cytokines. This data conflicts with the failure of B7-2 blockade to affect TNF-α levels in my experiments. Possibly the low levels of B7-2 expressed in T cell cultures cause the majority of CD28 molecules not to be bound. While some cytokines may only require minimal CD28-signaling for production, TNF-α may require more costimulation which could be supplied only by saturating levels of plate-bound anti-CD28 mAb.

The decrease in IL-2, IFN-γ, and IL-6 expression along with the decreased cytotoxicity of anti-B7-2-treated CTL agree with previous studies showing that neutralizing antibodies to these cytokines decrease CTL development in anti-CD3-activated T cell cultures (288,291,295). In contrast IL-10 inhibits CTL induction and neutralization of IL-10 bioactivity increases cytotoxic development (291). Therefore, one would expect the decrease in IL-10 levels in anti-B7-2-treated cultures to result in increased CTL activation. However, the already low levels of activation probably hide this effect, which would be marginal at best under these conditions. Neutralization of TNF-α has also been shown to decrease cytotoxicity in anti-CD3-activated cultures (288). The lack of an effect on TNF-α

levels by anti-B7-2 mAb suggests that TNF-α release in anti-CD3-activated T cell cultures is independent of CD28-signaling. It is interesting to note the very high concentrations of IL-6 in comparison to IL-2, IL-4, and IL-10 in anti-CD3-activated cultures (Table 2). It is possible that contaminating endotoxin is promoting IL-6 synthesis, however this is unlikely as sterile plastic culture plates and endotoxin free medium and FCS were used exclusively.

Since reduced production or bioactivity of IL-2, IFN-γ, IL-6, and TNF-α results in decreased development of cytotoxicity (288,291,295), it is possible that the addition of exogenous cytokines to anti-B7-2-treated T cell cultures could restore cytotoxicity. While the addition of exogenous IL-2, IFN-γ, IL-6, or IL-12 increased cytotoxicity in control cultures, only IL-2 and IL-12 were found to restore cytotoxicity in anti-B7-2-treated T cell cultures (Figure 25). The observation that all cytokines, except TNF-α, increased the basal level of P815 lysis by control CTL indicates that the cytokines were bioactive. In addition, TNF-α killed TNF-α-sensitive WEHI-164 cells (D. Hoskin, personal communication), indicating intact bioactivity of this cytokine as well. The huge increase in control CTL activity caused by IFN-γ can be explained by the finding that exogenous IFN-γ increases cytolytic mediator expression in anti-CD3-activated CTL (291). Although IL-2 and IL-6 are also known to be important for optimal expression of Gzm B and perforin (288,295), addition of these cytokines did not increase CTL activation to the extent as seen with IFN-γ. This suggests that IFN-γ is having other effects on anti-CD3-activated CTL. A recent report has shown that IFN-γ upregulates B7-1 expression on human monocytes after only 24 h of culture (321). It is, therefore, possible that IFN-γ and the other cytokines are

exerting their effects, in part, by affecting the levels of costimulatory molecules expressed by APC. The restoration of cytotoxicity in anti-B7-2-treated T cell cultures by IL-2 was expected since IL-2 production is mediated by CD28-signaling (7). This suggests that either IL-2R-signal transduction can mediate all the events necessary for the development of anti-CD3-induced CTL or IL-2R-signaling results in the expression of the other cytokines that are important for CTL activation. These conclusions agree with prior reports showing that CTL do not necessarily require CD28-signaling in order to become activated (225).

The restoration of cytotoxicity in anti-B7-2-treated T cell cultures by IL-12 was surprising considering the lack of cytotoxicity in anti-B7-2 plus IFN-γ-treated cultures. IL-12 is known to strongly upregulate IFN-γ expression by NK cells and T cells (143). However, CTL activated in the presence of anti-B7-2 mAb plus exogenous IL-12 in combination with neutralizing anti-IFN-γ mAb and blocking anti-IFN-γR mAb still had very high levels of cytotoxicity (Figure 26). This suggests that IL-12R-signaling on T cells induces other necessary CTL genes to be expressed, in addition to IFN-γ. The neutralizing IFN-γ and blocking IFN-γR antibodies were bioactive as shown by their ability to slightly inhibit CTL activation (Figure 26). This agrees with prior studies showing that IFN-γ-IFN-γR interactions are required for optimal anti-CD3-induced CTL activation (288). A likely product of IL-12 stimulation was IL-2. Interestingly, cultures treated with anti-B7-2 mAb plus exogenous IL-12 in combination with neutralizing anti-IL-2 mAb and blocking anti-IL-2R mAb had lower levels of cytotoxicity compared to cultures receiving only to anti-B7-2-treatment (Figure 27). Although this suggests that IL-12 is inducing IL-2 production,

other investigators have reported that the addition of IL-12 does not induce IL-2 production by T cells in a mixed tumor reaction (322). On the other hand, my data indicate that IL-12 substitutes for B7 costimulation during CTL activation (Figure 25). The results of Gajewski *et al.* do not necessarily contradict my own because they assayed IL-2 production after only 24 h, while CTL activity was assayed after 6 d of culture with allogeneic tumor cells (322). Perhaps IL-2 production occurs more slowly when T cells are stimulated by IL-12 in MLR versus anti-CD3 activation.

IL-12R signaling activates the STAT4 transcription factor which is known to enhance expression of the IFN-γ gene (323). Recently, it has been shown that the IL-12/STAT4 signal transduction pathway is necessary for T cell activation in a model of experimental colitis (324). However, IFN-γ production by these T cells was not required for their activation (324). This report, along with my finding that IL-12 can substitute for CD28 costimulation, indicate that APC have alternative methods to induce T cell activation, in addition to B7 costimulation. APC may be able to costimulate T cells by an IFN-γ-independent, IL-2-dependent IL-12R signaling pathway. The importance of IL-12 production by APC for direct CTL development is doubtful since it is unlikely that most target cells will produce IL-12 for CTL activation. This is perhaps another indirect route of CTL activation via APC-activated Th cell secretion of cytokines.

It is interesting to note that not all cytotoxicity is lost when CTL are activated in the presence of anti-IL-2 and anti-IL-2R mAbs (Figure 27). This suggests that a minor part of the activation signals which CTL receive are IL-2-independent. Because the low levels of cytotoxicity are similar in anti-B7-2-treated and anti-IL-2-treated CTL cultures (Figure 27),

it is tempting to speculate that all IL-2-dependent activation is caused by B7-2-induced CD28 signaling. However, this hypothesis is questionable as IL-2 levels in the supernatants of anti-B7-2-treated CTL cultures were only decreased by approximately 50%. This suggests that while B7-2/CD28 interactions are responsible for a large portion of IL-2 production, other mechanisms are also working to induce expression of this cytokine. Therefore, the residual cytotoxicity in anti-B7-2-treated CTL is not necessarily IL-2-independent.

An obvious reason for the decreased cytotoxicity in anti-B7-2-treated T cell cultures would be decreased production of cytolytic mediators. RT-PCR analysis of control CTL and anti-B7-2-treated CTL showed that blockade of B7-2 with specific mAb resulted in decreased levels of Gzm B mRNA (Figure 28A). In my experience, every treatment which is inhibitory for the production of CTL has also reduced Gzm B levels. Because of this, Gzm B is the best marker for CTL activation. The inhibitory effect of anti-B7-2 mAb seems to be operating at the level of transcription since Gzm B mRNA from anti-B7-2-treated CTL showed a half-life similar to Gzm B mRNA from control CTL (Figure 29). Furthermore, the decrease in Gzm B mRNA was confirmed at the protein level by aspase-specific enzymatic assay (D. Hoskin, personal communication). Taken together, the results from the RT-PCR and enzymatic assays suggest that the reason anti-B7-2-treated CTL cannot kill efficiently is due to a lack of Gzm B protein. This agrees with the reduced killing efficiency seen for Gzm $B^{-/-}$ murine CTL in a $^{51}Cr$-release assay (56).

