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The lipases of T cells and their function in cytotoxicity

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Abstract

Cytotoxic T lymphocytes (CTLs) eliminate virally infected cells and tumor cells through the use of cytotoxic proteins and death-inducing ligands. Through decades of research, scientists have identified and characterized many of these cytotoxic proteins but spent little time on phospholipases and lipases as cytotoxic enzymes released during CTL mediated killing. Pancreatic lipase related protein 2 (PLRP2) is an interesting candidate lipase found in CTLs. This dissertation has three parts: (1) determination of the role of T cell-derived lipases in tumor cell membrane degradation, (2) characterization of the induction of PLRP2 and determination as to how PLRP2 affects CTL mediated killing, and (3) investigation if CTL-derived lipases can mediate indirect toxicity toward tumor cells. During part (1), using tumor cells with radioactive lipids in their membranes, I found no evidence for gross membrane damage mediated directly by CTL lipases. In part (2), using quantitative RT-PCR I found that PLRP2 is consistently induced in CTLs by IL-4. With consistent induction of this lipase in wild type (WT) CTLs, it was possible to compare their activity with PLRP2^{-/-} (“knock out”) CTLs that completely lack PLRP2. Cytotoxicity of the WT CTLs was higher than the PLRP2^{-/-} CTLs. Attempts to match the elevated cytotoxicity with PLRP2 lipase activity left the issue unanswered. In fact, the results even suggest that PLRP2, as a nutritional enzyme in early mouse development, may affect differentiation of CD8 CTLs. During part (3), when I monitored cytotoxicity mediated directly by pure PLRP2 or indirectly by PLRP2 plus lipid substrate, there was cytotoxicity only indirectly, when lipid was present. Live CTLs expressing PLRP2 lacked lipid-dependent killing. There was a notable exception in which live CTLs could mediate lipid-dependent cytotoxicity. This exception was for CTLs repeatedly induced

with a unique lot of IL-4. Despite my many efforts, the critical properties of this lot are still unknown. In conclusion, the possibility of PLRP2 performing a direct role in T cell mediated cytotoxicity has been eliminated. My studies into indirect lipase toxicity revealed a novel lipid-dependent cytotoxic pathway induced by a yet to be identified factor. At the end of this project, an intriguing new cytotoxic mechanism awaits future investigation.

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Table of Contents

Abstract	i
Acknowledgments	iii
Chapter I: Introduction	1
Bibliography	14
Chapter II: Hydrolysis of tumor cell lipids after cytotoxic T lymphocyte (CTL)-mediated death	28
Abstract	29
Introduction	30
Material and Methods	33
Results	39
Discussion	47
Bibliography	51
Chapter III: Pancreatic lipase-related protein 2 (PLRP2) induction by IL-4 in cytotoxic T lymphocytes (CTLs) and evidence that effects of PLRP2 on cytotoxic immune function are independent of lipase-mediated damage during killing	67
Abstract	68
Introduction	69
Material and Methods	71
Results	79
Discussion	87
Bibliography	91
Chapter IV: Triglyceride lipids can enhance the cytotoxicity of induced cytotoxic T lymphocytes (CTLs)	108
Abstract	109
Introduction	110
Material and Methods	113
Results	118
Discussion	126
Bibliography	129
Chapter V: Perspectives and Conclusions	144
Bibliography	149
Appendix	150

List of Tables

1.1	Methods of Tc2 induction and Tc1 and Tc2 cytotoxicity comparison	27
2.1	Cytotoxic activities of the Tc1 and Tc2 CTLs	56
2.2	Fatty acid released from P815s under varying conditions	57
4.1	The triglyceride-enhanced cytotoxicity of IL-4 induced CTLs	133
4.2	The alternative methods of CTL induction tested	134

List of Figures

2.1 CTL release of triglyceride lipases	58
2.2 Release of ³ H-oleic acid from tumor cells after attack by cytotoxic T cells	60
2.3 The cell-free supernatant contained soluble fatty acids after CTL attack	61
2.4 Release of the oleic acid-derived radiolabel was independent of PLRP2	62
2.5 CTL release of fatty acid was perforin-dependent	64
2.6 Oleic acid release was sensitive to the lipase inhibitor tetrahydrolipstatin, regardless of the WT or PLRP2 ^{-/-} status of the CTLs	65
3.1 Induction of PLRP2 by IL-4	95
3.2 IL-4 induced CD8 CTLs contain little IL-4 or IL-5 and thus differ from the phenotype associated with type 2 (Tc2) CTLs	98
3.3 CTLs from WT PLRP2 ^{+/+} mice have more cytotoxic activity compared to PLRP2 ^{-/-} CTLs in short term killing assays, but similar cytotoxic activity in long term assays for apoptosis	100
3.4 The reduced cytotoxic activity of PLRP2 ^{-/-} CTLs is independent of Grz B production and CD8 CTL frequency	102
3.5 PLRP2 ^{+/+} and PLRP2 ^{-/-} CTLs are phenotypically indistinguishable by cell surface markers	104
3.6 mRNA encoding perforin was unaffected by PLRP2 KO in IL-4 induced CTLs	106
3.7 The cytotoxic activity of IL-4 induced PLRP2 ^{-/-} CTLs remained unchanged after rPLRP2 addition	107
4.1 PLRP2 with triglyceride lipid substrate mediated indirect toxicity toward tumor cells	135
4.2 CTLs secreted triglyceride lipase(s)	137
4.3 Triglyceride lipids enhance the cytotoxic activity of CTLs in 8 hour 51Cr-release assays	138
4.4 Triglyceride lipid enhanced the cytotoxic activity of IL-4 lot 60506 induced CTLs in long term tumor viability	139

4.5 Triglyceride enhanced CTL activity was perforin independent	140
4.6 Lot 60506 IL-4 induced CTLs produced toxic products in the presence of triglyceride lipids	141
4.7 The growth of CTLs was similar among different lots of IL-4	142
4.8 Purified colipase increased lipid-dependent cytotoxicity mediated by PTL but was without effect on the activity of IL-4-induced CTLs	143

Chapter I
Introduction

Introduction

This introduction is divided into three parts and is designed to introduce the reader to the topic of the role of lipases in cytotoxic T lymphocyte killing. Part one describes cytotoxic T lymphocytes (CTLs) and then focuses on type 2 CTLs (Tc2) which express a lipase called pancreatic lipase related protein 2 (PLRP2). Part two reviews the lipases of CTLs, including PLRP2. Part three describes how fatty acids released by lipases such as PLRP2 can kill cells. The introduction concludes with a rationale for the studies presented and a summary of results found in the following chapters of the dissertation.

Part 1. Immune control growth of tumors and viruses

Cancer is the second leading cause of death in the United States behind only heart disease and is responsible for almost one quarter of all of deaths (American Cancer Society 565,650 for 2008). Although some successful treatments for certain cancers have been developed, many cancers still have low survival rates. One area of cancer therapy, known as immunotherapy, focuses on the enhancement of the immune system using stimulatory factors. Discovering new ways to enhance the cell-mediated branch of the immune system through the induction of more potent T lymphocytes (T cells)¹ and natural killer cells (NK) continues to be a focal point of research in the field of immunotherapy.

Cell-mediated immunity is carried out by the cytotoxic NK and cytotoxic T lymphocytes (CTLs). These cytotoxic cells utilize two pathways to induce death in tumor cells. One pathway involves receptors known as death receptors on the tumor cell surface

to activate programmed death pathways found within the cell. The second pathway induces tumor cell death through the release of cytotoxic proteins from NK and CTLs. Although many of the cytotoxic proteins are known, there remain undiscovered proteins that may play a role in how these cell types induce apoptosis. Two enzyme families with the potential to function as cytotoxic mediators are lipases and phospholipases. Having lipids and phospholipids as their natural substrate, members of these enzyme families may increase membrane permeability and/or mediate damage directly. The focus of this dissertation is to characterize the known T cell lipase, pancreatic lipase related protein 2 in immune cytotoxicity, and determine the function of other T cell lipases in tumor clearance.

Adaptive cell-mediated immunity

Our immune systems have developed two pathways to protect the body from infection and cancer. These two pathways are the innate immune system and adaptive immune system. Innate immunity is always active and begins eradication of infection upon the detection of an insult. Adaptive immunity is induced later in an infection when lymphocytes are activated by specific antigens presented by the antigen presenting cells of the body. Both the innate and the adaptive immunity work collectively to clear infection and cancer from the body.

The adaptive immune system is further divided into two branches, the humoral immune system and the cellular immune system. The humoral immune system focuses on the clearance of bacterial infections through the generation of antibodies and proteins of the complement system to destroy the invading pathogen. Cellular immunity targets viral

infections and tumor cells for destruction through the use of specific activated T cells known as CTLs.

T cells and their toxic mechanisms

There are two main types of T cells which can be distinguished by their surface markers: CD4 and CD8. In this dissertation, I have examined the cytotoxic activity of CD8 T cells after they are stimulated by a cytokine to express a lipase. The cytokine used to induce expression of PLRP2 is produced by CD4⁺ T lymphocytes. The CD4⁺ lymphocytes can be divided into main subsets, T helper cells and T regulatory cells, based upon their functional roles. The T helper cells receive their name because they release the growth and differentiation factor utilized by other cells involved in the immune response. Thus they 'help' the initiation and propagation of the immune responses to infections. The T helper CD4⁺ lymphocytes are further divided into additional subsets, namely T helper 1 cells (Th1), T helper 2 cells (Th2) and T helper 17 cells based upon their different cytokine production [1-9]. The main cytokines released by the different T helper cells subsets are interferon-gamma (IFN- γ), interleukin 2 (IL-2), interleukin 5 (IL-5), interleukin 4 (IL-4), interleukin 17 (IL-17) and interleukin 21 (IL-21). Each of these cytokines has a distinct role.

The Th1 cells produce large amounts of IL-2 and IFN- γ . IL-2 acts as a growth, activation and survival factor used by T cells, B cells and natural killer (NK) cells [10-18]. IFN- γ possesses both antitumor as well as antiviral functions [19]. Along with its antitumor effect, IFN- γ also acts as a differentiation factor for T cells, driving T helper cells toward a Th1 profile [19]. Th1 cells are distinguished from Th2 cells by their high

production of both IL-2 and IFN- γ , and are associated with the induction of a cell-mediated immune response and delayed type hypersensitivity [20].

Th17 cells receive their name from the high production of IL-17 associated with these cells [8,21]. IL-17 induces other inflammatory cytokines and plays a role in recruitment and activation of neutrophils during immune responses to extracellular bacteria [9,22]. In addition to their production of IL-17, Th17 cells also produce IL-21, which exhibits signaling activity toward CD8 T cells, B cells, NK cells and dendritic cells [23].

IL-4 is the main cytokine released by Th2 cells. In this dissertation, IL-4 is the cytokine responsible for the induction of the CTL lipase PLRP2. IL-4 is a growth factor for activated B-cells and T-cells [24-28] and has the ability to cause isotype switching in B cells [29-34]. In addition to its function as a T cell growth factor, IL-4 induces the differentiation of T₀ lymphocytes into Th2 and Tc2 subsets [25,35-39]. IL-5 from Th2 cells functions to stimulate B cells to undergo immunoglobulin class switching and acts as a mediator of eosinophil accumulation and activity [40-44]. Th2 cells when activated, secrete increased amounts of IL-4 and IL-5 and have been shown to mediate B cell production of IgG1 and IgE [32,45]. Through the release of these cytokines, Th2 cells direct the immune system towards a more humoral response [1,46]. Like IFN- γ , IL-4 has been shown to aid in tumor clearance. Tumor cell lines transfected to express IL-4 had enhanced clearance when compared to the parental tumor [47-50]. In further support of these findings, IL-4 gene ablation was shown to impair CTL immunity against several carcinoma cell lines [51]. It is important to note that in contrast to the above studies, other studies have found that IL-4 impairs tumor clearance [52-54]. For perspective, IL-

4 is also produced by activated mast cells and basophils [55,56], cells that can be found throughout the body, suggesting that this cytokine is available in several sites to induce lipase in CD8 T cells.

The CD8⁺ T cells, which upon activation become known as cytotoxic T lymphocytes (CTLs), are the cells responsible for clearance of virally infected or cancerous cells. Before activation, both CD4⁺ and CD8⁺ T cells exist as non-dividing “resting cells”. Upon recognition of a specific antigen by the T cell receptor on the T cell’s surface and with concurrent stimulation by released cytokines, both CD4⁺ and CD8⁺ cells become active. CD8⁺ T cells begin to produce several cytotoxic proteins which will be stored in granules within the cell [57]. The activated CD8⁺ T cells are now known as cytotoxic lymphocytes (CTLs) and may use several mechanisms to eliminate the cancerous or virally infected cells. One mechanism employed by T cells to induce death is through the engagement of death ligands such as Fas ligand (FasL) or tumor necrosis factor-related apoptosis inducing ligand (TRAIL) displayed on the CTLs surface [58-60]. In the case of these death receptors, specific ligands will bind to the Fas or TRAIL receptors displayed on the tumor cell surface, leading to a death signal transmitted into the target cells causing cellular death through apoptosis [58,60,61]. Although an efficient method for the elimination of infected cells, several viruses and cancers cause a down regulation of Fas receptor rendering the cells refractory to this mechanism. In this dissertation, we have used 2 day tumor survival assays, mediated by CTLs without perforin, to monitor the role of lipases in death-receptor triggered death.

The second mechanism utilized by T cells to induce cell death requires the release of granules that contain cytotoxic proteins generated during the activation of the CD8⁺ T

cells. Stored within the cytotoxic granules is a pore-forming protein called perforin and a family of serine proteases known as granzymes. Perforin at high concentrations will cause extensive damage to the plasma membrane of a targeted cell. This membrane damage will cause the targeted cells to rapidly die from osmotic shock and necrosis. Although such high concentrations can be reached *in vitro* it is not the case in the normal environment of CTLs. The granzymes A, B and C [62-67] which accompany perforin within the cytotoxic granule, act additively with perforin to cause apoptotic death of the targeted cell [68]. Members of the granzyme serine protease family lack cytotoxic activity in the absence of perforin because of the granzymes' inability to cross the plasma membrane of the targeted cell [69]. It was initially thought that perforin formed a pore through which the granzyme entered the cell marked for destruction. Studies suggest that both perforin and granzymes are endocytosed by the target cell and perforin facilitates the release of the granzyme from the endosome into the cytoplasm of the target cell [69,70]. In this dissertation, I have evaluated the ability of CTL-derived lipases and purified lipases to promote perforin-dependent cell death.

Tc1 and Tc2 cytotoxic T cell subsets

Like CD4⁺ T cells, CD8⁺ T cells are divided into two subsets: Tc1 and Tc2, which is based on their cytokine production [71-73]. The designation of Tc1 is given to CD8⁺ T cells which produce the cytokines of IL-2 and IFN- γ [74,75]. CD8⁺ T cells producing IL-4, IL-5 and IL-10 are designated as Tc2 cells [28,76-78]. The differentiation of CD8⁺ T cells to either a type 1 phenotype or type 2 phenotype is based upon the cytokine stimulation the T cell receives when in an undifferentiated state known as a T₀ T cells.

Differentiation into Tc1 CD8⁺ T cells is caused by *in vitro* induction with IL-2 or IL-12 and/or IFN- γ during activation, whereas Tc2 CD8⁺ T cells are induced with IL-4 in the absence of IFN- γ [36-39]. Generally, T lymphocytes show a preference to become Tc1 effector cells rather than Tc2 as supported by *in vivo* studies. *In vivo* identification of T cells displaying Tc1 phenotypes is common, yet detection of T cells displaying a clear Tc2 phenotype is only detected under certain conditions such as cancers, chronic viral infections and autoimmune diseases [74,79-85]. In this dissertation, the CD8 T cells that were induced by IL-4 to express lipase were monitored to determine if these CTLs were polarized into standard 'Tc2' cells. (Few of the cells treated with IL-4 for 5 days had the properties of genuine Tc2 cells.)

Cytotoxic ability of Tc1 vs. Tc2. Since their discovery, Tc2 cells have been studied *in vitro* to determine their role in cellular immunity. Similar to Tc1 cells, Tc2 cells have perforin and granzymes, yet Tc2 T cells have been shown to have varying levels of cytotoxicity. The cytotoxic activity of Tc2 cells has been found in some cases to be lower than the cytotoxic activity of Tc1 cells [78,83,86-88]. In other experiments, Tc2 cytotoxic activity was found to be equal to the cytotoxic activity of Tc1 cells [38,39,89]. This difference in killing seems to depend on the method used to generate the Tc2 cells *in vitro* [38] (Table 1.1). Although different cytokine patterns are displayed by Tc2 cells, the killing mediated by Tc2 cells (in the short time of 4 hours) is primarily carried out by perforin [90-92]. In addition to this primary method of killing, Tc2 cells display similar ability to mediate cell death through Fas ligand as Tc1 cells [93].

Anti-tumor activity of Tc1 vs. Tc2 cells. Several studies into the anti-tumor activity of Tc2 cells have been performed [38,87,88,91,92,94,95]. Although the adoptive transfer of tumor-specific Tc2 were shown to clear lung metastases, other *in vivo* studies show Tc2 CTLs to have less ability to clear tumors than their Tc1 counterparts [95]. Using the OVA-transfected B16 melanoma lung metastasis model, Dick Dutton and colleagues found that Tc2 cells are 5 fold less effective at tumor clearance when compared to Tc1 cells in adoptive transfer studies of OVA Ag-specific Tc1 and Tc2 cells [90-92,94]. In these studies, Tc2 cells accumulated at the site of the tumor and induced tumor regression, although both accumulation at the tumor site and regression were lower than that seen with Tc1 cells [90,91,94]. Another antitumor study, by Lenardo and colleagues, found Tc1 cells to have higher levels of *in vivo* survival after transfer than Tc2 cells, suggesting that the increased tumor rejection induced by the Tc1 cells may be due to long term survival [96].

Although the killing mechanism used by Tc2 cells for tumor clearance is still unknown, it has been previously shown that tumor eradication is greatly affected by the presence of IFN- γ in studies using IFN- γ deficient mice [38,88,92,94]. Since Tc2 cells express IFN- γ at much lower levels than Tc1, it is believed that the lack of IFN- γ coupled with the suppressive effects of type 2 cytokines are the reason for Tc2 cells slower rate of tumor eradication rather than direct cytotoxic activity of the Tc2 cells [88,92]. The cytotoxic activity of Tc1 vs. Tc2 CTLs is relevant to this dissertation because I found that in the presence of lipid, the Tc2 cells can actually be more activity than the Tc1 cells (see chapter 4).

Potential for different cytotoxic mechanisms by Tc1 versus Tc2 cells. Tc2 cells differ from Tc1 cells in the proteins they produce [97]. Early studies into the differing effects of IL-2 and IL-4 induction of T cells by Glimcher et. al (Cell 1990) discovered that isolated T cell lines induced with IL-4 had mRNA expression for a pancreatic lipase like protein [98]. The gene encoding this pancreatic lipase was cloned and compared to the genomic database where it was determined that it encoded the pancreatic lipase known as PLRP2 [99].

Part 2. The lipases of CTLs, including PLRP2.

Lipases of CTLs.

PLRP2

PLRP2 is a member of the pancreatic lipase family produced by acinar cells of the pancreas and by duodenal enterocytes of the gut. The pancreatic lipase family contains four members; pancreatic triglyceride lipase (PTL), pancreatic lipase related protein 1, pancreatic lipase related protein 2 and pancreatic lipase related protein 3 (PLRP1, 2, 3). Lipases of the pancreatic family are essential for the breakdown of major ingested dietary lipids. These lipases will hydrolyze triglycerides at the sn1 and/or sn3 positions releasing fatty acids during digestion [100]. Studies on PLRP2 enzymatic function found that during digestion PLRP2 is secreted by acinar cells into the gut where it degrades triglycerides and other dietary fats into fatty acids [101-105]. Since only fatty acids and not triglycerides can cross the gut into circulation, this lipid breakdown is essential for fats to be nutrients. Although PLRP2 has similar structure and homology to the other members of the pancreatic lipase family, PLRP2 differs from the other members of the

pancreatic lipase family enzymatically. Like other members of the pancreatic lipase family, PLRP2 will release fatty acids from positions 1 & 3 of attachment to the glycerol backbone [101,106]. PLRP2 also has phospholipase A1 activity toward, phospholipids [107-110], and galactolipids [110,111].

In addition to its high expression in the pancreas and enterocytes, PLRP2 expression has also been found in the testes and two cells types having immune function, Paneth cells and in T cells [98,101,112]. It is thought that PLRP2 presence in the testes may have antimicrobial effects in the urethra. Support for this hypothesis was found in studies of goat semen. PLRP2 was identified in goat semen and was shown to reduce spermatozoa viability during long term storage [113-115]. Studies focused on the identification of the mechanism responsible for the reduction in spermatozoa viability found that PLRP2 was incapable of direct toxicity. However, PLRP2 generated toxic concentrations of fatty acids released from lipids used in the extender storage solutions. It was these studies in goat sperm that caused us to speculate that PLRP2-positive lymphocytes might have cytotoxicity that would be mediated only in the presence of exogenous lipid substrates for PLRP2.

Studies directly focused on the T cell function of PLRP2 used PLRP2 deficient mice (PLRP2^{-/-} mice) [116]. T cells from these mice were tested *ex vivo* after restimulation with IL-2 and antigenic cells to determine what the effects of losing PLRP2 would be on the T cells ability to kill [116]. PLRP2^{-/-} T cells had less cytotoxicity toward tumor cells compared to the cytotoxicity of T cells from wild type (WT) mice in the same assay. In this dissertation, I demonstrate that T cells from PLRP2 deficient mice also had a reduced ability to affect the lipid membrane of P815 target cells labeled with ³H-oleic

acid, suggesting that PLRP2 has activity against the plasma membrane of cells targeted for destruction by CTLs (chapter 2). Since most of the cellular label was in phospholipid, it is probable that the WT PLRP2-encoded enzyme was working as a phospholipase rather than a triglyceride lipase in this situation. These results suggest that PLRP2, having phospholipase A1 activity, could have direct or indirect cytotoxic effects toward tumor cells.

Part 3. Fatty acids and their potential for toxicity toward cells

The studies of PLRP2 toxicity toward spermatozoa suggest that PLRP2 may mediate indirect toxicity through the generation of increased concentrations of fatty acids. For a lipase plus lipid to be toxic, one or more of the products must be toxic if the lipase alone is nontoxic. These products include soluble fatty acids. Fatty acids (carboxylic acids) are esterified in triglycerides membrane lipids or waxes to cholesterol esters [117,118]. Fatty acids can be found in unbranched and branched forms and can be further characterized based on the bonding patterns found within their carbon chain. If no alkenyl groups are present in the carbon chain the fatty acids are known as saturated fatty acids. Fatty acids with one (monosaturated), two (disaturated), or more (polyunsaturated, PUFAs) alkenyl groups are designated as unsaturated [117,118].

Early studies into the effects of fatty acid treatments on tumor cells showed that pretreatment of tumor 'target' cells with fatty acids made the tumors more susceptible to both complement-mediated cytolysis and NK cell-mediated cytotoxicity [119,120]. Later studies into the effects on tumors grown in the presence of fatty acids near physiological

concentrations revealed that the fatty acids could be toxic to several cancer cell lines [121-130]. These studies revealed increased apoptotic activity as well as increased necrosis of cells grown in the presence of PUFAs. Tumor cells incubated with fatty acids have been observed to undergo DNA fragmentation and chromatin condensation [121,122]. Pro-apoptotic protein activities such as caspase activation and P53 gene up-regulation are observed when tumor cells were incubated with fatty acids at increased concentrations [121,122,125,130]. Studies into the apoptotic effects of fatty acid have discovered an increase in the generation of reactive oxygen species and an increase in nitric oxide synthesis [121,122,131,132]. Although it has been well characterized that tumor cells grown in fatty acids undergo apoptosis, the mechanism through which fatty acids induce apoptosis is still under investigation. A potential pathway for the apoptotic effects of fatty acids maybe through the interaction of fatty acids with the peroxisome proliferator-activated receptors (PPAR) [133-136]. Tumor cells treated with agonists to the PPAR γ and PPAR α receptors were found to undergo apoptosis [137-140]. These findings further support the possibility of fatty acids using the same mechanism to induce apoptosis.

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Table 1.1. Methods of Tc2 induction and Tc1 and Tc2 cytotoxicity comparison.

Reference	T cell source	Activation method	Cytokines used	Tc2 cytotoxicity vs. Tc1 cytotoxicity	Method of measurement
38	C57B6 and transgenic mice for LCMV fragment	Anti-CD3/28 or LCMV Peptide	Tc2: 20 U/ml IL-2, 10ng/ml IL-6 and 10ng/ml (100 U/ml) IL-4. Tc1: 20 U/ml IL-2 and 10 ng/ml IL-6.	Tc2 ≥ Tc1	3 to 5hr JAM assay with EL4 cells
39	C57B6 mice	Irradiated allogeneic M12.4.1 tumor cells	Tc2: 25 ng/ml (250 U/ml) IL-4, 500 U/ml IL-2 and 50 µg/ml anti-IFN γ . Tc1: 500 U/ml IL-2, 5 ng/ml IFN γ and 50 µg/ml anti-IL-4.	Tc2 = Tc1	4 hr ⁵¹ Cr release assay
77	C57B6 mice	Plate bound antibody mixture of Anti-CD3/Anti-CD8/Anti-CD11a	Tc2: 120 U/ml IL-2 and 23 ng/ml (230 U/ml) IL-4 and 1 µg/ml anti-IFN γ . Tc1: 120 U/ml IL-2.	Tc2<Tc1	4 hr ⁵¹ Cr release assay
85	C57B6 mice	Plate bound antibody mixture of Anti-CD3/Anti-CD8/Anti-CD11a	Tc2: 20 U/ml IL-2, 23 ng/ml (230 U/ml) IL-4 and 1 µg/ml anti-IFN γ Tc1: 20 U/ml IL-2.	Tc2<Tc1	4 hr ⁵¹ Cr release assay
86	Ova Specific OT-I and OT-II	Irradiated OVA-DC cells	Tc2: 20 U/ml IL-2, 20ng/ml IL-4, 10µg/ml anti-IFN γ Tc1: 20 U/ml IL-2, 5ng/ml IL-12 and 5µg/ml anti-IL-4.	Tc2 < Tc1	6hr ⁵¹ Cr release assay against EG7 OVA targets
87	C57B6 and transgenic mice for LCMV fragment Tc2 induced with:	Anti-CD3/28	Tc2: 20 U/ml IL-2, 10ng/ml IL-6 and 2000 U/ml IL-4. Tc1: 10U/ml IL-2, 10ng/ml IL-6.	Tc2<Tc1	3 to 5hr JAM assay with EL4 cells
88	C57B6 mice	10 ng/ml PMA with or without 250 ng/ml Ionomycin	Tc2: 200 U/ml IL-2 and 500 U/ml IL-4 Tc1: 200U/ml IL-2	Tc2>Tc1	4 hr ⁵¹ Cr release assay
82	HIV seropositive patients	PHA + irradiated allogeneic spleen cells	CD8 ⁺ T cells were isolated from tissue (skin biopsy) and allowed to expand in 20 U/ml IL-2. Determination of Tc1 or Tc2 phenotypes determined by intracellular IFN γ and IL-4 production	Tc2<Tc1	4 hr ⁵¹ Cr release assay

Chapter II

Hydrolysis of tumor cell lipids after cytotoxic T lymphocyte (CTL)-mediated death

ABSTRACT

Contributions of lipases to cytotoxic T lymphocyte (CTL) function have been debated, including if T cell lipases damage target cells. Expression of pancreatic lipase related protein 2 (PLRP2) was previously found in IL-4 cultured lymphocyte cell lines but absent from IL-2 cultured lymphocytes. Here we evaluated IL-2 and IL-4 induced CTLs for hydrolysis of target cell lipids and killing. Using anti-CD3 redirected lysis of ^3H -oleic acid labeled P815 tumor cells, we detected the release of the radioactive fatty acid. When PLRP2^{+/+} and PLRP2^{-/-} CTLs were compared there was more killing by the PLRP2^{+/+} CTLs. However, ^3H -oleic acid release was similar per dead P815, suggesting that lipid hydrolysis was produced by the dead P815s rather than by PLRP2. The fatty acid release and death were completely dependent on perforin, and also occurred when P815s were killed by perforin-containing T cell granule extracts that lacked lipase activity. Death by the cytotoxic granules extracts was unaffected by the addition of lipases. A lipase inhibitor, tetrahydrolipstatin, blocked fatty acid release without affecting CTL-mediated cytotoxicity. Also, CTL-mediated death caused as much fatty acid release as death by disruption of cells by freeze-thawing. The released oleic acid may be sufficient to promote secondary apoptotic responses after CTL-induced trauma.

INTRODUCTION

Cytotoxic T lymphocytes (CTLs) function in the immune system to clear tumors or virally-infected cells. In their arsenal, CTLs possess biochemical weaponry for several methods of killing, including release of the potent proteins such as perforin and granzymes for the exocytosis-induced pathway [1] and engagement of the plasma membrane Fas ligand for the Fas death receptor pathway [2]. Contributions of lipases to CTL function have been debated since 1971 when Koren *et al.* [3] observed release of oleic acid from dying P815 target cells. Lipase activities that are activated only by perforin-dependent CTL killing remain to be characterized, while lipase activities activated by the Fas pathway have been characterized as phospholipases A2 and C as well as diacylglycerol lipase [4]. The possibility that lipases may be secreted as killing enzymes is supported by the expression of a lipase, pancreatic lipase-related protein 2 (PLRP2), that is induced in CTLs by IL-4 [5], a cytokine that supports type 2 cytotoxic T lymphocyte (Tc2) differentiation [6]. Additionally, knockout of the PLRP2 gene results in reduced killing capability [7]. As of yet, the exact function of PLRP2 in activated T cells remains unknown; however, the lipase is a promising candidate as a membrane-damaging enzyme because it can hydrolyze multiple lipids, including triglycerides [8], phospholipids [8-10] and galactolipids [11,12]. Furthermore, cell death produced by IL-2 induced CTLs, which lack PLRP2 expression [5], results in substantial hydrolysis of lipids of the tumor cells 'targeted' for killing [13]. Our paper extends the key observations by Kleinfeld *et al.*[13] concerning IL-2 induced CTL-initiated release of target cell fatty acids to the examination of IL-4 induced PLRP2-associated hydrolysis of the tumor cell lipids.