Interestingly, perforin mRNA expression in anti-B7-2-treated CTL was not decreased and may even have been increased, although this increase was not always

reproducible (Figure 28A). FasL mRNA levels in control and anti-B7-2-treated CTL were comparable at 4 h of culture (Figure 28B). It is interesting to note that RAP and anti-B7-2 mAb treatments had different effects on the levels of Gzm B and perforin (compare Figures 6A and B with Figure 28A). While both treatments decreased Gzm B mRNA levels, only RAP decreased perforin mRNA expression as well. It would seem that the RAP-sensitive signaling pathways contribute to perforin expression while CD28-signaling pathways do not. This differential expression can perhaps be explained in the following way. Since perforin is expressed constitutively in mouse CTL, the lack of costimulation through CD28 will not affect its expression. On the other hand, RAP will inactivate transcription factors such as CREB (194) in resting cells, thereby decreasing perforin expression. The lack of an inhibitory effect on FasL mRNA levels by either RAP or anti-B7-2 mAb is puzzling. It would be interesting determine whether this also holds true at the protein level. However, commercial anti-FasL antibody preparations have been reported to be unreliable for flow cytometry (325). A Fas$^+$ target cell used in a $^{51}$Cr-release assay in the presence of EGTA to inhibit granule exocytosis would perhaps allow one to indirectly compare FasL levels on anti-B7-2-treated CTL with normal CTL. Western blotting of FasL protein would be an alternative approach, but I am unaware of any reports of this kind using a commercially available antibody.

IL-12 was found to restore Gzm B mRNA levels in anti-B7-2-treated cultures to near control levels (Figure 30). Therefore, the restoration of killing in IL-12 plus anti-B7-2-treated CTL is probably a result of more Gzm B protein being produced. This data agrees with previous reports showing that IL-12 independently, and in synergy with IL-2, can

upregulate expression of Gzm B and perforin in NK cells (297). It is noteworthy that the decrease in Gzm B in anti-B7-2-treated cultures seen in Figure 30 is much more dramatic than that seen in Figure 28A. Figure 28A depicts a two step RT-PCR reaction (mRNA --> cDNA in one reaction and cDNA --> amplicon in the second reaction) while Figure 30 shows data from a newly available one step RT-PCR reaction procedure (mRNA --> cDNA --> amplicon, all in one reaction). The reason for the different Gzm B levels in anti-B7-2-treated CTL between the two figures lies in the fact that the cDNA loading control for Figure 30 is total RNA while the loading control for Figure 28A is GAPDH. While GAPDH and β-actin expression should not be affected by anti-B7-2 mAb to such a great extent as Gzm B, the transcription of these genes will be slightly increased or decreased depending on the proliferative/metabolic state of the T cell. Logically, this suggests that equalizing housekeeping gene mRNA levels may actually reduce the observable effect of a particular treatment. The true comparison for gene expression is to isolate mRNA from equal numbers of cells. Figure 30 shows that approximately equal numbers of cells were used because the level of ribosomal RNA is roughly the same for all treatments.

I have demonstrated that the major CD28 binding ligand during anti-CD3 activation of CTL is B7-2. In addition, B7-2 was shown to bind and activate $CD8^+$ T cells. Furthermore, only B7-2 expression on B cells was found to be effective in a costimulatory capacity. Although B7-1 has the potential to costimulate CTL, this molecule was not expressed at high enough levels for effective costimulation in anti-CD3-activated CTL cultures. Thus, B7-1 and B7-2 molecules are both capable of activating CTL activity but B7-2 is favoured because of its constitutive expression. The ability of CD28-knockout mice

to generate a normal CTL response *in vivo* (225) may have temporarily led investigators to doubt the importance of B7 molecules during CTL activation. However, the recent finding that B7 molecules bind CTLA4 in CD28-deficient mice to activate T cells has created new questions (236). The recent engineering of B7-1/B7-2 double-knockout mice will provide valuable insight into the contribution of these molecules to CTL activation. There is no doubt that understanding the ligand/cytokine requirements for CTL induction by anti-CD3 provides valuable insight into $CD8^+$ T cell activation. The data presented in this thesis may help to resolve some of the controversy regarding the contribution of B7-1 and B7-2 to CTL activation.

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February 23, 1998

Jane Davidson
Assistant Editor
The Journal of Immunology

Dear Ms. Davidson

I am writing to ask you for permission to use the figures which appeared in my article in the November 15th, 1998 issue of your journal (Inhibition of cytotoxic T lymphocyte induction by rapamycin: Interleukin-2 rescues granzyme B and perforin expression but only partially restores cytotoxic activity. Vol. 159, No. 10. p.4700). The work included in this publication was done by myself while undertaking in studies leading to a Ph.D. degree. I have started writing my thesis and would greatly appreciate the chance to use the same figures for my written thesis.

Looking forward to your response,

Sincerely,

A. Makrigiannis

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February 24, 1998

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# Inhibition of CTL Induction by Rapamycin

## IL-2 Rescues Granzyme B and Perforin Expression but Only Partially Restores Cytotoxic Activity[1]

Andrew P. Makrigiannis and David W. Hoskin[2]

**Rapamycin (RAP) is a potent inhibitor of CTL induction. Since RAP is known to block IL-2 signaling through the IL-2R, we hypothesized that RAP may interfere with CTL generation by inhibiting IL-2-induced expression of granzyme (Gzm) B, perforin, and/or Fas ligand (FasL). MHC-unrestricted mouse CTL induced in vitro with anti-CD3 mAb in the presence of RAP (1 ng/ml) exhibited dramatically reduced cellular proliferation and cytotoxicity against P815 tumor target cells. Gzm B mRNA expression and enzymatic activity in RAP-treated CTL were greatly reduced compared with those in control CTL. Perforin mRNA expression was also reduced by RAP. In contrast, RAP failed to inhibit FasL mRNA expression. RAP, therefore, inhibits induction of the perforin/Gzm B cytolytic pathway but spares Fas/FasL-mediated cytotoxicity. To determine whether RAP exerts a total blockade of the IL-2R signaling pathway, we induced CTL in the presence of both RAP and exogenous rIL-2 (100 U/ml). Under these conditions, Gzm B and perforin mRNA and protein expression as well as cellular proliferation were restored to at least control levels. Surprisingly, inhibition of cytotoxicity was only partially alleviated when CTL were induced in the presence of RAP plus rIL-2, even though CTL conjugated normally with target cells and had an intact granule secretory pathway. We conclude that 1) the inhibitory effect of RAP at the level of the IL-2R is incomplete; and 2) the suppressive effect of RAP on CTL induction is only partly due to inhibition of Gzm B and perforin gene expression.** *The Journal of Immunology,* **1997, 159: 4700–4707.**

Rapamycin (RAP),[3] an immunosuppressive macrolide derived from the soil microorganism *Streptomyces hydroscopicus* (1), delays the transition of eukaryotic cells from G1 to S phase of the cell cycle (2), thereby effectively retarding cellular proliferation. Lymphocytes are especially sensitive to the antiproliferative effect of RAP (3). IL-2-driven T lymphocyte proliferation is affected because RAP interferes with p70 S6 kinase ($p70^{S6K}$) activity, which is involved in one pathway of IL-2R signal transduction (4). RAP mediates $p70^{S6K}$ inhibition by complexing with the FK506 binding protein (FKBP12) located in the cytoplasm of mammalian cells (5). The RAP-FKBP12 complex, in turn, binds to the mammalian homologue of yeast TOR protein (mTOR) (6), preventing mTOR from activating $p70^{S6K}$ (7). Activation of $p70^{S6K}$ by mTOR is required for the phosphorylation of a wide array of proteins involved in cell cycle control, transcription, and translation initiation (reviewed in Ref. 8).

Signaling through the IL-2R is necessary for T cell activation and differentiation after antigenic stimulation (reviewed in Ref. 9). In addition to promoting T cell proliferation, IL-2 up-regulates CTL expression of perforin, granzyme (Gzm) B, and Fas ligand (FasL) molecules (10, 11). Gene knockout studies and naturally occurring mutations have revealed the importance of perforin, Gzm B, and FasL in target cell destruction by CTL (12, 13). Moreover, the presence of perforin and Gzm B in transplanted tissues is an indicator of impending graft rejection by immune effector cells (14, 15). Once CTL recognize and bind to target cells, perforin is exocytosed and polymerizes in the target cell membrane, forming transmembrane channels through which Gzm B is thought to enter and initiate an apoptotic signaling cascade mediated by endogenous latent proteases (16). FasL is able to induce a similar death signal in Fas-bearing target cells (17). However, recent studies have shown that FasL-mediated apoptosis may be more important in immune regulation and tolerance induction than as a mechanism of immune surveillance (18, 19).