Despite the cited observations, the connection between general lipase-mediated membrane hydrolysis and T cell-mediated cytotoxicity remains unclear. Several tantalizing, but unexplored, ideas in T cell immunology could potentially be explained by lipases. For example, it is still unknown exactly how perforin and granzymes are transferred from a CTL into the target cell cytoplasm. Traditional models involve perforin-dependent plasma membrane pores through which granzymes can diffuse. However, at physiological conditions, perforin alone is unable to produce pores with sufficient diameter to allow granzyme entry [14]. Lipases from the CTLs may be able to alter these pores and facilitate granzyme entry into cells. Other models involving perforin-induced cell death are based on evidence that granzymes can enter cells through receptor-mediated endocytosis, but the internalized granzymes cannot be released from the endosomes without a permeabilizing agent such as perforin [14]. Again, lipases from the CTLs could have a cytotoxic function, potentially co-localizing in and disrupting the granzyme-laden endosomes. Lipases of the target cells may also be directly involved in the induction of apoptosis. For example, TNF- α may induce apoptosis in part by activation of target cell phospholipase A₂ enzymes, although there is conflicting evidence on the connection between lipid hydrolysis and apoptosis [15,16]. Thus, lipases contributing to cell death can be from either the CTL or the target (or from both). In this study, we tested this possibility using the lipase inhibitor tetrahydrolipstatin.

We have selected immunological and biochemical approaches to address the potential for lipases of different CTLs to damage tumor cell membranes. We activated mouse T cells with concanavalin A to induce CTLs from all T cells regardless of their antigen-restricted specificity and cultured the cells with either interleukin 2 or interleukin

4. Also, we detected lysis by CTLs regardless of their antigen specificity, by using an anti-CD3 antibody redirected method to initiate lysis [17]. Thus, by using concanavalin A activation and redirected lysis, we were able to characterize the rapidly induced lipases of CTLs and avoid the variables associated with long-term differentiation that occurs during the time needed *in vitro* to produce large numbers of T cells specific for a single antigen. Wild type, PLRP2^{-/-}, or perforin (Pfn^{-/-}) CTLs were used to evaluate T cell-derived lipase. The PLRP2^{-/-} CTLs were used to determine the role of this lipase in CTL killing and the perforin^{-/-} CTLs were used to determine if perforin lesions were required for target cell lipid hydrolysis.

CTL released lipases were detected two ways. The first was to monitor the release of any triglyceride lipases (including PLRP2) by T cells after stimulation to secrete proteins and to release granules [18]. The second approach was to assay both triglyceride and phospholipase activities by monitoring hydrolysis of lipids of P815 cells, using radiolabeled oleic acid that is incorporated into many different triglycerides and phospholipids. For the second approach it became necessary to extract the cell-free supernatants to distinguish between soluble fatty acids and intact lipids associated with small apoptotic vesicles that are also found in the supernatants [3,13,19]. Here we report that both IL-2 and IL-4 induced CTLs release triglyceride lipases. Cells dying after IL-2 induced CTL and IL-4 induced CTL attack release substantial amounts of fatty acid. However, it appears that during CTL-mediated killing, both CTL lipases and lipases of the target cells fail to contribute to tumor cell death.

MATERIALS AND METHODS

Cytotoxic T lymphocytes (CTLs) and tumor cells.

CTLs were generated from mouse splenocytes, from either BALB/c mice (Jackson Laboratories, Bar Harbor, ME, USA), PLRP2^{-/-} BALB/c [7] and their PLRP2^{+/+} littermate controls (from Dr. Mark Lowe at the University of Pittsburgh, USA), C57BL6 WT or Pfn^{-/-} mice (order #003505, *Pfn^{tm1Sdz}*, Jackson laboratories). T cells were stimulated and grown in complete RPMI 1640 media (Sigma, USA) containing 10 mM HEPES buffer (Sigma), 24 mM sodium bicarbonate (Fisher Laboratories, Waltham, MA, USA), 1% penicillin/streptomycin (Sigma), 10% fetal calf serum from Hyclone (Logan, USA), 25 μ M 2-mercaptoethanol (Sigma), 2.5 ug/ml concanavalin A (Sigma), and 500 units/ml of either mouse recombinant interleukins 2 (2.9×10^{-9} M) or 4 (1.4×10^{-9} M) from eBiosciences (San Diego, USA) or BD Biosciences (San Jose, CA, USA). The specific activity of r-IL-2 was 1×10^7 units/mg (1.7×10^{11} units/mmol); the specific activity of r-IL-4 was 2.5×10^7 units/mg (3.5×10^{11} units/mmol). Following culture for three days at a concentration of 0.5×10^6 cells per ml, CTLs were passaged into mitogen-free complete media with the respective cytokines. Cells were incubated at 37°C and 5% CO₂. eGFP-P815 mastocytoma tumor cells [20] were maintained in continuous passage in Dulbecco's modified Eagle/s medium, DMEM, (Gibco, USA) with 10% fetal calf serum.

Lipase assays.

Triglyceride lipase assays. Lipase activity was determined by release of fatty acid from ³H labeled triolein. An assay solution containing 30 mM Tris-HCl (Fisher), 1

mM CaCl₂ (Sigma) at pH 8.5 and 0.32 mM total triolein with 0.312 mM unlabeled (from 1.016 M stock, Sigma) mixed with ³H-triolein (from 0.1mM stock, spec. act. 52.6 Ci/mmol, Perkin Elmer (Waltham, USA) at a 50:1 ratio. The micelles were incubated with the cell-free supernatants or granule extracts of CTLs. Assays were incubated at 37°C and stopped at 1 hour. Fatty acids were extracted using chloroform/ methanol/ heptane (14.5 parts / 12 parts /10 parts) (Fisher) and made basic with a pH 10, 50 mM carbonate solution. The aqueous phase was formed by centrifugation at 2600 rpm (1040x g) for 5 minutes at room temperature. From the top phase 200 µls were collected, mixed with 500 µls of scintillation cocktail (MicroScint-20 Perkin Elmer) and counted in a Beckman scintillation counter for 3 minutes per sample. To calibrate and standardize the activity of different assays, a titration of rPLRP2 from 50 ng to 1.6 ng was carried out and stopped after 10 minutes to create a standard curve. Release of CTL lipases was triggered by either a combination of phorbol myristic acid (PMA) (100 ng/ml, Sigma) and ionomycin, (1 µg/ml, Sigma) or by bead-bound anti-CD3 antibody (clone 2C11, eBiosciences).

Inhibition of r-PLRP2 by tetrahydrolipstatin. Recombinant (r-) human PLRP2 [10] (a generous gift from F. Carriere, Ph.D.) was inhibited with the irreversible lipase inhibitor tetrahydrolipstatin [21] (Sigma). The inhibitor was dissolved in 100% dimethyl sulfoxide (DMSO, Sigma) and then added to r-PLRP2 in HEPES buffered saline with 1 µg/ml bovine serum albumin at varying concentrations up to 8 µg/ml and incubated for 10 minutes at 37°C. Following this incubation, the lipase was assayed with Di-FMU octanoate [22] (Invitrogen, Carlsbad, CA USA).

[³H]-oleic acid release from labeled P815 tumor cell lipids. A feature of P815 cells, as mast cell-derived tumors, facilitates T cell-mediated killing regardless of the CTLs' antigen specificity. P815s have immunoglobulin Fc-Ig receptors that bind antibodies, in this case specific to CD3 epsilon, a signaling component of the T cell receptor for antigen. Anti-CD3 epsilon bound simultaneously to both the T cell CD3 and to the P815 Fc-Ig receptors triggers CTL lysis, cytotoxic granule release and secretion of other T cell mediators. P815 cells were labeled overnight with 25 μCi ³H-oleic acid (Movarek Biochemicals, Brea, CA USA) per million cells in DMEM media containing 1 mg/ml delipidated BSA (Sigma). Labeled P815 cells were washed and mixed with 2 $\mu\text{g/ml}$ anti-CD3 clone 2C11 monoclonal antibody (eBiosciences) to support CTL-redirected lysis. The assay media used was complete RPMI media with 1 mg/ml delipidated BSA. In quadruplicate assays, 10^4 P815 cells were added in a 100 μl volume to wells of 96-well microtiter plates (Becton Dickinson, Fullerton, USA). CTLs were added to each well in a 100 μl volume at varying effector:target ratios. Following four hours of incubation, the cell-free supernatant was separated by centrifugation at 1200 rpm and the released ³H-oleic acid (and any labeled lipids in small apoptotic bodies) was measured by liquid scintillation. The total CPM labeled was measured by adding 1% SDS detergent to labeled tumor cells. Spontaneous release was measured with an equal volume of media in place of CTLs. Percent specific release of oleic acid was calculated as follows:

% specific release = [(Experimental CPM – Background CPM) / (Total CPM – Background CPM)] * 100.

For lipase inhibition, tetrahydrolipstatin (Sigma) was first dissolved in DMSO and then added to the assays at 10 µg/ml final concentration. To determine maximal endogenous lipase activity in the tumor cells, 10^5 radiolabeled P815 cells in 1 ml of RPMI + 1 mg/ml BSA were 2x frozen in liquid N₂, thawed, and then incubated for 4 hours as below for the cytotoxicity assays.

To monitor labeled fatty acid release following death caused by extracts of isolated cytotoxic granules, we used extracts of cytotoxic lymphocyte granules from rat RNK-16 cells [23] at a concentration of 0.057 mg/ml (final 280 hemolytic units/ml) and oleic-acid labeled P815 cells. The cells were incubated with the perforin-containing granule extracts for 4 hours in the presence of the metabolic inhibitors 5 mM 2-deoxyglucose (Sigma), 1.5 mM NaN₃ (Sigma) and 1 mM KCN (Sigma) to increase the cellular susceptibility to isolated perforin [24].

Purified bovine pancreatic triglyceride lipase was obtained from Sigma, and bovine lipase colipase was obtained from AbD Serotech (Raleigh, USA). These lipases were used in combination with cytotoxic granule extras. Without granule extracts, these proteins lack cytotoxicity and the ability to hydrolyze lipids of intact target cells.

⁵¹Cr cytotoxicity assays.

Lysis of target cells was determined by redirected lysis in a similar manner to the ³H-oleic acid release assays with several differences: P815s were labeled with Na₂⁵¹CrO₄

(Perkin-Elmer) [17,25] and 100 μ l of supernatant was collected for detection in a Cobra-II auto gamma counter. In order to determine if the oleic acid release assay was proportional to the dead cells (as opposed to the lymphocyte cell density), in some experiments we normalized fatty acid release to the number of dead cells, using the ratio of WT and PLRP2^{-/-} CTL lytic activities (in lytic units) as a correction factor to make the number of dead cells equivalent. Lytic units were calculated as number of lytic units per 10×10^6 lymphocytes, with 1 lytic unit defined as the number of lymphocytes required to lyse half (5×10^4) the target cells [26].

Determination of fatty acids vs. apoptotic membrane fragments released after CTL-mediated killing.

The ³H radiolabel released into the cell free supernatants could be either fatty acids or lipid vesicles from apoptotic cells[27]. Lipids and fatty acids were separated by extraction with organic solvents [28,29]. In 24-well plates (Corning), ³H-oleic-acid labeled, 10^5 tumor cells and 8×10^5 CTL effectors were incubated in 1 ml volumes with or without anti-CD3 antibodies. Following a four hour incubation, the cells were collected, centrifuged at 28,000x g in a microcentrifuge, and the supernatant and cell pellet were separated by decanting. Cell pellets were resuspended in 1 ml of chloroform. The supernatant or the cell pellet (200 μ l) was added to a fresh mixture of chloroform:methanol:heptane (12.5/10/14) in 4 ml glass vials and vortexed. Fifty millimolar carbonate pH 10 (250 μ l) was added to the mixture and vortexed. The glass vials were centrifuged using a Thermo IEC-7000M (International equipment company, Needham, USA) at 1040 x g for 5 minutes to separate the phases. The aqueous (upper)

and organic (lower) phases (100 μ l) were removed for scintillation counts using a Beckman scintillation counter instrument.

Correction factors were made for the fatty acids remaining in the chloroform phase. For determination of fatty acid and triglyceride partitioning, 5 μ Ci 3 H-oleic acid (Movarek Biochemicals) or 5 μ Ci 3 H-triolein (Perkin Elmer) were brought up in 1 ml RPMI containing 1 mg/ml BSA and extracted as above. Corrections were made to the experimental aqueous phase scintillation data by adding the percentage of oleic acid predicted to remain in the organic layer. The equations are listed below:

1. Total CPM as fatty acids = (Experimentally determined CPM in the aqueous layer) / (the fraction of total oleic acid normally partitioning into the aqueous layer)
2. Total CPM as lipids = (Experimentally determined CPM in the organic layer) – (the oleic acid partitioned into the organic layer)

Statistical analyses.

Results are expressed as mean \pm SD of the collected data. Statistical evaluations of the data were performed with Excel and Sigma plot software. The differences in mean values were evaluated by Student's T-test or when required ANOVA One-Way analysis.

Differences were considered significant if the P was <0.05 .

RESULTS

CTLs release lipases.

IL-2 induced CTLs (Tc1 CTLs) have higher cytotoxicity than IL-4 induced CTLs (Tc2 CTLs) and might also differ in their release of lipases. Evidence supporting a difference is found with the presence of the pancreatic lipase, PLRP2, which is induced with the type 2 cytokine, IL-4. We have determined that IL-4 induced CTLs rapidly and selectively express PLRP2 within 4 days of activation, while IL-2 induced CTLs lack PLRP2 expression (chapter 3).

To determine if CTLs release lipase activity, day 6 IL-2 induced CTLs and IL-4 induced CTLs were stimulated to undergo exocytosis via stimulation with solid phase anti-CD3 antibodies. At 0 and 2.5 hours, the T cell-free supernatants were collected and assayed for triglyceride lipase activity. Both IL-2 induced CTLs and IL-4 induced CTLs released lipase activity (Figure 2.1). There was measurable activity in the tissue culture media alone and in the media removed from the CTLs at the initiation of culture (T_{zero}). The IL-2 induced CTLs secreted activity without anti-CD3 stimulation, which was decreased in recoverable activity after anti-CD3 induced stimulation. In contrast, the IL-4 induced CTLs secreted more recoverable lipase activity after anti-CD3 stimulation. In 3 of 6 experiments with IL-4 induced CTLs from WT and PLRP2^{-/-} littermates, the released lipase was greater for the WT; however, the differences had a P values >0.05. Thus, we were unable to detect recoverable lipase attributable to PLRP2 and the identities of the lipases are unknown. The anti-CD3 stimulation was accompanied by release of granzyme proteases (not indicated).

IL-2 induced CTLs and IL-4 induced CTLs release radiolabeled fatty acids and/or lipids from tumor cells during killing.

The next issue was whether death mediated by CTLs would be accompanied by major hydrolysis of tumor target cell lipids, particularly since perforin damage occurs at the plasma membrane and apoptosis might leave organelles like lipase-containing lysosomes intact. Tumor cell lipids were labeled with ^3H -oleic acid and used to monitor release of oleic acid during killing. Parallel assays were performed with CTLs and P815 tumor targets labeled with ^{51}Cr to monitor cell death. IL-2 induced CTLs and IL-4 induced CTLs effected substantial release of ^3H -oleic acid from tumor cell membranes that paralleled cytotoxicity (Figure 2.2 A & 2B). As might be expected, the IL-2 induced CTLs were more active killers than the IL-4 induced CTLs cells [30]. Both oleic acid release and cytotoxicity required the presence of anti-CD3 antibody that was needed to support the redirected lysis. The release of oleic acid suggests that both types of CTLs can release lipid products as either fatty acids, generated by lipases and/or as lipids associated with apoptotic bodies or membrane vesicles. The IL-2 induced CTLs had more lytic activity per T cell (Figure 2.2A). IL-2 induced CTLs also released more oleic acid-radiolabel than the IL-4 induced CTLs (Figure 2.2B) when compared at the same effector to target cell (E:T) ratios.

The ^3H -oleic acid radiolabel released into the supernatants represents both fatty acid products and membrane-associated lipids.

Zhang, et al. showed that released radiolabel in the supernatant of apoptotic cells (killed by etoposide stimulus) that was purported to represent detection of fatty acids, was

actually reflective of floating apoptotic bodies with intact lipids as well as reflective of soluble fatty acids that were produced by cellular PLA₂ [27]. We wanted to distinguish oleic acid released by lipase activities from the membrane fragments and vesicles of dead cells. To determine the fraction of the cell-free supernatant that was truly fatty acid, we performed chloroform:methanol:heptane extractions to isolate the aqueous phase fatty acids. In preliminary studies, we found that radiolabeled triolein and phospholipids were completely retained in the organic phase and that oleic acid partitioned 84-90% in the aqueous layer and 10-16% in the organic phase. Extractions of the cell-free supernatant indicate that roughly half of the released radiolabel was soluble fatty acids (Figure 2.3A). We speculate that the other half of the released label, the radiolabel retained in the organic phase, might be apoptotic bodies. When we used centrifugation to precipitate apoptotic bodies and membrane vesicles from the cell-free supernatant, about 50% of the radiolabel released by IL-2 induced CTLs and tumor cells at an E:T of 8:1 with anti-CD3 was precipitated (not illustrated), consistent with half of the released radiolabel as membrane-bound vesicles. An increase in effector to target ratio to 32:1 resulted in an enhanced fatty acid to lipid ratio present in the supernatant (not illustrated). Extractions of the cell pellet demonstrated that the majority of biologically incorporated fatty acid radiolabel (95% or greater in all conditions) partitioned into the organic layer, reflecting label incorporated into phospholipids or triglycerides (Figure 2.3B). CTL-mediated cytotoxicity resulted in loss of radiolabel from cell pellet concurrent with the release of soluble radiolabel from dead cells into the supernatant. The dead cells and cell fragments of the targets incubated with CTLs and anti-CD3 in Figure 2.3B retained less radiolabel than the targets without CTLs or without anti-CD3. Thus, the cell-free supernatants

contained substantial fatty acids, as originally reported by A. Kleinfeld, as well as substantial membrane fragments associated with cell death that contained un-degraded lipids. Similar results were observed with cells killed by IL-4 induced CTLs. Based on the specific activity of the oleic acid, the soluble fatty acid concentrations reached 1.7×10^{-9} M concentrations after 2.5 hours of killing at lymphocyte to P815 ratios of 8:1. The volume of the soluble fatty acids was 1 ml for 5×10^5 target cells, while physiologically these cells would probably be surrounded by 2 μ l or less tissue fluid creating the potential for local oleic acid production to exceed 200 μ M in 2.5 hours. (This concentration becomes significant for fatty-acid induced apoptosis, see discussion.)

³H-oleic acid release is proportional to the number of dead P815 cells and is PLRP2-independent.

An important issue is whether the release of the fatty acid-derived radiolabel was mediated in part by the CTL lipase, PLRP2. To address this issue, we questioned whether ³H-oleic acid radiolabel released would differ between PLRP2^{+/+} and PLRP2^{-/-} CTLs when normalized to a constant number of dead P815 cells. More activity by the WT CTLs would be consistent with PLRP2 activity. PLRP2^{+/+} CTLs generally displayed more ³H-oleic acid release when compared to the oleic acid released by the PLRP2^{-/-} CTLs at similar E:T ratios, regardless of whether the CTLs in question were IL-2 induced CTLs or IL-4 induced CTLs (Figure 2.4 A, 4B). Reduction in cytotoxicity by the PLRP2^{-/-} CTLs, as previously reported, was quantified for this experiment and other representative experiments, using short-term ⁵¹Cr release assays and lytic units (Table 2.1). Differences in cell death mediated by IL-4 induced PLRP2^{+/+} CTLs vs. PLRP2^{-/-}

CTLs were greater than for WT compared to KO IL-2 induced CTLs, and were 3.5 fold better for WT IL-4 induced CTLs than PLRP2^{-/-} cells (Table 2.1). To address the issue of whether the lipases came from the IL-4 induced CTLs or from P815s (or from both cells), we normalized the ³H-oleic acid release to the number of dead P815 cells with the idea that PLRP2 activity would be reflected by more lipid hydrolysis by PLRP2^{+/+} IL-4 induced CTLs than PLRP2^{-/-} IL-4 induced CTLs. When the correction factor (of 3.5) was applied to the ³H oleic acid release mediated in IL-4 induced CTL killing in Figure 2.4D, there was similar release of label per dead P815 cell, killed by WT or by PLRP2^{-/-} CTLs. Thus, overall, the data of Figure 2.4 are consistent with the majority of lipase being independent of PLRP2, and potentially being from P815 cells rather than T cells. Later we illustrate that dead P815s have substantial auto-degrading lipase activity.

The fatty acids released from P815 cells may be caused by endogenous lipase activities.

Both apoptosis and necrosis can activate endogenous lipases. To assess further whether the lipase activity was derived from CTLs or from dead tumor cells, we evaluated an extreme model for endogenous lipases released using cells that were 100% disrupted at the start of incubation and then incubated for the same time as the CTL assays. We compared the ³H-oleic acids released by live P815 cells alone, by 2X frozen and thawed P815 tumor cells, by cytotoxic granules with P815 cells, and by tumor cells after CTL-mediated killing (Table 2.2). The fatty acid released by live P815 cells alone were ~2% of all the ³H-oleic acid incorporated, while in the extreme case of 100% cell death caused by freezing and thawing the P815s, another 2% of free fatty acids were

released by endogenous lipases. When CTLs mediated the killing (at 8:1), a total of ~5% free fatty acids were released, with similar release mediated by either IL-2 induced CTLs and IL-4 induced CTLs. Using granules isolated from cytotoxic lymphocytes, we tested if fatty acids release could be carried out by the action of extracts of cytotoxic lymphocyte granules containing perforin that alone will disrupt plasma membranes and induce tumor death. To increase P815s tumor cells susceptibility to the cytotoxic granule extracts, the P815 cells were treated with 5 mM 2-Deoxy-D-glucose, 1.5 mM NaN_3 and 1 mM KCN to hinder cellular repairs of cytotoxic granule mediate damage [24]. Granules at a concentration of 0.0574 mg/ml resulted in 74% cell death in the pretreated P815s and mediated a release of 9% of the total fatty acid. Treated P815s alone released 6% of the total fatty acid, indicating that cytotoxic granule-mediated cell death yielded a 3% specific release of fatty acids. The granule extracts lacked lipase activities as evaluated by 2 assays. Furthermore, addition of 1 $\mu\text{g/ml}$ of rPLRP2 or 2 $\mu\text{g/ml}$ pancreatic triglyceride lipase (PTL) in combination with excess colipase (10 $\mu\text{g/ml}$) failed to increase the cytotoxicity of the cytotoxic granules although these lipases did increase the oleic acid release (data not illustrated). Thus the death by CTL attack activates endogenous P815 lipases and there would have been sufficient un-hydrolyzed substrate to observe additional lipases.

CTL-associated P815 lipase activity was perforin dependent.

To determine if the observed lipase release is a result of CTL-mediated cellular death of the tumor cell, we used $\text{Pfn}^{-/-}$ CTLs to prevent target cell lysis (and release of endogenous P815 lipases). In the absence of perforin, triggering of CTL cell receptors

for antigen causes cytotoxic granule proteins, including granzymes, to be exocytosed but the tumor cells remain intact (as measured by ^{51}Cr release, not illustrated). Lipase activities are released by these cells upon anti-CD3 stimulation (data not illustrated). We compared the release of fatty acids using both the IL-2 induced and IL-4 induced Pfn^{-/-} CTLs. There was no detectable release of radiolabel (over that secreted by the live P815 cells) by either IL-2 induced or IL-4 induced Pfn^{-/-} CTLs. These data indicate that the IL-2 induced CTL and IL-4 induced CTL lipases were unable to mediate substantial hydrolysis of lipids within intact tumor target cells (Figure 2.5).

Tetrahydrolipstatin, an inhibitor of the pancreatic α/β hydrolase fold-containing subclass of lipases, affects the lipases of P815 cells and the lipase activity associated with CTL-mediated cytotoxicity.

We next asked whether the release of P815 fatty acids was reflective of a lipase activity and if tetrahydrolipstatin (THL) inhibition of lipase activity would decrease the cytotoxic activities of either IL-2 induced CTLs or IL-4 induced CTLs. THL, (also known by the pharmaceutical name Orlistat^R) is an irreversible lipase inhibitor that binds to Ser₁₅₂ in the catalytic triad of lipases of the α/β hydrolase fold containing lipases [31-33]. THL has been also shown to inhibit PLRP2 activity [9] and under the pH of our tissue culture media, at 8 $\mu\text{g}/\text{ml}$ (3.2×10^{-7} M) THL inactivated 88% of r-PLRP2 in 10 minutes (not illustrated). Both endogenous P815 lipase activities and CTL-initiated lipase activities were inhibited by THL. When ^3H -oleic acid radiolabeled P815 cells were disrupted by freeze-thawing, and then incubated for 4 hours with or without 10 $\mu\text{g}/\text{ml}$ THL, the lipase activity, which released 4% of total radiolabel, was inhibited 43%

(not illustrated). At 10 $\mu\text{g/ml}$, THL dramatically inhibited the release of radiolabel mediated by either IL-2 induced or IL-4 induced CTLs of either WT or PLRP2^{-/-} origin (Figure 2.6 A-D). Thus, there were active lipases of P815 cells or CTLs, other than PLRP2, that were sensitive to THL. The cytotoxic activity of IL-2 induced CTLs and IL-4 induced CTLs was unaffected by treatment with THL (Figure 2.6 E-F). It should be noted that the induction of cytotoxicity occurs in only minutes [34] and that by comparison, THL was a slow inhibitor. Thus, THL was able to inactivate the lipases that substantially degraded P815 lipids but might have been insufficient to reduce any rapidly induced biological functions that might be dependent upon the T cell lipases. Furthermore, THL was unsuitable as a probe to distinguish PLRP2 from other cellular lipases.

DISCUSSION

In this study, we centered our attention on lipase damage to tumor cell membranes after CTL-mediated cytotoxicity and on the function of the IL-4 induced CTL-associated lipase, PLRP2. We found evidence showing the release of lipase activity by both IL-2 induced CTLs and IL-4 induced CTLs. Using ^3H -oleic acid labeled P815 targets we observed the release of fatty acids after CTL mediated killing by both IL-2 induced CTLs and IL-4 induced CTLs. The contribution of PLRP2 from IL-4 induced CTLs to the hydrolysis of the lipids of the tumor cells was undetectable. We determined that a significant proportion of the lipid hydrolysis could be produced by endogenous tumor lipases when cells were disrupted in the absence of CTLs and that, when cells remained intact after anti-CD3 conjugation with $\text{Pfn}^{-/-}$ CTLs, the tumor cell lipids were refractory to the CTL lipases. These two observations suggest that CTL lipases have modest (if any) general lipase activity toward the P815 cell lipids. It should be noted that we found that r-PLRP2 is stable in the presence of granule extracts containing active granzyme proteases (not illustrated), so it is likely that released PLRP2 lipase would remain intact. PLRP2 activity would also be expected to be low as the tumor lipids contain only low amounts of the triglycerides and phosphatidylethanolamine (compared to phosphatidylcholine), which are the lipids that PLRP2 favors as substrates [8,35]. Thus the issue remains open whether PLRP2 mediates any lipid hydrolysis when IL-4 induced CTLs deliver their cytotoxic mediators.

Our data suggest the presence of another CTL lipase or multiple lipases that are secreted by IL-2 induced CTLs and by IL-4 induced CTLs; however, the observations with the $\text{Pfn}^{-/-}$ CTLs also suggest that these lipases are also unable to hydrolyze the lipids

within the membranes of intact P815 cells. We observed oleic acid release from disrupted tumor targets, when burst by freezing and thawing or when attacked by CTLs. However, the major questions that persist are (I) whether all the lipase actually originates from the dead tumor cells and (II) whether CTL lipase(s) play a role in cytotoxicity or have another yet-to-be-discovered function. Answers may be attainable when the tumors are derived from animals that are genetically ablated for the endogenous lipase(s). These animals remain to be derived. Although our tetrahydrolipstatin experiments indicate that tumor death is unaffected even by an inhibitor concentration sufficient to block the released lipase activity by 8-fold, which would suggest that the lipase is irrelevant to death, caution is needed before concluding that the CTL lipases are totally irrelevant to cell death. CTL killing can occur within minutes following T cell receptor for antigen (TCR) engagement [34], and inactivation of CTL lipase may be too slow to affect cytotoxicity. Tetrahydrolipstatin is a slow acting inhibitor [36] and thus may be an inadequate probe. When new lipase-deficient tumors and better lipase inhibitors become available, the issue of the roles of lipases in CTL cytotoxicity should be reevaluated.

The fact that perforin was required for detectable lipase activity suggests that the lipases may be acting in conjunction with cytotoxic CTL molecules. Perforin may act to initiate cell membrane damage by forming foci as start sites for lipid hydrolysis. We have found that PLRP2 is located separately from the dense cytotoxic granules containing perforin (chapter 3) and so synchronized release of PLRP2 and cytotoxic granules would be necessary if PLRP2 were one of the lipases. If release were synchronized, the sulfated proteoglycans of the cytotoxic granules [37] would be available to bind PLRP2, which binds well to heparin [38].

In this dissertation, we detected lipases that are released by IL-2 induced CTLs and IL-4 induced CTLs and noted differences in that only the IL-4 induced cells seemed to release lipases after T cell antigen-receptor stimulation. Little of this lipase appears to be PLRP2, which means that our experiments with the PLRP2-deficient CTLs provide no insight into functions of this lipase. Of note is the observation that perforin^{-/-} IL-4 induced CTLs were effective against tumors *in vivo* [39]. It is known that IL-4 induced Tc2 cells secrete a different cytokine profile from IL-2 induced Tc1 cells, secreting IL-4, IL-5, IL-6, and IL-10 [6]; however, cytotoxicity is independent of these cytokines. Furthermore, adoptively transferred immune anti-tumor IL-4 induced Tc2 CTLs were able to increase the primary host IL-2 induced Tc1 CTL response to tumors [39], indicating that Tc2 CTLs may orchestrate immune events as well as kill tumor cells. The IL-4 induced T cell lipase(s) may have a role in these events. Previous studies have been focused on lipases of IL-2 induced CTLs, partly because IL-2 supports T cell growth and partly because IL-4 will depress expression of perforin and granzymes [40], proteins that support short term lytic assays that can be easily measured *in vitro*.

The biological significance of PLRP2 found in IL-4 induced CTLs remains a mystery. It may be relevant that PLRP2 of goat semen mediates toxicity, but only when provided exogenous lipid substrates are available to support generation of toxic byproducts such as oleic and linoleic acid. The goat spermatids remained viable in the presence of PLRP2 alone, and triglycerides alone, but their acrosomal membranes were disrupted when PLRP2 was co-incubated with triglycerides [41,42]. The fatty acid products of physiologically available triglycerides, oleic and linoleic acids, can mediate apoptosis of tumor cells but these fatty acids required more than 24 hours for death to

occur [43-47]. Thus it is possible that PLRP2 released by CTLs could produce lipid byproducts to affect tumor cell survival indirectly, provided that the appropriate triglyceride substrates are available *in situ*. Our future experiments will explore this possibility.