Allograft rejection is mediated by alloantigen-specific Th cells and CTL as well as by nonspecific inflammatory cells such as macrophages (reviewed in Ref. 20). Recently, there has been considerable interest in the use of RAP as one component of an overall immunosuppressive treatment strategy for organ transplant patients. In large part, this interest has resulted from evidence that RAP is an immunosuppressant of remarkable potency. In some model systems of transplantation, RAP effectively prevents allograft rejection at doses 10- to 100-fold lower than the dose of cyclosporin required to mediate a comparable effect (21). Furthermore, RAP effectively suppresses the activation of alloantigen-specific CTL (22), which are believed to play a major role in allograft rejection (20). However, the mechanism by which RAP interferes with CTL induction is poorly understood. In the present study we investigate the effect of RAP on the expression of genes

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[3] Abbreviations used in this paper: RAP, rapamycin; m, mammalian; Gzm, granzyme; FasL, Fas ligand; ActD, actinomycin D; CRE, cyclic adenosine 3',5'-monophosphate-responsive element; MAPK, mitogen-activated protein kinase; SSPE, standard saline phosphate/EDTA (0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA).



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associated with mouse CTL function, as well as the effect of exogenous rIL-2 on RAP-mediated inhibition of mouse CTL induction.

## Materials and Methods

### Mice

Male 6- to 8-wk-old C57BL/6 mice were purchased from Charles River Canada (Lasalle, Quebec, Canada). Mice were maintained on standard laboratory chow and water supplied ad libitum in our animal care facilities.

### Medium and reagents

RPMI 1640 medium (ICN Biomedicals Canada Ltd., Mississauga, Ontario, Canada), hereafter referred to as complete RPMI 1640 medium, was supplemented with 10 mM L-glutamine, 100 μg/ml streptomycin, 100 U/ml penicillin (all from ICN Biomedicals Canada), 5 mM HEPES buffer (Sigma Chemical Co., St. Louis, MO; pH 7.4), and 5% heat-inactivated (at 56°C for 30 min) FCS (Life Technologies, Burlington, Ontario, Canada). Human rIL-2 (active in the murine system) was obtained from Cetus Corp. (Emeryville, CA). Sp. act. is expressed as Cetus units per milliliter. Mouse rIFN-γ and rTNF-α were purchased from Genzyme Corp. (Cambridge, MA). Sp. act. is expressed as Genzyme units per milliliter. The hybridoma (clone 145-2C11) that produces hamster anti-mouse CD3 mAb (23) was provided by Dr. J. Bluestone (University of Chicago, Chicago, IL). Rat anti-mouse CD2 mAb (clone RM2-1) (24) was a gift from Dr. H. Yagita (Juntendo University, Tokyo, Japan). A stock solution of RAP (Research Biochemicals International, Natick, MA), was prepared in DMSO and stored at −20°C. Actinomycin D (ActD), PMA, and ionomycin were purchased from Sigma Chemical Co. ActD was dissolved in ethanol, and the stock solution was stored at −20°C.

### Generation of anti-CD3-activated CTL

C57BL/6 spleen cells (depleted of RBC by osmotic shock) were passaged through a nylon wool (Cellular Products, Inc., Buffalo, NY) column to remove most B lymphocytes and macrophages (25). Residual FcR-bearing B cells and macrophages are required as accessory cells in the anti-CD3 activation protocol (26). Nylon wool-nonadherent spleen cells were depleted of NK cells by a two-step treatment with polyclonal anti-asialoGM1 Ab (1/40; Wako BioProducts, Richmond, VA) and rabbit C (1/12; Cedarlane Laboratories, London, Ontario, Canada). The resulting T lymphocyte-enriched cell preparation is typically 91% $CD3^+$ and <0.1% $NK1.1^+$ by flow cytofluorometric analysis (27). T cells were adjusted to a concentration of 4 to 5 × $10^6$ cells/ml in complete RPMI 1640 medium containing soluble anti-CD3 mAb (1/20 hybridoma culture supernatant), with or without RAP (1 ng/ml, unless otherwise indicated) and/or IL-2 (100 U/ml). Cultures were maintained for 40 to 45 h at 37°C and 5% $CO_2$ in a humidified atmosphere. Anti-CD3-activated CTL were then collected for use.

### T cell proliferation assay

After 48 h of stimulation with anti-CD3 mAb alone or in combination with RAP and/or IL-2 (as described above), a 200-μl volume of T cells was transferred to triplicate wells of a 96-well round-bottom microtiter plate (Sarstedt, Inc., St. Leonard, Quebec, Canada). The cultures were pulsed with 0.5 μCi of [$^3$H]TdR (sp. act., 65 Ci/mmol; ICN)/well and maintained at 37°C in a 5% $CO_2$ humidified atmosphere for 6 h to measure DNA synthesis. Cultures were harvested onto glass fiber mats (ICN) using a Titer-Tek multiple sample harvestor, and [$^3$H]TdR incorporation was determined in a Wallac 1410 liquid scintillation counter (Wallac, Turku, Finland). Data are expressed as mean counts per minute ± SD.

### $^{51}$Cr release cytotoxicity assay

MHC-unrestricted CTL induced with anti-CD3 mAb were washed extensively with PBS, resuspended in complete RPMI 1640 medium, and added to wells of a 96-well V-bottom microtiter plate (Sarstedt)in graded dilutions to obtain the desired E:T ratios. P815 mastocytoma cells were labeled for 1 h at 37°C with 100 μCi of sodium [$^{51}$Cr]chromate (ICN), washed three times, resuspended in complete RPMI 1640 medium, and added to the microtiter plate at a concentration of 5 × $10^3$ cells/well. The plates were incubated for 4 h in a humidified atmosphere at 37°C and 5% $CO_2$, then centrifuged, and a 100-μl aliquot of the supernatant was removed for measurement in a Beckman Gamma 8000 counter (Beckman, Fullerton, CA). The percent lysis was determined by the following equation:

$$\% \text{ lysis} = (E - S)/(M - S) \times 100$$

where E is the release from experimental samples, S is the spontaneous release, and M is the maximum release upon lysis with 10% SDS. Data are presented as the mean percent lysis of triplicate samples ± SD.

### Northern blot analysis

CTL were centrifuged, and the cell pellet was resuspended in TRIzol reagent (Life Technologies) to isolate total RNA according to the manufacturer's instructions. RNA was electrophoresed across a 1.2% agarose/7% formaldehyde gel and then transferred onto a Nytran membrane (Schleicher and Schuell, Keene, NH) by vacuum blotting. The Nytran membrane was fixed by exposure to UV light and baked at 80°C for 90 min. Next, the RNA blot was prehybridized for 2 h at 42°C in 5× SSPE, 50% formamide, 5× Denhardt's reagent, 0.1% SDS, and 100 mg/ml denatured low m.w. DNA. The blot was hybridized for 12 h at 42°C in fresh buffer containing $^{32}$P (DuPont Canada, Inc., Mississauga, Ontario, Canada) random prime-labeled cDNA probes for Gzm B (28) (provided by Dr. R. C. Bleackley, University of Alberta, Edmonton, Alberta, Canada) or β-actin. The blot was washed twice for 30 min each time in 6× SSPE at room temperature followed by a 30- to 60-min wash at 65°C in 1× SSPE. Finally, the blot was exposed to Amersham Hyperfilm for various lengths of time at −70°C. Nytran membranes were stripped as suggested by the manufacturer and reprobed as needed.