The most striking finding of our studies is the amount of fatty acid release from cells killed by lymphocytes. The findings with IL-2 induced CTLs corroborate the findings of Anel A[48] and Kleinfeld *et al.*, [13,19]. Our data with the IL-4 induced CTLs are novel and indicate similar release per dead cell, regardless of whether it was killed by an IL-2 induced or an IL-4 induced CTL. Our interpretation, shared by Dr. Kleinfeld [13] who also used oleic acid-radiolabeled targets cells, is that the lipases are of target cell origin. These lipases appear to be activated by cell death, even when the original injury started at the plasma membrane. Thus, CTL-killing releases fatty acids that will become immediate mediators of inflammation. Elevated concentrations of oleic and linoleic fatty acids above 100 uM (with albumin from 10% serum) cause cells to undergo apoptosis [45-47,49]. Thus fatty acids released by cells dying from specific CTL attack, provided that the fatty acids exceed the buffering capacity of localized albumin [50], have potential to promote additional death of adjacent cells.

Chapter 2 bibliography

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Table 2.1. CytoCytotoxic activities of the IL-2 induced CTLs and IL-4 induced CTLs

Cytotoxic activity of IL-2 induced CTLs and IL-4 induced CTLs (PLRP2 ^{+/+} vs. PLRP2 ^{-/-})					
Lytic Units/10M PLRP2 ^{+/+} CTLs		Lytic Units/10M Balb/c PLRP2 ^{-/-} CTLs		Ratios PLRP2 ^{+/+} CTLs vs PLRP2 ^{-/-} CTLs	
<i>PLRP2^{+/+} IL-2 induced</i>	<i>PLRP2^{+/+} IL-4 induced</i>	<i>PLRP2^{-/-} IL-2 induced</i>	<i>PLRP2^{-/-} IL-4 induced</i>	<i>IL-2 induced</i>	<i>IL-4 induced</i>
2049	386	1276	110	1.6	3.5

*The lytic activities of IL-2 induced CTLs and IL-4 induced CTLs generated from PLRP2^{+/+} and PLRP2^{-/-} mice were measured by 4 hour ⁵¹Cr release. Ratios of the lytic activity were used to determine the fold difference in lytic activity exhibited by PLRP2^{+/+} and PLRP2^{-/-} for both the IL-2 induced CTLs and IL-4 induced CTLs. This experiment is representative of 5 experiments.

Table 2.2. Fatty acid released from P815s under varying conditions

Fatty acid released from P815s under varying conditions			
	IL-2 induced CTLs	IL-4 induced CTLs	cytotoxic granule extract
Control ^a	1.80%	2.00%	6.00%
2x Freeze/thaw ^b	4.16%	4.02%	N/D
8:1 ^c	4.97%	5.18%	N/D

*The fatty acids were extracted from the cell-free supernatants at the end of 4 hour incubations of the cells. ^aP815 were labeled with ³H-oleic acid and allowed to incubate alone for 4 hours. ^bRelease of fatty acids by P815 cells after 2x freeze/thaw cycles

^cRedirected lysis was at an effector to target ratio of 8 to 1. For IL-2 induced cells 83% of the P815 cells were killed and for IL-4 induced cells 67% of the P815 cells were killed. The data are representative of two similar experiments. ^dCytotoxic granule extract (0.0574 µg/ml) was added to P815 targets that were treated with 5 mM 2-deoxyglucose, 1.5 mM NaN₃ and 1 mM KCN 5 mM to increase tumor cell sensitivity to perforin. The lysis by granule extracts was 74%, respectively.

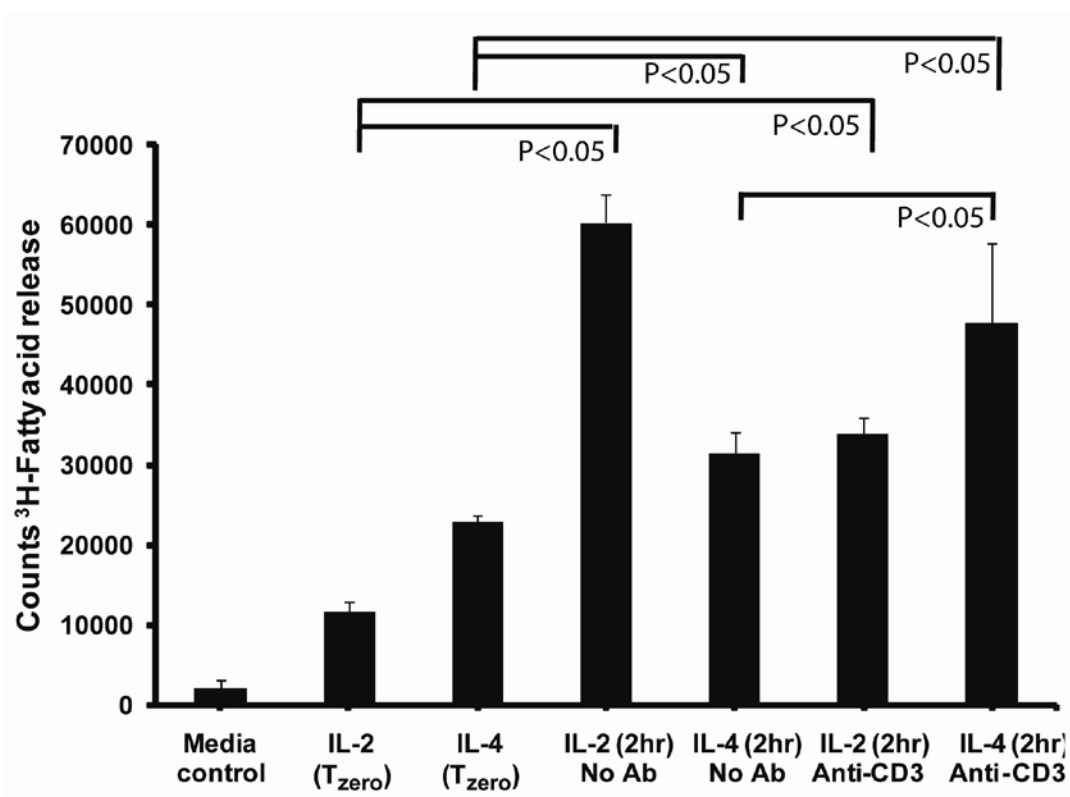


Figure 2.1. CTL release of triglyceride lipases. Splenocytes were activated and cultured with 500 u/ml of either murine r-IL-2 or r-IL-4. CTLs were incubated for 2.5 hours with immobilized anti-CD3 to stimulate the T cell receptors for antigen and trigger exocytosis of cytotoxic granules and protein secretion. Each lipase assay contained 3.2 million cell equivalents. As a control for endogenous lipase activity present in the media and contributed by the washed CTLs, cell-free supernatants were taken at the zero time (T_{zero}) points. The cell-free supernatants from the T_{zero}, IL-2 induced cells and IL-4 induced cells after stimulation, were concentrated and immediately assayed for lipase activity using radioactive triolein for 60 minutes at 37°C. IL-2 induced CTLs secreted a constitutive lipase activity that was decreased after anti-CD3 stimulation. In contrast, IL-

4 induced cells secreted detectable lipase activity after anti-CD3 induced exocytosis. All P-values were determined using ANOVA One-Way Analysis.

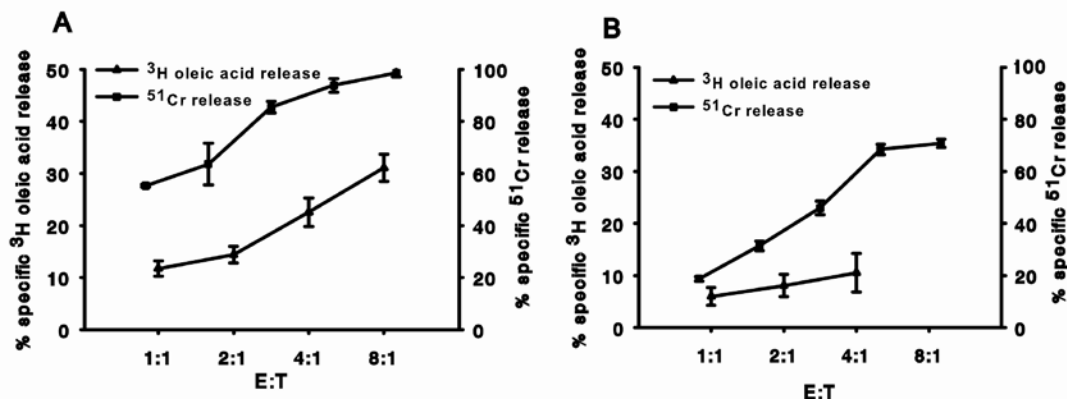


Figure 2.2. Release of ^3H -oleic acid from tumor cells after attack by cytotoxic T cells. P815 cells were incubated overnight with ^3H -oleic acid to label their lipids or labeled with ^{51}Cr for 4 hours to monitor cell death. The ^3H -radiolabel released into the cell-free supernatant was monitored as an initial indication of lipase activity (that would release oleic acid). Subsequently we found that apoptotic bodies and membrane vesicles contributed to ~50% of the ^3H -radiolabel in these supernatant (see Figure 2.3). Release was proportional to the ratio of effector CTLs to P815 target cells (E:T). IL-2 induced CTLs or IL-4 induced CTLs, derived from BALB/c spleens and stimulated with either 500 U/ml IL-2 (A) or 500 U/ml IL-4 (B), respectively, were used to redirect lysis of ^3H -oleic acid-labeled (triangles) or ^{51}Cr labeled P815 tumors (squares).

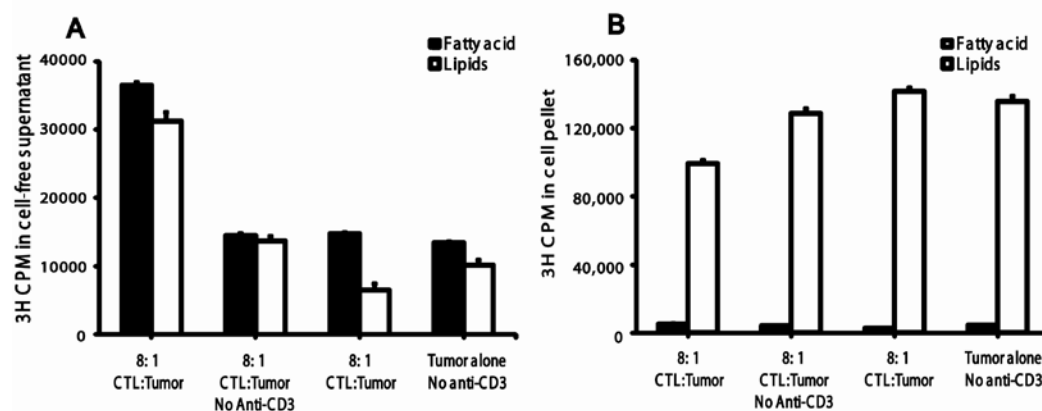


Figure 2.3. The cell-free supernatant contained soluble fatty acids after CTL attack.

A: Extractions of the cell-free supernatant indicated that roughly half of the released label was water-soluble oleic acid and that oleic-acid-containing lipids were also elevated in the supernatants after cell death. B: Extractions of cell pellets yielded nearly all oleic acid into the organic phase, where lipids partitioned. A loss in the cell-associated lipid counts was observed after redirected lysis.

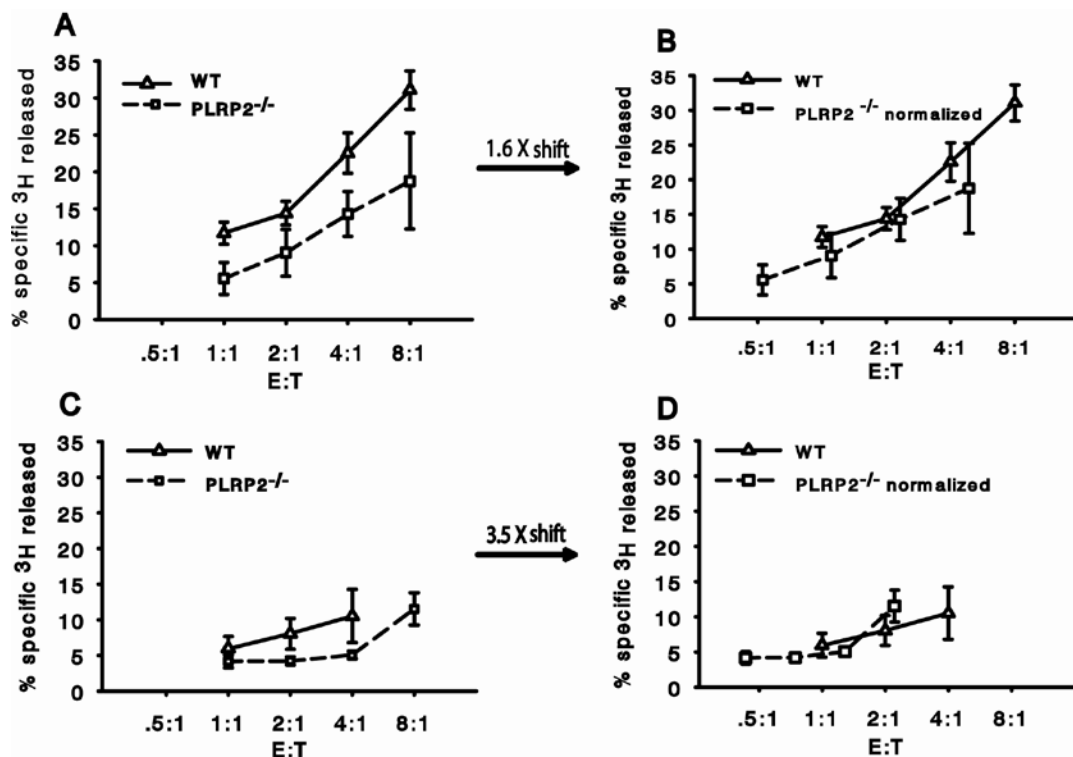


Figure 2.4. Release of the oleic acid-derived radiolabel was independent of PLRP2.

CTLs derived from either PLRP2^{+/+} (solid line) or PLRP2^{-/-} (dashed line) mice were cultured with 500 U/ml IL-2 (A and B) or 500 U/ml IL-4 (C and D) and used for redirected lysis of ³H oleic acid-labeled P815 cells (A-D). In the right column (B & D), the oleic acid release has been normalized to the death of the tumor cells, such that the graphs represent the amount of fatty acid released relative to the number of cells killed determined by ⁵¹Cr release assays. The arrows between the left and right columns indicate the correction factor used to normalize the oleic acid release to the number of dead cells. The correction factor was determined from the fold reduction in death exhibited by PLRP2^{-/-} CTL when compared to PLRP2^{+/+} CTLs (Table 1). The WT CTLs with the PLRP2 gene were more lytic than the PLRP2^{-/-} CTLs, particularly after culture

with IL-4 to induce PLRP2 (3.5X more lytic). However, when the oleic acid release was normalized to cell death (see 3B and 3D), PLRP2 had insignificant effects on the amount of lipid radiolabel released during killing.