### Semiquantitative RT-PCR

Total cellular RNA was isolated from CTL using the TRIzol reagent as recommended by the manufacturer (Life Technologies). Single-stranded cDNA was synthesized from 0.5 μg of RNA in the presence of 5 mCi of [$^{32}$P]dCTP (DuPont) with 200 U of Moloney murine leukemia virus-reverse transcriptase (Life Technologies). Amplification of equal levels of cDNA was insured by monitoring [$^{32}$P]dCTP incorporation during reverse transcription. PCR was conducted in an automatic DNA thermal cycler (MJ Research, Inc., Watertown, MA). PCR reactions were run with the following primers (obtained from Life Technologies): β-actin: 5′ primer, CTG GAGAAGAGCTATGAGC; and 3′ primer, TTCTGCATCCTGTCAG CAATG (provided by A. Stadnyk, Dalhousie University, Halifax, Nova Scotia, Canada); perforin: 5′ primer, GTCACGTCGAAGTACTTGGTG; and 3′ primer, ATGGCTGATAGCCTGTCTCAG; Gzm B: 5′ primer, GC CCACAACATCAAA GAACAG; and 3′ primer, AACCAGCCACATAG CACACAT (29); and FasL: 5′ primer, ATGGTTCTGGTGGCTCTGGT; and 3′ primer, GTTTAGGGGCTGGT TGTTGC (30). Primer specificity was verified by Southern blot analysis using $^{32}$P-labeled cDNA probes for FasL (31) (provided by Dr. S. Nagata, Osaka Bioscience Institute, Osaka, Japan), perforin (32) (generously provided by Dr. K. Okumura, Juntendo University, Tokyo, Japan), Gzm B (28), and β-actin. Each reaction used 5 × $10^4$ cpm of cDNA, 2.5 U of Taq polymerase (Life Technologies), 200 M deoxynucleotide triphosphates (Life Technologies), and gene-specific 5′ and 3′ primers (50 nM for β-actin; 500 nM for Gzm B, perforin, and FasL) in a final volume of 50 μl. The conditions used for PCR were: β-actin (22 cycles), perforin (29 cycles), and Gzm B (22 cycles): 92°C for 30 s, 57°C for 30 s, and 72°C for 1 min; FasL (34 cycles): 94°C for 1 min, 64°C for 1 min, and 72°C for 1.5 min. The number of PCR cycles chosen for FasL, Gzm B, perforin, and β-actin amplifications were previously determined to generate PCR product during the exponential phase of amplification. RT-PCR performed under these conditions has been shown to be semiquantitative, providing reliable detection of twofold or greater differences in mRNA levels without the use of especially prepared internal standards (33). PCR products were resolved on a 1.5% agarose gel containing ethidium bromide and visualized by UV light illumination. The detected PCR amplicon was compared with a 100-bp ladder (Promega Corp., Madison, WI) and quantified by densitometric scanning with a Macintosh Color OneScanner (Apple Computer, Inc., Cupertino, CA) and National Institutes of Health Image Software (version 1.6). PCR product levels, as determined by densitometric scans, were normalized relative to the steady state expression of β-actin.

### Colorimetric Gzm B assay

Gzm B activity in the cytosolic fraction of CTL was measured by colorimetric enzyme assay as previously described (27) using the Gzm B-specific synthetic substrate Boc-Ala-Asp thiobenzyl ester (34) purchased from Enzyme Systems Products (Dublin, CA). An absorbance of 0.01 at 405 nM was arbitrarily defined as 1 U of esterolytic activity.

### Detection of perforin protein using immunostaining

Postnuclear proteins were obtained from 5 × $10^6$ CTL by treatment with 200 μl of ice-cold lysis buffer (1% Nonidet P-40, 5 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin/pepstatin, and 10 μg/ml aprotinin) for 30 min. Cell lysates were cleared by centrifugation at 14,000 × g for 6 min, and the

Table I. *Dose-dependent inhibitory effect of RAP on CTL induction by anti-CD3 mAb*

| Additions to Culture[a] | % Lysis P815[b] E:T 50:1 | E:T 25:1 |
|---|---|---|
| Anti-CD3 alone | 57 ± 3 | 37 ± 5 |
| Anti-CD3 + DMSO vehicle | 68 ± 3 | 45 ± 3 |
| Anti-CD3 + RAP (0.1 ng/ml) | 29 ± 3 | 16 ± 4 |
| Anti-CD3 + RAP (1 ng/ml) | 6 ± 2 | 2 ± 2 |
| Anti-CD3 + RAP (10 ng/ml) | 6 ± 2 | 2 ± 2 |
| Significance[c] | $p < 0.001$ | |

[a] CTL were induced with anti-CD3 mAb as described in *Materials and Methods*.
[b] Cytotoxicity against P815 target cells was evaluated in a 4-h $^{51}$Cr-release assay. Data from triplicate samples of a representative experiment ($n = 4$) are expressed as mean percent lysis ± SD.
[c] Determined by one way ANOVA followed by the Bonferroni test.

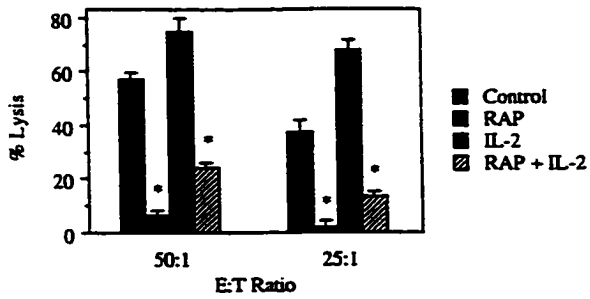


FIGURE 1. Effect of exogenous rIL-2 on RAP-mediated suppression of CTL induction. T cells were stimulated with anti-CD3 mAb in the presence of RAP (1 ng/ml), rIL-2 (100 U/ml), or RAP plus rIL-2. Following 48 h of culture, cytotoxicity against P815 mastocytoma cells was evaluated in a 4-h $^{51}$Cr release assay. Data from a representative experiment ($n = 3$) are expressed as the mean percent lysis ± SD. * indicates $p < 0.001$ compared with the control, as determined by Student's *t* test.

supernatant was immediately stored at −20°C. Samples were diluted 1/1 with 2× Laemmli sample buffer containing 2-ME, boiled for 5 min, and electrophoresed across a 12% SDS-polyacrylamide gel at a constant voltage setting of 200 V/60 mA. Separated proteins were transferred onto a 0.45-μm nitrocellulose membrane (Mandel Scientific Co., Guelph, Ontario, Canada) using a Bio-Rad Mini Trans Blot Module for 1 h at 100 V/250 mA under the conditions recommended by the manufacturer. After transfer, the nitrocellulose membrane was allowed to air-dry, blocked with 3% BSA (Boehringer Mannheim, Laval, Quebec, Canada) in 10 mM Tris (pH 7.5), 100 mM NaCl, and 0.1% Tween-20 for 1 h at room temperature. All blocking and washes were performed with gentle agitation. Modified blocking buffer (1% BSA in 10 mM Tris (pH 7.5), 100 mM NaCl, and 0.1% Tween-20) containing 2.5 μg/ml of rat anti-mouse perforin mAb (Kamiya Biomedical Co., Tukwila, WA) was next applied to the membrane overnight at 4°C. The blot was washed with 10 mM Tris (pH 7.5), 100 mM NaCl, and 0.1% Tween-20 and immersed in modified blocking buffer containing 1.6 μg/ml of alkaline phosphatase-conjugated AffiniPure mouse anti-rat IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for 2 h at room temperature. Following additional washes, perforin protein bands were visualized by the addition of 150 μg/ml of 5-bromo-4-chloro-3-indolyl phosphate and 335 μg/ml of nitro blue tetrazolium in substrate solution (200 mM Tris, pH 9.5, containing 10 mM $MgCl_2$).

### *Flow cytofluorometric analysis*

CTL were incubated for 30 min at 4°C with a 1/2 dilution of hybridoma supernatant containing mAb specific for mouse LFA-1 (clone FD441.8, American Type Culture Collection, ATCC, Rockville, MD), CD8α (clone 2.43, ATCC), or CD4 (clone GK1.5, ATCC) in PBS containing 0.2% sodium azide and 2.5% BSA. Next the CTL were washed and incubated for 30 min at 4°C with 10 μg/ml FITC-conjugated mouse anti-rat IgG (Jackson ImmunoResearch) in PBS containing 0.2% sodium azide and 2.5% BSA. The cells were then thoroughly washed with PBS containing 0.2% sodium azide and 1% BSA. The percentage of fluorescent cells was determined by analysis of $10^4$ cells with a FACScan (Becton Dickinson Canada, Mississauga, Ontario, Canada).

### *Conjugate formation assay*

This procedure was performed as previously described (35).

### *Statistical analysis*

Statistical comparisons of data were performed using the Instat statistics program (GraphPad Software, Inc., San Diego, CA). Student's *t* test or one-way ANOVA and the Bonferroni test were used where appropriate. $p < 0.05$ was considered statistically significant.