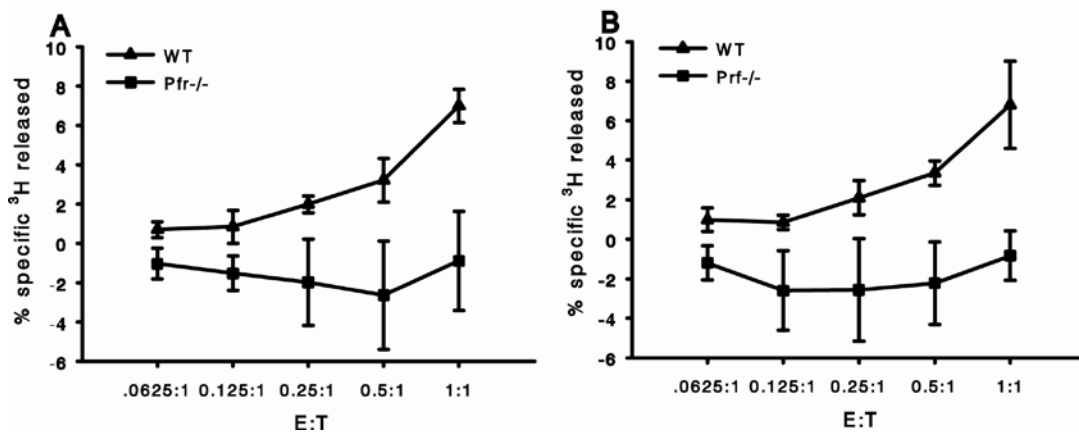


Figure 2.5. CTL release of fatty acid was perforin-dependent. CTLs were derived from WT and perforin knockout mice of the same C57BL/6 background, using conditions described for Figure 2.2 and assayed for redirected lysis of ³H-oleic acid-labeled or ⁵¹Cr labeled P815 cells. There was substantial ⁵¹Cr lytic activity for the WT IL-2 induced CTLs and IL-4 induced CTLs that was totally absent from the Pfn^{-/-} CTLs (not illustrated). (A & B): In the absence of perforin, release of oleic acid was also undetectable for both IL-2 induced CTLs and IL-4 induced CTLs even at the highest ratio E:T ratio of 1:1.

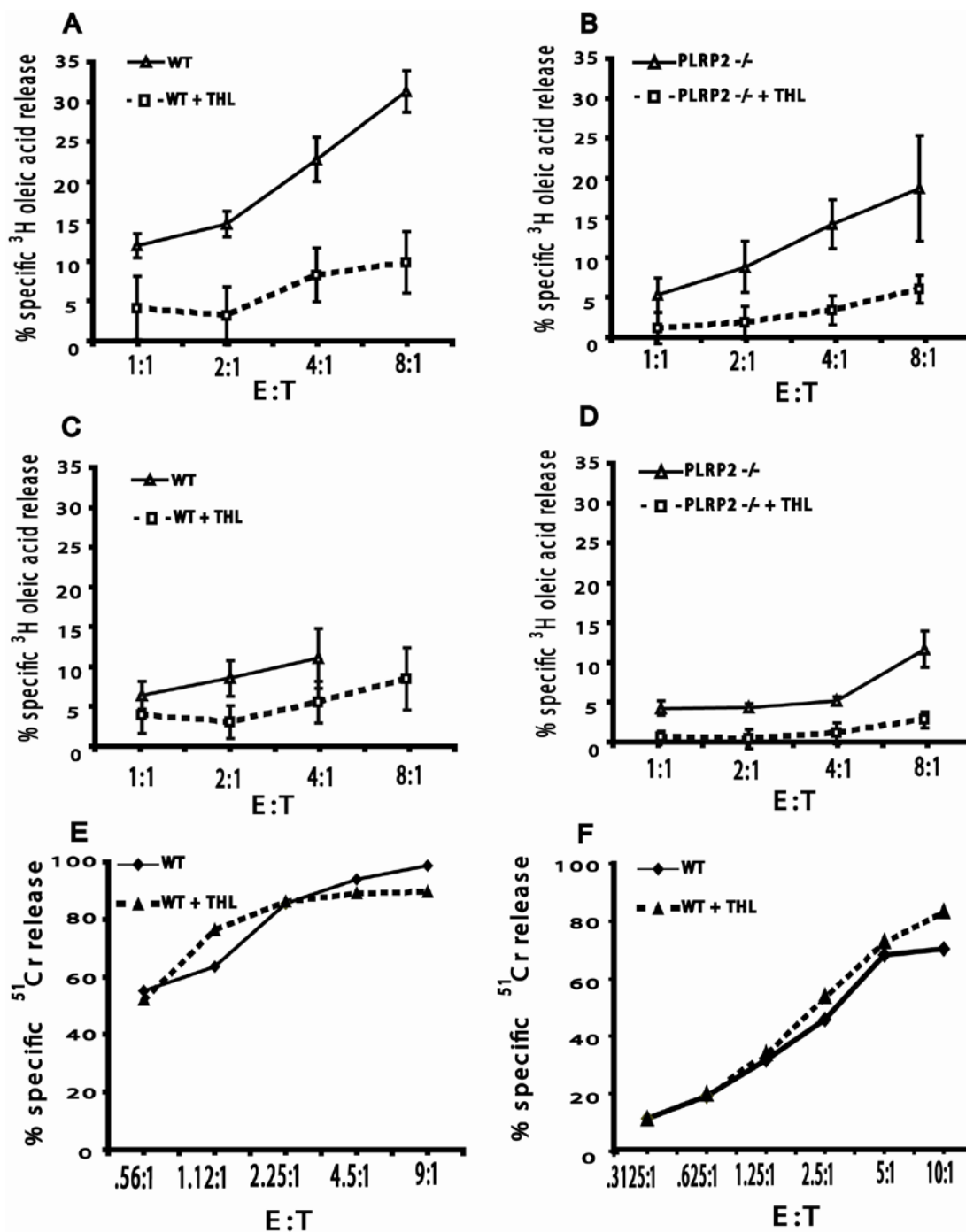


Figure 2.6. Oleic acid release was sensitive to the lipase inhibitor tetrahydrolipstatin, regardless of the WT or PLRP2^{-/-} status of the CTLs. However, tetrahydrolipstatin lacked effects on CTL-mediated cytotoxicity. CTLs were derived and tested as in Figure

2.2. Inhibited assays received 10 $\mu\text{g/ml}$ tetrahydrolipstatin (dashed line), while control assays received DMSO as a control for the solvent added with THL (solid line). Both WT (A & C) and PLRP2^{-/-} CTL release of oleic acid (B & D) were affected by THL. In (E & F), the P815s were labeled with ⁵¹Cr to monitor CTL-mediated lysis in the presence or absence of 10 $\mu\text{g/ml}$ tetrahydrolipstatin.

Chapter III

Pancreatic lipase-related protein 2 (PLRP2) induction by IL-4 in cytotoxic T lymphocytes (CTLs) and evidence that effects of PLRP2 on cytotoxic immune function are independent of lipase-mediated damage during killing.

ABSTRACT

Based on observations that pancreatic lipase related protein 2 (PLRP2) expression is induced by IL-4 in cytotoxic T lymphocyte (CTL) clones and that *in vivo* immunized wild type (WT) PLRP2^{+/+} CTLs are more cytotoxic than PLRP2^{-/-} CTLs, we investigated how the lipase PLRP2 might influence CTL functions. First, PLRP2 was induced in splenocytes by 3.5×10^{-8} M IL-4 by day 6 after activation and was restricted to CD8⁺ T cells. PLRP2 expression was detected inconsistently (and at low levels) with IL-2. IL-4 driven PLRP2 was unaffected by IFN- γ . Next, we monitored other variables that might affect cytotoxicity. Granzyme B levels, activation markers and CD8⁺ T cell frequencies were similar for WT vs. PLRP2^{-/-} CTLs (with either cytokine). Cytotoxicity in 4 hour ⁵¹Cr assays of WT CTLs was ~3 fold the activity of PLRP2^{-/-} CTLs cultured with IL-4 and was also ~2 fold the activity of PLRP2^{-/-} CTLs cultured with IL-2. Thus, disruption of the PLRP2 gene affected short term perforin-dependent cytotoxicity, even under the IL-2 conditions. Addition of recombinant PLRP2 to IL-4 induced PLRP2^{-/-} CTLs failed to restore lysis, suggesting that the missing mediator is more than exocytosed lipase alone. Notably, cytotoxicity of WT and PLRP2^{-/-} CTLs was similar in 2 day tumor survival assays with either IL-2 or IL-4, indicating that the genetic lesion applied only to perforin-dependent killing. Our results are inconsistent with lipase function during killing and suggest that juvenile malnutrition (caused by lack of PLRP2) could affect generation of preCTLs.

INTRODUCTION

Digestive lipases are pancreatic enzymes that hydrolyze triglycerides and phospholipids to generate fatty acids for transport from the gut into the body for subsequent energy utilization. There are four members of the pancreatic lipase family, the classical pancreatic triglyceride lipase (PTL) and the more recently characterized pancreatic lipase-related proteins (PLRP1, PLRP2 as well as the newly recognized human PLRP3). PTL hydrolyzes triglycerides into fatty acids, while PLRP2 hydrolyzes multiple substrates including triglycerides, phospholipids and galactolipids [1-5]. In addition to its hydrolysis of multiple lipid substrates, PLRP2 has activity against some substrates in the absence of a colipase, a pancreatic protein which is required for PTL activity [4-7].

While previous studies of pancreatic lipases have mainly focused on their role in digestion, other cells also express PLRP2. Gene expression of PLRP2 was previously reported in cytotoxic T lymphocyte cell lines when stimulated with IL-4 [8] and in Paneth cells which are secretory epithelial cells in the intestinal crypts [9]. Both T and Paneth cells promote anti-microbial resistance, suggesting that PLRP2 may participate in protective responses to invading pathogens. In studies involving PLRP2^{-/-} mice, T cells taken *ex vivo* 10 days after immunization with EL4 tumor cells had reduced EL-4 specific cytotoxicity when compared to that of wild type (WT) T cells [10]. Increased WT cytotoxic activity was also found after *in vitro* re-stimulation in cultures supplemented with IL-2 [10]. In both approaches, the CTLs' phenotype, the frequencies of the cytotoxic CD8 T cells in the population and the extent of their development of cytotoxic granules were undefined, raising the possibility that these variables rather than PLRP2 deficiency might account for the differences in cytotoxic activity.

In this paper, we sought to account for the effect of PLRP2 deficiency and in the process eliminated several reasonable possibilities. We presented the first evidence of PLRP2 protein expression in fresh isolated T cells cultured with IL-4. We found PLRP2 expression was localized to the CD8⁺ T cell subset which advances the case for PLRP2's direct involvement in CTL activity. The addition of the type 1 cytokine, IFN- γ (to polarize T cells to perforin-dependent killers) had little effect on the expression of PLRP2 induced by the type 2 cytokine IL-4. This result suggests that the lipase is retained even when other very active killing mechanisms are present. Our investigation found that WT CTLs cultured with either IL-2 or IL-4 demonstrated more cytotoxicity than PLRP2^{-/-} CTLs in short term ⁵¹Cr assays where perforin is essential, suggesting that the lipase might augment a perforin-dependent mechanism. The phenotypes of WT and PLRP2^{-/-} CTLs had similar granzyme B levels and activation markers. In addition, the distribution of CD8⁺ and CD4⁺ T cells was unperturbed by the PLRP2 gene deficiency. The short term lysis by IL-4 induced PLRP2^{-/-} CTLs was unchanged by the addition of recombinant PLRP2, which suggests that the lipase performs a separate function other than to increase the damage to target cells initiated by the pore-forming protein perforin. In contrast, cytotoxicity of WT vs. PLRP2^{-/-} CTLs was similar in 2 day tumor survival assays regardless of the cytokine used to induce the CTLs. We interpret the results to mean that PLRP2 has a positive impact on perforin-dependent cytotoxic T cell functions but that the lipase is unlikely to directly damage tumor cells.

MATERIALS AND METHODS

Cytokines. Recombinant mouse IL-2 was purchased from eBioscience (San Diego, CA, 1×10^7 units/mg; 1.7×10^{11} units/mmol). Recombinant mouse IL-4 was purchased from BD Biosciences (San Jose, CA, 2.5×10^7 units/mg; 3.5×10^{11} units/mmol). Recombinant mouse IFN- γ was from Peprotech (Rocky Hill, NJ, 1×10^7 units /mg; 1.5×10^8 units/mmol).

Animal use. The animal protocols for this study were approved by the University of Nevada, Reno Animal Care and Use Committee.

Cell culture. Splenocytes were harvested from the spleens of Balb/c mice wild-type (Jackson Labs, Bar Harbor, MN), or Balb/c PLRP2^{-/-} mice [10] and their littermate controls from Dr. Mark Lowe, bred at the University of Pittsburgh. For *in vitro* cultures, T cells were cultured at 5×10^5 cells/ml in T-75 flasks (Sarstedt) with complete RPMI-1640 media (Sigma Chemical Co, St. Louis, MO) containing 10% fetal calf serum (HyClone, Logan, UT), 10 mM HEPES buffer (Fisher Scientific, Waltham, MA), 1% Pen-Strep (Sigma), 24 mM sodium bicarbonate (Fisher), 25 μ M 2-mercaptoethanol (Sigma), 2.5 μ g/ml concanavalin A (conA) (Sigma) and varying cytokine conditions indicated in the results. Following an initial 3 day culture with conA and cytokines, the T cells were washed to remove the mitogen conA and re-cultured in complete tissue culture medium with renewal of the respective cytokines. Cells were re-cultured in fresh cytokines (500 u/ml) on day 5 if they were maintained longer. EGFP-P815 mastocytoma cells [11] were maintained in Dulbecco's Modified Eagle Medium, DMEM, (Gibco,

Grand Island, New York) with 180 mM sodium bicarbonate (Fisher) and 10% FBS (HyClone). Cell cultures were incubated at 37°C and 5% CO₂.

RNA extraction and analysis. Gene expression was monitored by non-quantitative and quantitative RT-PCR. *Non-quantitative RT-PCR.* T cells were counted and extracted for total RNA using TRIzol (Sigma). The extracts were flash frozen in liquid nitrogen for storage and to protect against RNase activity. The RNA was dissolved in nuclease-free water and the concentration determined with a NanoDrop ND-1000 Spectrophotometer. Reverse transcription was performed with the Ambion Retroscript Kit. Half to 1.0 µg of total RNA was transcribed with oligo-dT primers following the manufacturer's directions. Briefly, the RNA and primers were heated at 80°C for 3 min and placed on ice. A master mix of deoxynucleotides, RT buffer, RNase inhibitor, and reverse transcriptase was added according to the directions in a final volume of 20 µl and the reaction was incubated in a Robocycler (Stratagene, La Jolla, CA) for 1 cycle at 80°C for 3 min and 1 cycle at 44°C for 60 min followed by 92°C for 10 min. The product was amplified by PCR in an Applied Biosystems 9800 Fast Thermal Cycler. 0.4 to 1.0 µl was added to 10 µl of GeneAmp Fast PCR Master Mix (Applied Biosystems, Foster City, CA). The primer pair to amplify mouse PLRP2 was 5'-ATCTTGGTCGCTCTGT-ACGGATGTAACG-3' and 5'-GTCTTTAGCGCGTTGCAGTGTGA-3'. The primer pair to amplify mouse cyclophilin, was suggested for use by our collaborator Mark E. Lowe at the University of Pittsburgh and used as an internal control to monitor differences in initial amounts of RNA amplified, was 5'-ATGGTCAACCCACCGTGTT-3' and 5'-CGTGTGAAGTCACCACCCT-3'. PLRP2

was amplified with 34 cycles of 94°C for 15 seconds and 67°C for 15 seconds and cyclophilin was amplified with 30 cycles of 94°C for 15 seconds and 65°C for 15 seconds. The products were separated by electrophoresis in a 2% agarose gel using 1x TBE buffer. The bands were stained with ethidium bromide and a digital picture was taken. The density of the bands was quantified with SigmaGel analysis software (Systat Software, San Jose, CA).

TaqMan® quantitative PCR. Total RNA was isolated from TRIzol samples by organic extraction with chloroform (200 µl per ml of TRIzol initial) and vortexing the mixture. After 15 minutes at room temperature each sample was centrifuged at 14,000 rpm for 15 minutes at 4°C. The aqueous phase was collected and -20°C isopropanol (500 µl per ml initial TRIzol) was added and allowed to incubate for 10 minutes at room temperature followed by 1 hour at -80°C. The samples were next centrifuged at 14,000 rpm for 30 minutes at 4°C to pellet the extracted RNA. The supernatant was removed from the each RNA pellet and 75% ethanol added (1 ml per ml initial TRIzol) and centrifuged at 14,000 rpm for 5 minutes at 4°C to wash the pellet. Ethanol was removed and the remaining residual ethanol was evaporated away. The RNA was re-suspended in 20µl RNase-free H₂O and then (5 units) DNase I (Promega, Madison, WI) and RNase inhibitor, RNAsin (Promega), were added to each sample. The samples were incubated at 37°C to allow for degradation of DNA and then incubated at 65°C to inactivate the DNase I. The RNA concentration for each sample was determined by measuring absorbance at 260 nm using Beckman DU 650 Spectrometer (Beckman Instruments, Fullerton, CA). First strand cDNA synthesis was performed from 2 µg of each RNA sample using 250 ng random

primers and nuclease-free H₂O in a final volume of 12 μ ls. This mixture was heated to 70°C for 10 minutes and quickly cooled using an Applied Biosystems 2770 Thermal Cycler. Then a master mix containing 5x First Strand buffer (Invitrogen), 10 mM DTT (Invitrogen), and 0.125 mM dATP, dTTP, dGTP and dCTP mM dNTP mix (Promega) was added to each sample to create a volume of 18 μ ls. Each sample was gently pipetted to mix and incubated at 25°C for 10 minutes followed by 2 minutes at 42°C. The reaction was paused and 1 unit SuperScript RT III (Invitrogen) was added to each sample and reverse transcription reaction was carried out with 1 cycle at 50°C for 60 minutes and 1 cycle at 72°C for 15 minutes followed by a hold at 4°C. Remnant RNA was removed with the addition of 20 units RNaseH and incubation at 37°C for 20 minutes. Each resulting cDNA sample was diluted 1:5 in RNase free H₂O to a final volume of 100 μ ls. For the TaqMan® quantitative PCR assay, each reaction was performed in triplicate in a final volume of 20 μ ls containing a 1x concentration of TaqMan® PCR Master Mix as follows: 2 μ ls of the cDNA were added to 10 μ ls TaqMan® Fast Universal PCR Master Mix (2x) (Applied Biosystems, Foster City, CA) and 7 μ l RNase free H₂O. The 20x TaqMan® FAM-dye labeled probes to detect and amplify the cDNAs were mouse PLRP2, Mm00448214_m1; mouse cyclophilin, Mm02342429_g1; and mouse perforin, Mm00812512_m1, from Applied Biosystems. For each reaction 1 μ l of primer was added to the 1x concentration of TaqMan® PCR Master Mix to create the final volume of 20 μ ls. The sample amplification was performed on a 7500 ABI real-time PCR System (Applied Biosystems) with an initiation by incubation at 95°C for 20 seconds, followed by 40 cycles of 95°C for 1 second and 60°C for 20 seconds. The production of polymerase chain reaction products was followed by monitoring the increase in the

fluorescence passed a fixed detection threshold. Standard curves were generated for each target gene by serial diluting the cDNA of a control sample and by plotting the log of the initial copy number of the standard vs. the threshold cycle (C_t) value. Resulting C_t values from each experimental sample were fit to the standard curve and used to calculate the quantity of target genes relative to the standard curve. All samples were normalized to cyclophilin mRNA amplified from the same samples to control for variation in sample quality.

Western blots: Splenocytes, grown in 500 u/ml IL-4, were disrupted using nitrogen bomb cavitation. The resulting cell homogenate were layered onto a 54% Percoll® (Sigma) gradient and centrifuged at 45,000 x g for 20 mins at 4°C. The resulting density gradient was divided into 6 fractions. Percoll® was removed from each fraction by centrifugation at 145,000 x g for 4 hours at 4°C. Granules and vesicles were collected from above the Percoll® pellet. Dry NaCl was added to bring each fraction up to a 1.15M concentration and the samples were extracted by freeze-thawing. Each fraction was loaded onto a 10%-SDS-PAGE (BioRad) and run. Following transfer of the proteins to a nitrocellulose sheet, each blot was probed with the polyclonal chicken anti-recombinant fragment of human PLRP2 antibody (Abcam ab17740). Unfortunately, the antibody used in Figure 3.1 to PLRP2 is currently unavailable, so only one experiment is presented. We also generated weak positive results with another chicken antibody (Abcam ab37599) using extracts of un-fractionated IL-4 cultured cells or the IL-4 responsive cell line, CT-4R, used to clone PLRP2 cDNA [8,12] (not illustrated). The probed blots were developed with secondary affinity purified donkey anti-chicken IgG

labeled with IR-Dye800 and analyzed using the Odyssey infrared imaging system (LiCor biosciences, Lincoln, NE). To detect perforin, we used rabbit anti-rat perforin (Fitzgerald Industries, MA) with secondary affinity purified goat anti-rabbit IgG labeled with AF680 (Molecular Probes, OR).

Cytotoxicity assays: ⁵¹Cr release assays. T cell lysis was detected using redirected lysis assays with P815 mastocytoma cells as radiolabeled targets. T cells were redirected to the Fc receptor-bearing P815 cells with 1 µg/ml anti-mouse CD3 mAb (clone 145-2C11) (eBioscience, San Diego, CA). P815 target cells were labeled with Na⁵¹CrO₄ (Perkin Elmer) [13] and assays run in quadruplicate with 10,000 radiolabeled cells per well for 4 hours. In experiments where recombinant PLRP2 was added to the CTLs, 1 µg/ml final PLRP2 was added at the beginning of each 4 hour assay.

Specific release was calculated by the formula:

$$\% \text{ Specific Release} = [(Experimental \text{ CPM}) - (Spontaneous \text{ Release})] / [(SDS \text{ Total Release}) - (Spontaneous \text{ Release})].$$

Cytometric tumor outgrowth assays. On day 5, CTLs were plated out with eGFP-P815s [11] at specified effector to target ratios in the presence of 1 µg/ml anti-CD3 (clone 145-2C11) (eBioscience) or with 1 µg/ml of the IgG control antibody, Golden Syrian hamster anti-mouse IgG (eBioscience, San Diego, CA). The assay was incubated at 37°C with 5% humidity for 48 hours. Upon completion of the 48 hour incubation, cells were harvested and measured with a Beckman Coulter XL/MCL Flow Cytometer. Live and dead eGFP-P815s were distinguished from one another based on GFP fluorescence and

forward scatter properties. Because dead cells can disintegrate into debris and become undetectable, we counted live cells in constant volumes that were determined using true count calibrated cell count beads (Polymer Labs, Amherst, MA). The beads were added to each sample and used to normalize the samples to a constant volume per sample. The data were analyzed with FlowJo software (Tree Star, Inc., Ashland, OR).

T cell separation. Anti-CD4 and anti-CD8 magnetic particles were purchased from BD Biosciences (Cat #551539 and #551516). Cells were placed into 4 ml tubes (Falcon #352052) and incubated with beads at a concentration of 50 μ l per 10^7 cells. The cells were incubated at 4°C for 20 minutes and then each tube was placed in the magnetic apparatus (BD Biosciences iMagnet) for 10 minutes at 4°C. Supernatant containing the negatively enriched cells was carefully removed and placed in a separate tube. The tubes containing the negatively enriched cells were further enriched through 3 repetitions of the magnetic separation described above. Cells were stained and assayed by flow cytometry as described below.

Flow cytometry analyses of cellular proteins. Cells were harvested and epitopes detected with the following mAbs: PE-Cy7-anti-mouse CD3 IgG (clone 145-2C11, eBioscience), PE-Cy5-anti-mouse CD4 IgG2b (clone GK1.5, eBioscience), and FITC-anti-mouse CD8 IgG2a (clone 53-6.7, eBioscience), PE-anti-mouse CD25 IgG1L (clone PC61.5, eBioscience), PE-anti-mouse CD44 IgG2b (clone IM7, eBioscience), PE-anti-mouse CD45 IgG2b (clone 30-F11, eBioscience) and PE-anti-mouse CD137 Syrian hamster IgG (clone 17B5, eBioscience). After external antibody staining, the cells were washed, fixed, and permeabilized (IntraPrep Kit, Beckman Coulter, Fullerton, CA). Grz

B was detected with PE anti-human Grz B mAb (clone GB-12, Caltag Laboratories, Burlingame, CA) that cross-reacts with mouse Grz B and fails to react with Grz B^{-/-} T cells (data not shown).

Intracellular cytokine staining: T cells after culture for 5 days were stimulated with 80 ng/ml PMA (Sigma) and 800 µg/ml ionomycin (sigma) in the presence of Golgi plug® used at a concentration of 1:1000 as per manufacturer's instructions (BD biosciences) for 4 hours. Following this incubation, cells were washed with PBS containing 1% FBS. The resulting cells were fixed and permeabilized using the Beckman Coulter IntraPrep intracellular staining kit and staining was carried out per manufacturer's instructions (Beckman Coulter, Fullerton, CA). IFN-γ, IL-4 and IL-5 was detected using AF647-anti mouse IFN-γ mAb (clone XMG1.2, eBioscience), PE-anti-mouse IL-4 (clone 11B11, eBioscience) and PE-anti-mouse IL-5 (clone TRFK5, eBioscience), respectively. Samples were measured with a Beckman Coulter FC500 Flow Cytometer with optical filters to detect 525, 575, 670, and 740 nm. The data were analyzed with FlowJo software (Tree Star, Inc., Ashland, OR).

Statistical Analyses: Results are expressed as mean +/- SD of the collected data. Statistical evaluations of the data were performed with Excel and Sigma plot software. The differences in mean values were evaluated by Student's T-test. Differences were considered significant if the P was <0.05.

RESULTS

IL-4 induces pancreatic lipase related protein 2 (PLRP2) in CD8+ T cells

Grusby et al. [8] showed that PLRP2 mRNA expression was induced in long term CD8 T cell lines cultured with IL-4. To set the foundation for examination of the role of the PLRP2 lipase in CTL killing, we first wanted to validate that the PLRP2 mRNA and the protein are present in the CTLs that we would later examine for function. We first determined PLRP2 induction by IL-4 in freshly isolated T cells by performing a time course at various concentrations of IL-4. Induction of PLRP2 mRNA in splenocytes stimulated with the lectin concanavalin A and cultured with exogenous IL-4 was first detected on day 4 in the presence of 250 units/ml IL-4, with increased PLRP2 mRNA expression on day 5 (Figure 3.1A). Incubation with 500 u/ml IL-4 resulted in earlier detection of PLRP2 mRNA, starting on day 3 without significantly increasing the mRNA expression days 4 and 5 (data not shown). T cells exhibited a dose response for the expression of PLRP2 mRNA assayed on day 6 with induction at the lowest IL-4 concentration of 125 u/ml (3.5×10^{-8} M, 5 μ g/ml of this lot of IL-4) (Figure 3.1B). PLRP2 mRNA increased in a dose dependent manner, plateauing at 250 units and higher quantities of IL-4. For perspective, this concentration of IL-4 is attainable: 5 μ g/ml of IL-4 can be produced by memory Th2 cells in culture [14] and *in vivo*, Th2, mast cells and eosinophils all produce IL-4.

To assess the role of PLRP2 on immune function, we assessed if IL-2 induced CTLs would lack PLRP2. If the IL-2 cultured CTLs lacked PLRP2 mRNA, these CTLs would be useful as negative controls to examine if the PLRP2 influenced events other

than the actual CTL killing. For example, PLRP2^{-/-} mice have stunted growth until after weaning and this nutrition effect might affect immunity. IL-2 was much less effective than IL-4 at inducing PLRP2 expression (Figure 3.1A). In 3 of 4 non-quantitative RT-PCR experiments, weak signals were observed in cells cultured with only IL-2 (Figure 3.1A, and data not shown). Further investigation of PLRP2 using quantitative TaqMan RT-PCR failed to detect signal in IL-2 induced CTLs in 3 out of 3 additional experiments (one included in Figure 3.1C). We consider it likely that occasional PLRP2 expression could be supported by endogenous IL-4 being produced by the activated CD4⁺ T cells within the cultures. Our findings clearly indicate that CTLs generated with IL-2 have low or no PLRP2 mRNA.

To determine if PLRP2 expression was affected by cytokines other than IL-2 and IL-4, the type 1 helper T cell cytokine IFN- γ was added to the IL-4 induced splenocytes. High concentrations of IFN- γ support type 1 responses and suppressed type 2 lymphocyte responses even when the type 2-polarizing cytokine IL-4 is present [15-18]. Splenocytes were activated with conA and induced with IL-4 alone or with IL-4 combined with increasing concentrations of IFN- γ . On day 5, as expected, PLRP2 expression was observed in the cells induced with IL-4 alone. For analysis of the effects of IFN- γ , the PLRP2 expression of the IL-4 induced CTLs was set as the 100% control. The additions of IFN- γ had a slight enhancing effect on the expression of PLRP2 (Figure 3.1C). The data show that although PLRP2 is induced by the type 2 cytokine IL-4, its expression remains unaffected by IFN- γ . Thus the presence of type 1 IFN- γ -secreting CD4⁺ T helper cells would be unlikely to counteract the effects of IL-4 provided by type 2 CD4⁺ T helper cells or by IL-4 secreting by mast cells.

In previous studies, only CD8⁺ (and not CD4⁺) T cell lines expressed PLRP2 mRNA [8], but the studies lacked cytotoxic CD4⁺ CTL lines. To test whether it was the CD8⁺ T cells or the induction of cytotoxicity that led to PLRP2 expression, we stimulated cells with conA, a mitogen that activates CD4⁺ cytotoxic T cells as well as CD8⁺ cytotoxic T cells. ConA activated CTLs were cultured with IL-4, and separated on day 6 into CD4⁺ or CD8⁺ by magnetic bead enrichment. The degree of cellular enrichment was assessed by flow cytometry. In the experiment illustrated (Figure 3.1D), the starting population was 34% CD4⁺ 53% CD8⁺; the CD4⁺ enriched cells were 96% CD4⁺ 1.5% CD8⁺ and the CD4-depleted cells were 1% CD4⁺ 95% CD8⁺, while the CD8⁺-enriched cells were 2% CD4⁺ 78% CD8⁺ and the CD8-depleted cells were 69.4% CD4⁺ 9% CD8⁺. The semi-quantitative PCR analyses indicated that only the populations with CD8⁺ T cells were PLRP2-positive and that the CD4⁺-enriched T cells lacked PLRP2 (Figure 3.1D). In additional experiments, >95% of the IL-4 cultured CD4⁺ T cells were observed to contain granzyme B, a cytotoxic granule protein and marker of cytotoxic function albeit in lower amounts per cell than in CD8⁺ cells (results not illustrated). These results show that the CD8⁺ T cell subset expresses PLRP2 and that even when CD4⁺ T cells contain cytotoxic granzymes the CD4⁺ T cells lack PLRP2.