## Results

### *RAP inhibits MHC-unrestricted CTL induction by anti-CD3 mAb*

To study the effect of RAP on CTL induction, mouse T cells were stimulated with mitogenic anti-CD3 mAb in the absence or the presence of various concentrations (0.1–10 ng/ml) of RAP. Previous studies have established that mouse T cells activated with anti-CD3 mAb acquire potent MHC-unrestricted cytotoxic activity against a range of tumor targets, including P815 mastocytoma cells, which peaks at 48 h of culture (36). Anti-CD3-activated T cells harvested at 48 h of culture were added at various E:T ratios to $^{51}$Cr-labeled P815 cells. As shown in Table I, RAP inhibited the induction of cytolytic activity against P815 tumor cells in a dose-dependent manner. Maximal inhibition occurred at RAP concentrations of 1 ng/ml and above. The DMSO vehicle did not affect CTL induction. RAP at a concentration of 1 ng/ml or lower had no discernible effect on T cell viability, as determined by trypan blue dye exclusion. However, at a concentration of 10 ng/ml, RAP proved to be moderately toxic to T lymphocytes. Therefore, unless otherwise noted, subsequent experiments used RAP at a final concentration of 1 ng/ml.

### *Exogenous rIL-2 restores cellular proliferation, but not cytotoxicity, in anti-CD3-activated T cell cultures treated with RAP*

Because IL-2 is known to up-regulate the expression of CTL-associated cytotoxic effector molecules (10, 11), we were curious to determine whether exogenous rIL-2 could reverse the suppressive effect of RAP on CTL induction. Not surprisingly, the addition of 100 U/ml rIL-2 to anti-CD3-activated T cells at the initiation of culture augmented the subsequent development of cytotoxicity (Fig. 1). However, the addition of exogenous rIL-2 to CTL induced with anti-CD3 mAb in the presence of RAP resulted in only a partial restoration of cytolytic function, which still remained well below control levels.

It was possible that RAP was preventing the synthesis of IL-2-induced gene products such as IFN-γ that are required for CTL development (27). In addition, induction of CTL with anti-CD3 mAb can occur through IL-2-independent as well as IL-2-dependent means (37). In this regard, TNF-α is known to be involved in the development of CTL (38). We, therefore, tested whether exogenous rIFN-γ or rTNF-α could counteract RAP-mediated inhibition of CTL induction. As shown in Figure 2, the addition of 100 U/ml exogenous rIFN-γ to anti-CD3-activated T cell cultures performed in the presence of RAP had an effect similar to that of 100 U/ml IL-2, increasing cytotoxicity but failing to completely reverse the inhibitory effect of RAP. The addition of 100 U/ml

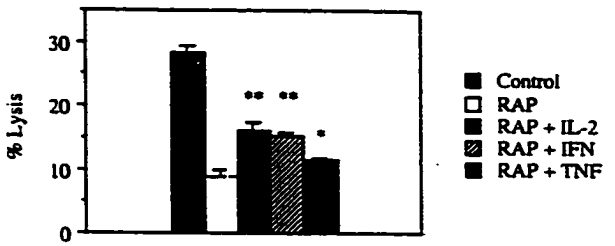


FIGURE 2. Effect of exogenous rIL-2 vs exogenous rIFN-γ or rTNF-α on RAP-mediated suppression of CTL induction. T cells were stimulated with anti-CD3 mAb in the absence or the presence of RAP (5 ng/ml) or of RAP plus rIL-2, rIFN-γ, or rTNF-α (all 100 U/ml). Following 48 h of culture, cytotoxicity against P815 mastocytoma cells was evaluated in a 4-h $^{51}Cr$ release assay. The effects of RAP on CTL viability were minimal. Data from a representative experiment ($n = 3$) are expressed as the mean percent lysis ± SD at an E:T ratio of 50:1. * indicates $p < 0.02$; ** indicates $p < 0.002$ (compared with the values for RAP-treated CTL, as determined by Student's $t$ test).

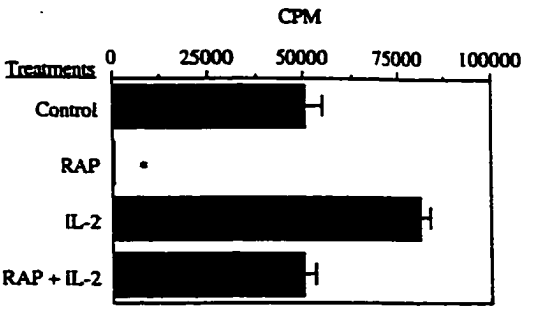


FIGURE 3. Effect of RAP and/or exogenous rIL-2 on anti-CD3-induced T cell proliferation. T cells were stimulated with anti-CD3 mAb in the presence of RAP (1 ng/ml), rIL-2 (100 U/ml), or RAP plus rIL-2. Following 48 h of culture, T cell proliferation was determined by [$^3$H]TdR incorporation as described in *Materials and Methods*. Data from a representative experiment ($n = 3$) are expressed as the mean counts per minute ± SD. * indicates $p < 0.0001$ compared with the control, as determined by Student's $t$ test.

rTNF-α resulted in a slight increase in cytotoxicity in RAP-treated CTL cultures.

Since RAP is known to delay cell cycle progression from G1 to S phase (2), one possible explanation for decreased cytotoxicity by CTL induced in the presence of RAP is that generation of cytotoxic activity is dependent on T cell proliferation. To test this hypothesis, we used anti-CD3 mAb to induce CTL in the absence or the presence of 1 ng/ml RAP, with or without 100 U/ml exogenous rIL-2. Forty-eight hours later, cellular proliferation in these cultures was measured by [$^3$H]TdR incorporation. As shown in Figure 3, RAP dramatically inhibited DNA synthesis, while exogenous rIL-2 augmented DNA synthesis in response to anti-CD3 mAb stimulation. Interestingly, exogenous rIL-2 restored cellular proliferation to control levels in anti-CD3-activated T cell culture performed in the presence of RAP. Since [$^3$H]TdR incorporation does not always reflect actual cell division, we also performed trypan blue cell counts of T cells stimulated with anti-CD3 mAb in the absence or the presence of 1 ng/ml RAP, with or without 100 U/ml exogenous rIL-2. At 48 h of culture, the cell counts were consistent with the values for [$^3$H]TdR incorporation obtained under the same experimental conditions (data not shown). Taken together, these data indicate that RAP-induced suppression of CTL induction cannot be attributed to a blockade of the cell cycle.

### *Exogenous rIL-2 rescues Gzm B and perforin gene expression from inhibition by RAP*

To better understand the mechanism by which RAP inhibits CTL induction, we examined the effect of RAP on the expression of genes associated with T cell-mediated cytotoxicity. Total cellular RNA was isolated from CTL induced in the absence or the presence of 1 ng/ml RAP, with or without 100 U/ml exogenous rIL-2, and Gzm B, perforin, and FasL gene transcription was assessed by a semiquantitative RT-PCR protocol. Time-course studies using RNA obtained at 2, 4, 8, 16, 24, and 48 h of culture revealed that in control cultures as well as in cultures treated with RAP and/or rIL-2, maximum expression of Gzm B and perforin genes occurred at 48 h of culture, while FasL mRNA synthesis peaked at 4 h of culture (data not shown). Figure 4*A* shows that 48-h expression of both Gzm B and perforin mRNA was enhanced when CTL were induced in the presence of exogenous rIL-2. Addition of RAP to anti-CD3-stimulated T cell cultures led to a marked reduction in Gzm B and perforin mRNA expression. Surprisingly, exogenous rIL-2 was able to restore Gzm B and perforin mRNA levels in RAP-treated CTL to at least control levels, even though exogenous rIL-2 could only partially reverse the inhibitory effect of RAP on cytotoxicity (Fig. 1). The same results were obtained when Gzm B gene expression in CTL induced in the absence or the presence of RAP, with or without exogenous rIL-2, was measured by Northern blot analysis (data not shown), establishing that in our hands RT-PCR yields reliable semiquantitative data. Figure 4*B* shows that RAP failed to diminish CTL expression of FasL mRNA measured at 4 h of culture. IL-2 also had no effect on FasL mRNA expression at this time point.

One possible explanation for diminished Gzm B and perforin mRNA levels in CTL induced in the presence of RAP is that RAP treatment might shorten the $t_{1/2}$ of these CTL-associated mRNA species. We, therefore, examined the effect of RAP on the kinetics of Gzm B and perforin mRNA decay in the presence of ActD to inhibit de novo RNA synthesis. Six sets of cultures (T cells plus anti-CD3 mAb with or without 1 ng/ml RAP) were set up, and RNA was extracted from the first set of cultures 48 h after initiation of culture. At this point ActD was added at 10 μg/ml to the remaining cultures, which were incubated further for 1, 2, 4, 8, and 12 h before RNA extraction. Gzm B and perforin mRNA levels were then determined by semiquantitative RT-PCR. As shown in Figure 5, the rates of decay of Gzm B (Fig. 5*A*) and perforin (Fig. 5*B*) mRNA were very similar in the presence or the absence of RAP, suggesting that decreased Gzm B and perforin mRNA levels in CTL treated with RAP are not due to the accelerated decay of these mRNA species.

Since RAP is known to prevent the translation of certain mRNA species (39) it was important to determine whether exogenous rIL-2 is able to rescue Gzm B and/or perforin protein expression by CTL induced with anti-CD3 mAb in the presence of RAP. A colorimetric enzyme assay was used to measure Gzm B activity in postnuclear cell lysates obtained from CTL induced with anti-CD3 mAb in the absence or the presence of 1 ng/ml RAP, with or without 100 U/ml exogenous rIL-2. The results of this experiment, shown in Figure 6, are in close agreement with those obtained by RT-PCR and Northern blot analysis. Gzm B activity was significantly reduced in lysates obtained from CTL induced in the presence of RAP and was elevated when CTL were induced in the presence of exogenous rIL-2. CTL treated with RAP in combination with rIL-2 exhibited greater than control levels of Gzm B enzymatic activity. Western blot analysis with a perforin-specific mAb was used to determine perforin protein levels in CTL lysates.

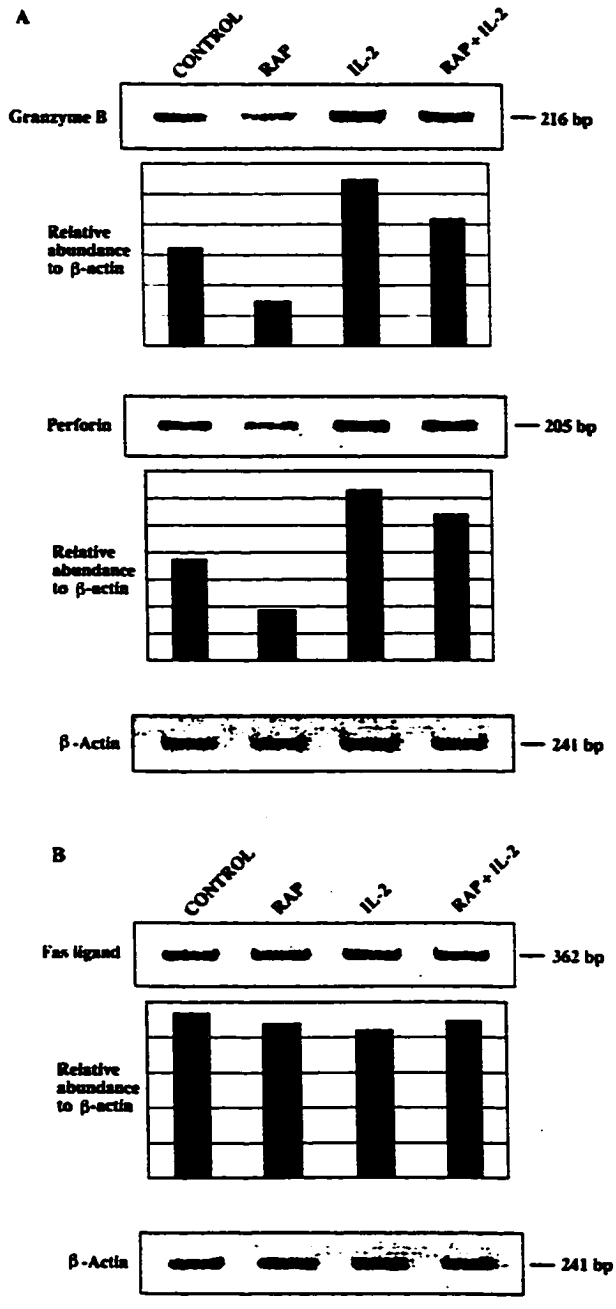


FIGURE 4. Effect of RAP and/or exogenous rIL-2 on Gzm B and perforin (*A*) and FasL (*B*) expression by anti-CD3-induced CTL. T cells were stimulated with anti-CD3 mAb in the presence of RAP (1 ng/ml), rIL-2 (100 U/ml), or RAP plus rIL-2. Following 4 h of culture, total RNA was isolated, and FasL and β-actin mRNA levels were determined by semiquantitative RT-PCR as described in *Materials and Methods*. Gzm B and perforin mRNA expression was measured after 48 h of culture. Densitometric analysis was performed to quantitate Gzm B, perforin, and FasL expression relative to the steady state expression of β-actin. Data are representative of three independent experiments.

As depicted in Figure 7, RAP inhibited perforin expression, while exogenous rIL-2 enhanced perforin expression. Perforin protein was present at greater than control levels in CTL induced in the presence of RAP plus exogenous rIL-2.

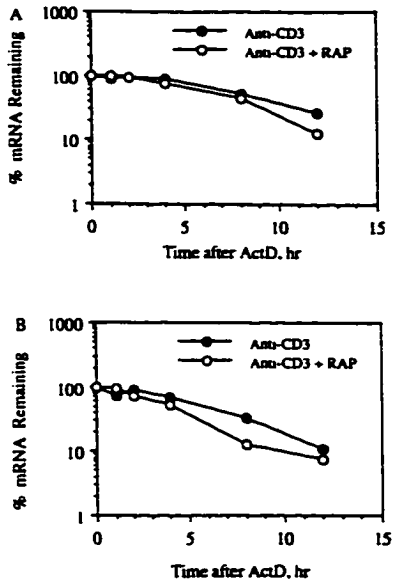


FIGURE 5. Effect of RAP on Gzm B (*A*) and perforin (*B*) mRNA stability. T cells were stimulated with anti-CD3 mAb in the absence or the presence of RAP (1 ng/ml) for 48 h. Total RNA was then extracted from the first set of cultures, and ActD (10 μg/ml) was added to the remaining sets of cultures. Gzm B and perforin mRNA expression by equivalent numbers of T cells was analyzed at the indicated time points by semiquantitative RT-PCR as described in *Materials and Methods*. Densitometric values are expressed over time as a percentage of the normalized measurements obtained at time zero (100%).

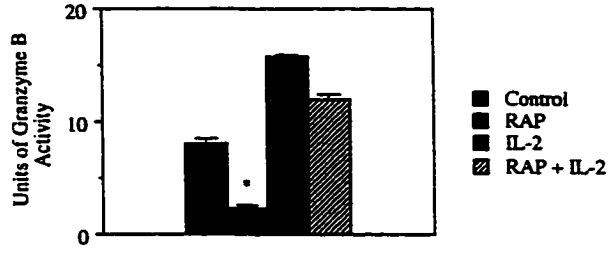


FIGURE 6. Effect of exogenous rIL-2 on RAP-mediated suppression of Gzm B protein expression. T cells were stimulated with anti-CD3 mAb in the presence of RAP (1 ng/ml), rIL-2 (100 U/ml), or RAP plus rIL-2. Following 48 h of culture, postnuclear lysates were prepared from equal numbers of T cells and added to a colorimetric reaction mixture containing synthetic Gzm B substrate as described in *Materials and Methods*. Data from a representative experiment ($n = 3$) are expressed as the mean units of esterolytic activity ± SD. * indicates $p < 0.0001$ compared with the control, as determined by Student's *t* test.

### *The percentage of CD8⁺ cells in anti-CD3-activated T cell cultures is not altered by RAP and/or exogenous rIL-2*

We have previously shown that the cytotoxic activity present in anti-CD3-activated T cell cultures resides primarily in the CD8⁺ T cell fraction (35). To rule out the possibility that the modulating effects of exogenous rIL-2 and RAP on Gzm B/perforin expression and cytotoxicity in anti-CD3-activated T cell cultures might be due to changes in the overall percentage of CD8-bearing T cells, we induced CTL in the absence or the presence of RAP and/or exogenous rIL-2 and used flow cytofluorometric analysis to determine the proportion of CD4- and CD8-bearing lymphocytes at 48 h of culture. We did not observe any change in the proportion of CD4⁺

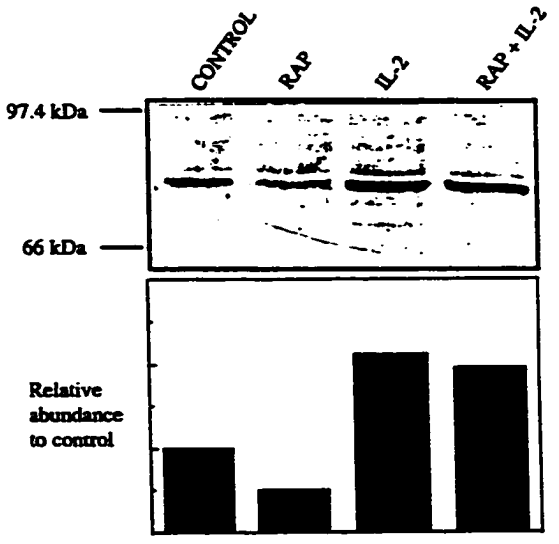


FIGURE 7. Effect of exogenous rIL-2 on RAP-mediated suppression of perforin protein expression. T cells were stimulated with anti-CD3 mAb in the presence of RAP (1 ng/ml), rIL-2 (100 U/ml), or RAP plus rIL-2. Following 48 h of culture, postnuclear lysates were prepared from equal numbers of T cells, and perforin expression was analyzed by Western blotting as described in *Materials and Methods*. Data are representative of two independent experiments.

or $CD8^+$ T cells in anti-CD3-activated T cell cultures performed under the conditions used in this study (Table II).

It was also possible that RAP might be affecting the ability of CTL to adhere to target cells, and that this effect was not reversible by exogenous rIL-2. However, cell conjugation assays revealed that CTL induced with anti-CD3 mAb in the presence of RAP plus exogenous rIL-2 adhered normally to P815 target cells (48 ± 13% conjugation vs 53 ± 5% conjugation for control untreated CTL, in one experiment representative of two independent experiments). This is consistent with the observation that control CTL and CTL induced in the presence of RAP plus IL-2 exhibited no detectable differences in cell surface LFA-1 expression by flow cytofluorometric analysis (data not shown). LFA-1 is an adhesion molecule that plays a major role in P815 target cell binding by MHC-unrestricted CTL induced with anti-CD3 mAb (40). The possibility that RAP might induce a defect in the granule exocytotic pathway that is refractory to the effect of exogenous rIL-2 was examined using a colorimetric assay that detects granule exocytosis (40). Treatment with PMA and ionomycin resulted in the normal secretion of cytoplasmic granule contents by CTL induced with anti-CD3 mAb in the presence of RAP plus rIL-2 (data not shown). Similar results were obtained when more proximal signaling molecules were activated by exposing RAP- plus rIL-2-treated CTL to anti-CD2 mAb immobilized on plastic (data not shown). Taken together, these data suggest that the cellular machinery involved in granule exocytosis is intact in these CTL.

## Discussion

It is well established that RAP is able to block certain signal transduction pathways coupled to the IL-2R of T cells by interfering with $p70^{S6K}$ activation (4). Recently, RAP has also been shown to inhibit membrane expression of the IL-2R α-chain (CD25) by activated T lymphocytes (41). Since IL-2 induces CTL to express perforin and Gzm B (10), it follows that RAP might prevent CTL

Table II. *Effect of RAP and/or exogenous rIL-2 on percentages of $CD4^+$ and $CD8^+$ T cells*

| Additions to Culture[a] | % $CD4^+$[b] | % $CD8^+$ |
|---|---|---|
| Anti-CD3 alone | 43 | 38 |
| Anti-CD3 + RAP | 42 | 38 |
| Anti-CD3 + rIL-2 | 46 | 40 |
| Anti-CD3 + RAP + rIL-2 | 45 | 40 |

[a] CTL were induced with anti-CD3 mAb in the absence or presence of RAP (1 ng/ml) and/or rIL-2 (100 U/ml).

[b] The percentage of CD4-bearing or CD8-bearing T cells was determined by flow cytofluorometric analysis as described in *Materials and Methods*. Data shown are representative of two independent experiments.

responses by down-regulating the synthesis of these cytotoxic effector molecules. In the present study we have demonstrated that CTL induced in the presence of RAP exhibit reduced levels of Gzm B and perforin mRNA. A similar reduction in Gzm B and perforin mRNA levels is observed when CTL are induced with anti-CD3 mAb in the presence of immunosuppressive concentrations of cyclosporin (36) (our unpublished observations). Since the rates of Gzm B and perforin mRNA decay in the presence or the absence of RAP were comparable, altered gene transcription is the most likely explanation for decreased Gzm B and perforin mRNA levels in RAP-treated CTL. On the basis of RT-PCR analysis and protein expression assays, RAP appears to exert a quantitatively similar inhibitory effect on perforin and Gzm B expression. However, it is noteworthy that the inhibition of gene expression was only partial (~70 and ~50% inhibition at the protein level for Gzm B and perforin, respectively), suggesting that there may be a threshold level of expression that is refractory to RAP-mediated inhibition. Nevertheless, the inhibitory effect of RAP on the expression of two important CTL-associated cytotoxic effector molecules is likely to contribute to the striking efficacy of RAP therapy in prolonging organ allograft survival in animal models of transplantation (42, 43), since CTL play an important role in allograft rejection (20), and the expression of both perforin and Gzm B by graft-infiltrating lymphocytes has been implicated in allograft rejection (14, 15).

On the other hand, RAP did not decrease FasL mRNA levels in MHC-unrestricted CTL induced with anti-CD3 mAb. This is in sharp contrast to results obtained with cyclosporin, which inhibits FasL mRNA expression by both MHC-restricted and -unrestricted CTL (44) (our unpublished observations). Interestingly, we consistently observed a low, but significant, level of cytotoxicity in RAP-treated anti-CD3-activated T cell cultures that might be mediated by the Fas/FasL cytolytic pathway, since P815 target cells are known to express low levels of cell surface Fas (45). Alternatively, low level cytotoxicity by CTL induced in the presence of RAP might simply be due to the activity of residual Gzm B and perforin. The failure of RAP to suppress FasL gene transcription may have important implications for the clinical use of RAP in situations where Fas-bearing tissues, such as heart and liver, are being transplanted (11). However, at the present time the role of Fas/FasL interactions in allograft rejection is uncertain, since cardiac allografts from Fas-deficient *lpr/lpr* mice are rejected just as rapidly as hearts from animals with normal Fas expression (46).

Our studies reveal that T cell proliferation and the expression of cytotoxic gene products following T cell activation with anti-CD3 mAb are associated with but are not directly linked to the acquisition of cytotoxic effector function. Thus, addition of exogenous rIL-2 at the initiation of culture was able to completely reverse the inhibitory effects of RAP on anti-CD3-induced T cell proliferation and Gzm B and perforin expression, but brought about only a

partial restoration of cytotoxicity. Addition of exogenous rIFN-γ or, to a lesser extent, rTNF-α at the start of culture also partially reversed the inhibitory effect of RAP on CTL development. These findings suggest that RAP is not acting by interfering with IL-2-induced synthesis of IFN-γ by T cells and are consistent with the observation that IL-2-independent as well as IL-2-dependent pathways are involved in the generation of CTL with anti-CD3 mAb (37). Interestingly, IFN-γ and TNF-α have been shown to up-regulate Gzm B and perforin expression in mouse CTL (10, 27). The addition of exogenous rIL-2 to RAP-treated anti-CD3-activated T cell cultures at the start of culture may compensate for a reduced stimulatory effect by endogenous Th cell-derived IL-2 due to a RAP-induced reduction in CD25 expression by responding T cells (41). Our findings are also in line with recent evidence that RAP-resistant signaling pathways linked to the IL-2R are sufficient to drive cellular proliferation (47). In addition, our data provide evidence, for the first time, that RAP-resistant signaling pathways are involved in Gzm B and perforin gene induction. However, at least one IL-2R-associated signal that is necessary for the development of cytotoxic effector function is RAP sensitive, since CTL induced in the presence of both RAP and exogenous rIL-2 failed to effectively kill P815 target cells despite the fact that perforin and Gzm B were expressed at greater than control levels. Although the precise nature of the activity controlled by this RAP-sensitive signal is not yet clear, we have ruled out a RAP-induced defect in the ability of CTL to bind to target cells. CTL treated with RAP in the presence of exogenous rIL-2 were also able to effectively exocytose the contents of their cytoplasmic granules following stimulation with phorbol ester and ionophore or by cross-linking CD2 with anti-CD2 mAb. We conclude that RAP does not affect signaling molecules required for granule exocytosis induced by these stimuli. However, it is still possible that RAP might interfere with the expression or the function of signaling components associated with the as yet unidentified tumor recognition receptor employed by MHC-unrestricted CTL induced with anti-CD3 mAb, and that this effect is refractory to IL-2.

There are several possible mechanisms by which RAP might interfere with Gzm B and perforin gene expression. Ligation of the IL-2R leads to activation of signal transduction pathways mediated by $p56^{lck}$ (48), c-*myc* (49), $p70^{S6K}$ (4), STAT5 (50), and *bcl-2* (47). However, only signaling pathways involving $p70^{S6K}$ and *bcl-2* are known to be sensitive to inhibition by RAP (4, 47). In this regard, $p70^{S6K}$ is known to regulate the activity of a cAMP-responsive element (CRE) binding protein (51), and CRE binding activity induced by IL-2 has been shown to be inhibited by RAP (52). Interestingly, both murine Gzm B and perforin genes contain CRE and CRE-like binding motifs in their 5′-flanking sequence (53, 54), suggesting a possible mechanism by which RAP could reduce Gzm B and perforin gene transcription. This is in line with the finding that mutations in the CRE-binding motif in the human Gzm B gene upstream region result in reduced promoter activity (55). However, a recent report indicates that CRE binding proteins are also activated via the mitogen-activated protein kinase (MAPK) signaling pathway (56). Since activation of MAPK is not affected by RAP (57), IL-2-induced activation of CRE binding proteins by MAPK, in addition to RAP-sensitive $p70^{S6K}$ kinase, may account for residual Gzm B and perforin mRNA expression by CTL induced in the presence of RAP as well as enhanced Gzm B and perforin expression in T cell cultures activated with anti-CD3 mAb in the presence of RAP and exogenous rIL-2.

It is clear that the inhibitory effect of RAP at the level of IL-2R signaling is not absolute, since RAP only partially blocked Gzm B and perforin expression, and exogenous rIL-2 added at the start of culture was able to restore Gzm B and perforin expression to at least control levels in CTL induced in the presence of RAP. These findings suggest that certain IL-2-responsive elements involved in Gzm B and perforin transcriptional control are refractory to inhibition by RAP. The IL-2R-inducible transcription factor, activating protein-1, which is unaffected by RAP (49), can bind to the 5′ regulatory region of both the mouse perforin and Gzm B genes (54, 58) and may therefore account for the failure of RAP to completely block Gzm B and perforin expression. In humans, activating protein-1 has been shown to be important in activating Gzm B gene transcription (55). IL-2R ligation is also known to activate STAT5 DNA binding proteins (50) independently of $p70^{S6K}$ activation (59). Since the IL-2 inducible DNA binding activity of STAT5 is resistant to inhibition by RAP (60), STAT5 may be involved in regulating perforin and/or Gzm B expression. In this regard, we have identified putative STAT5 binding motifs in the 5′-flanking sequence of the murine perforin gene. The possibility that mouse perforin gene transcription is regulated by STAT5 elements is currently under investigation in our laboratory.

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# IMAGE EVALUATION
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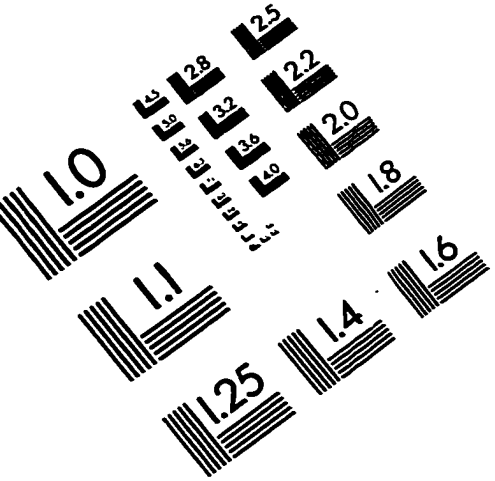


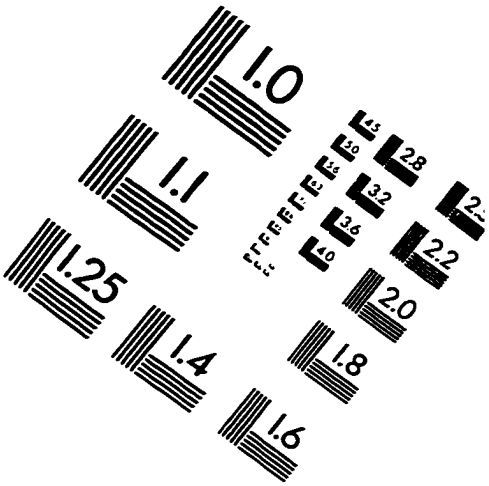


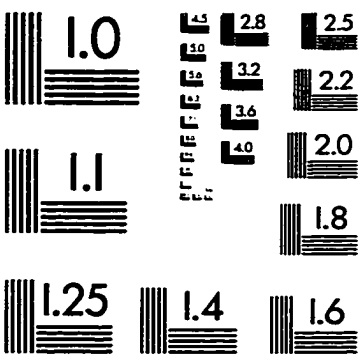


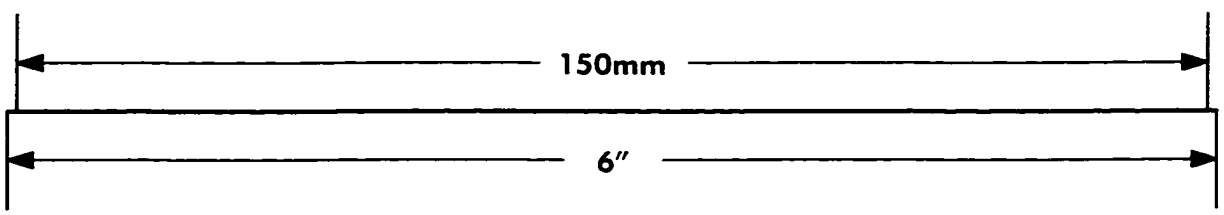


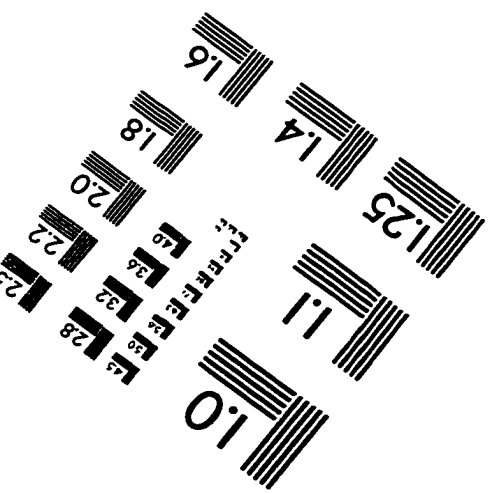


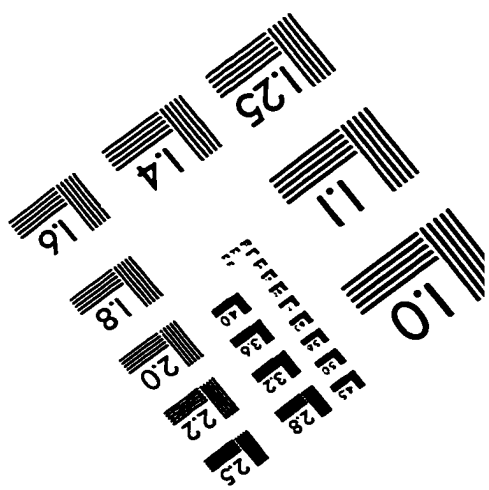


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