To verify that the PLRP2 mRNA was translated into protein, we probed for PLRP2 using immunoblots of splenocytes grown in 500 u/ml IL-4 and fractionated into Percoll^R fractions of different densities. The proteins were run on SDS-PAGE gels. The blots were probed for PLRP2 using chicken antibodies elicited with a human PLRP2 protein fragment (Abcam ab17740), antibodies that cross react with mouse PLRP2 and with antibodies to perforin as a positive control. An immunoreactive band corresponding

to PLRP2 at 52 kDa was observed in the least dense fraction of T cells stimulated with IL-4 (Figure 3.1E) while perforin was located in the dense granule fraction. Specificity controls with non-immune chicken IgY were negative (data not shown). PLRP2 was undetectable in IL-2 cultured cells. Thus PLRP2 is expressed as a lymphocyte protein, though the available reagents limit our ability to detect any low PLRP2 that might be in IL-2 induced cells. For perspective, we examined how far the CTLs of this study were polarized to a type 2 phenotype by IL-4 [19-21]. We used intracellular IL-4 and IL-5 as markers of type 2 CTLs and simultaneously measured intracellular IFN- γ , which is the hallmark of type 1 polarized CD4⁺ T cells. We found that only 2-3% of all CD8⁺ T cells expressed IL-4 or IL-5 and that about ~60% of other CD8⁺ expressed high levels of IFN- γ (Figure 3.2). The CD4⁺ T cells of the same culture served as positive controls for intracellular IL-4 and IL-5, with about ~25-30% of CD4⁺ cells staining for IL-4 or IL-5 (Figure 3.2). There was a small population of ~8% of IFN- γ and IL-4/5 double expressing cells. Similar intracellular IL-4, IL-5 and IFN- γ staining compared to IL-4 conditions was observed when the CD8⁺ and CD4⁺ T cells were induced with IL-2 instead of IL-4, but a reduced amount of IFN- γ and IL-4/5 double expressing cells (Figure 3.2). Thus the PLRP2⁺ CD8⁺ T cell population induced with IL-4 lacks the IL-4 and IL-5 production that could classify it as type 2 CTLs (Tc2 cells).

Knockout of PLRP2 reduced the rapid cytotoxicity of T cells

To test if PLRP2 participates in killing mediated by CTLs representing a polyclonal spectrum of naturally occurring lymphocytes, we performed ⁵¹Cr release assays using T cells from both PLRP2 knockout and littermate WT Balb/c mice. Splenic

T cells were activated by the polyclonal activator concanavalin A. IL-4 induced CTLs from the WT mice had 3.5 fold the lytic activity of the PLRP2^{-/-} CTLs (Figure 3.3A). Unexpectedly, IL-2 induced CTLs from the WT mice had ~2 fold the lytic activity of the PLRP2^{-/-} CTLs (Figure 3.3B). The IL-2 results confirm previous finds by M. Lowe et al.[10]. The activity of both the IL-2 and IL-4 induced CTLs in these ⁵¹Cr release assays is more than 95% perforin-dependent (unpublished data).

The low expression or lack of PLRP2 in the IL-2 induced CTLs raises several possibilities. First, since the IL-4 cells show a greater difference, there might be a component of the lysis that is affected by PLRP2 induced by IL-4, which we tested later by adding r-PLRP2 to the PLRP2^{-/-} CTLs. Second, the fact that the IL-2 CTLs had a difference between WT and KO (when the PLRP2 gene is barely expressed if expressed at all in the IL-2 CTLs) creates a need to consider an effect of PLRP2 prior to CTL activation. Furthermore, the IL-2 effects prompt consideration of chromosomal effects of the gene ablation on transcription of other proteins involved in CTL killing, which we illustrate later in the results.

Knockout of PLRP2 was without effect on long term CTL-mediated apoptosis

We questioned if the PLRP2-associated cytotoxic activity which was observed in the perforin-dependent 4 hour assays would also be detected in longer assays that monitor apoptosis. The flow cytometry assays of apoptosis measure tumor cell survival after 48 hours and used fluorescent eGFP P815 cells as ‘target’ cells. In this assay low effector to target ratios were used in redirected lysis of eGFP-P815 cells and the lytic activity was detected by the number of viable “green” P815 cells that remain after the extended

incubation [11]. The lytic activity was similar for WT and perforin^{-/-} C57Bl/6 mice (unpublished results with both IL-2 and IL-4 activated CTLs), which suggests that it can detect perforin-independent apoptosis. We know that these assays monitor cell death because at the higher E:T ratios we recover fewer target cells than were plated (by monitoring cell recovery determined by the cell count beads). Furthermore, when the P815 cells failed to survive, there was debris detected by flow cytometry that was characteristic of dead cells. Interestingly, the cytotoxic activities of PLRP2^{+/+} and PLRP2^{-/-} IL-4 induced CTLs were similar (Figure 3.3C). This observation suggests that different CTL cytotoxic mechanisms are dominant in the 4 hour vs. 2 day assays, and that PLRP2 affects only short term, perforin-dependent mechanisms.

PLRP2^{+/+} and PLRP2^{-/-} CTLs were phenotypically indistinguishable and the CD8+ CTLs were represented in similar frequencies within WT and KO cell cultures

Perforin-dependent cytotoxicity is affected by the amounts of perforin per cell, the amounts of other perforin-dependent mediators per cell (such as granzyme B), and by the activation of T cells which will affect adhesion and other T cell surface proteins. We questioned if the difference in the lytic ability of PLRP2^{-/-} CTLs could be attributed to less granzyme B expression, differences in activation proteins or differences in the distribution of CD4 T cells compared to CD8 CTLs. Using flow cytometry to determine granzyme B expression, the production of granzyme B was similar in T cells from PLRP2^{-/-} T cells when compared to T cells from WT mice (Figure 3.4A). Analysis of the ratio of CD8 and CD4 cells showed similar ratios when one compares the PLRP2^{-/-} and WT population grown under the same conditions (Figure 3.4B). Investigation of the

activation and cell surface markers CD25, CD44, CD45 and CD137 found that the phenotypes of IL-2 and IL-4 induced CTLs from both PLRP2 WT and KO mice were virtually identical (Figure 3.5 A and B). Thus expression of the high affinity IL-2 receptor CD25, the adhesion molecule for hyaluronan CD44 that is up-regulated with the memory T cell phenotype, the tyrosine phosphatase CD45 associated with the T cell receptors for antigen and a co-stimulatory molecule for antigenic responses in IL-4 treated cells CD137 [22,23], were comparable. These results eliminate the possibility of a difference in CTL frequency, granule formation, or the assessed surface phenotypes being responsible for the differences in cytotoxic activities observed between PLRP2^{-/-} and PLRP2^{+/+} CTLs.

Rapid lysis is unchanged by the addition of r-PLRP2

To determine if restoration of PLRP2 could augment the deficiency in short term cytotoxic function observed for IL-4 induced CTLs, we added recombinant human PLRP2 to ⁵¹Cr redirected lysis assays. The recombinant PLRP2 alone with the P815 target cells caused no cytotoxicity. The cytotoxic activity of the IL-4 induced PLRP2^{-/-} CTLs was unchanged by the addition of 1µg/ml recombinant PLRP2, indicating that restoration of PLRP2 alone to the CTLs was insufficient to improve lysis (Figure 3.7). Lysis was also unchanged when IL-2 induced PLRP2^{-/-} CTLs received recombinant PLRP2 (data not illustrated). We have established that the r-PLRP2 lipase activity is stable in the presence of the granzyme proteases that are exocytosed during killing (data not illustrated). These results suggest that the function of PLRP2 appears to be independent of direct cytotoxic effects on tumor cells.

Perforin mRNA levels were comparable in IL-4 induced WT and PLRP2^{-/-} CTLs

It is possible that ablation of the PLRP2 gene could unexpectedly affect perforin expression and thereby account for the differences in ⁵¹Cr-release short term lysis. The best available method to monitor perforin at this time is to quantify the mRNA because the antibodies available to mouse perforin are unsuitable to detect perforin by flow cytometry and immunoblotting. Thus, we applied TaqMan^R RT-PCR to quantify mRNA to perforin. We found that perforin mRNA levels were similar for IL-4 induced WT and KO CTLs (Figure 3.6A). This observation suggests that perforin differences are unlikely to account for PLRP2 KO effects in the IL-4 induced CTLs. The IL-2 induced WT CTLs had 20-fold more perforin mRNA than the IL-4 CTLs, consistent with the greater ⁵¹Cr lytic activity of IL-2 CTLs compared to IL-4 CTLs (Figure 3.3 A&B). Unexpectedly, we observed that in IL-2 treated CTLs, the perforin mRNA was markedly greater in the WT than in the PLRP2 KO. As a result, the differences in the amounts of perforin mRNA correlate with the differences in short term lytic activity of the IL-2 but not the IL-4 when comparing WT vs. KO CTLs. In summary, we have two unexpected observations with IL-2 to indicate that the PLRP2 gene ablation has effects. This situation is surprising since the PLRP2 gene is hardly expressed in the WT CTLs cultured with IL-2.

DISCUSSION

Since the discovery of PLRP2 in cytotoxic T cells by Grusby *et al.* [8] and the later findings that PLRP2^{-/-} T cells have reduced cytotoxic activity compared to WT T cells [10], it has been hypothesized that PLRP2 helps destroy cells targeted for death by T cells [9,24,25]. Although this idea has been accepted, we demonstrated for the first time that freshly isolated T cells under IL-4 stimulation express PLRP2 mRNA and protein. PLRP2 is expressed in only CD8⁺ T cells after induction with IL-4, even under conditions in which CD4⁺ T cells also contain cytotoxic granules with granzyme B and in which CD4 T cells are able to mediate cytotoxicity in perforin dependent assays. Since CD8 T cells are the main mediators of killing by T cells, these data strengthen the idea that PLRP2 plays a role in cytotoxic activity of CD8 T cells.

We showed that IL-4 and IL-2 induced PLRP2^{-/-} CTLs had reduced short term cytotoxic activity when compared with WT T cells grown under the same conditions. The short term assays were perforin-dependent, however, the difference in the cytotoxic activity was lost in long term 48 hour killing assays. The long term assays can function without perforin, indicating that there was a perforin-independent secondary mechanism that can terminate target cells if perforin fails to do so. The simplest interpretation of our results was that a perforin-independent killing mechanism was also PLRP2-independent. Our best efforts to account for the differences in the 4 hour cytotoxic activities included searches for differences in frequency of CD8⁺ CTLs, differences in granzyme and

perforin production, differences in activation states (as indicated by surface marker proteins) and restoration of PLRP2 to the PLRP2-deficient CTLs. These possibilities were then eliminated from consideration. The addition of PLRP2 failed to enhance the cytotoxic activity of IL-4 induced CTLs, suggesting that the function of PLRP2 was independent of direct cytotoxic functions on tumor targets. A function of PLRP2 that was independent of direct cytotoxicity was further supported by our unpublished experiments that show the inability of r-PLRP2 to augment cytotoxicity mediated by perforin-containing cytotoxic lymphocyte granule extracts.

We were left with the question: how was the loss of the PLRP2 gene affecting the short term cytotoxic function of CTLs under certain conditions when it may not even be actively expressed? We consider the possibility of upstream or downstream attenuation of other genes. Gene ablation can have unanticipated effects on the expression of neighboring genes [26-28]. First, we considered proteins known to be important to CTL killing to see if they were on mouse chromosome 19 with PLRP2. However, granzymes B-H and N were on chromosome 14; granzymes A and K on chromosome 13; granzyme M, perforin and CD18 (part of LFA-1) on chromosome 10. The other heterodimeric protein of the adhesion molecule LFA-1 (CD11a) was on chromosome 7; and the TCR co-receptor CD8 was on chromosome 2, indicating that these proteins important for cytotoxicity were very unlikely to be perturbed. Activation of the IL-4 receptor of CD8 T cells induces a wide spectrum of intracellular signaling through two cascades [29] that will undoubtedly affect many genes other than the ones considered above. We also examined the genes flanking the PLRP2 site using NCBI Mapviewer for candidate genes within a 3,000 kB site. The 12 genes upstream and the 17 genes downstream of PLRP2

appear unrelated to cytotoxicity, although 10 of these 19 genes have currently unidentified functions and could be recognized in the future as important for cytotoxicity.

If the effect is really due to loss of PLRP2 rather than extraneous chromosomal effects, it is possible that cytotoxic activity could be developmentally affected by the malnutrition that the PLRP2^{-/-} mice experience during suckling. Dietary triglycerides provide ~90% of the energy during the first 6 months of human development after birth [30,31]. To utilize the energy stored in these triglycerides lipases must hydrolyze the fatty acids. The main lipase responsible for the breakdown of triglycerides in neonates is PLRP2 [32-34]. The PLRP2^{-/-} mice had a decreased weight gain of 0.33 g/day when compared to the WT suckling mouse who gained weight at 0.44 g/day [10]. Their body mass was decreased by ~20% by 23 days of age. With the onset of PTL lipase expression near weaning, the PLRP2^{-/-} mice exhibited catch-up growth and their adult weights became indistinguishable from wild-type littermates. The PLRP2^{-/-} mice were malnourished at just the time of T cell development. Nutritional deficiencies have been associated with impairment of immune function. Malnourishment during development results in prolonged impairment of cell mediated immunity [35-37]. A reduction in the number of T lymphocytes and their ability to respond to mitogens has been found in both human and animal models of malnourishment [35,36,38]. Although many of the observed effects of malnourishment on immune function were reversible in adults with restoration of proper nutrition, temporarily malnourished infants who regained weight later continued to exhibit impaired cellular immune functions. The effects included higher risk of upper and lower respiratory infections for years after proper nutrition was restored [39], infections that require CTLs for control.

There was another model for a function of what PLRP2 may perform in T cells, based on research in another area, artificial insemination. PLRP2 was a product of the goat bulbourethral gland and found in semen [40]. PLRP2 was the enzyme responsible for the reduction of sperm viability but only when milk lipids were included in cryopreservation [41,42]. Assessing this potential, we found that r-PLRP can be toxic to P815 cells *provided* that the triglycerides triolein or trilinolein were also added and *provided* that pancreatic co-lipase, a co-factor, was added (unpublished results with the 2 day tumor survival assays). We also found that the potential products, free fatty acids are toxic in these 2 day assays. However, the PLRP2 lipase-cofactor-lipid combination was *without* effect at 4 hours on P815 cells as were unbound oleic and linoleic acids [43,44]. Consequently, the assay affected by the gene deficiency in CTLs was unaffected by the potential mediators of purified PLRP2 lipase, co-lipase and lipid! Also the long term CTL assays were performed without the lipid supplementation that is required to generate the indirect cytotoxicity produced by fatty acids. At this time, our attempts to identify a role for PLRP2 in T cell cytotoxicity have addressed some critical issues, raised new issues as to when in animal development the effect may occur, and set the stage for defining the role of PLRP2 in T cell function in the future.

Chapter 3 Bibliography

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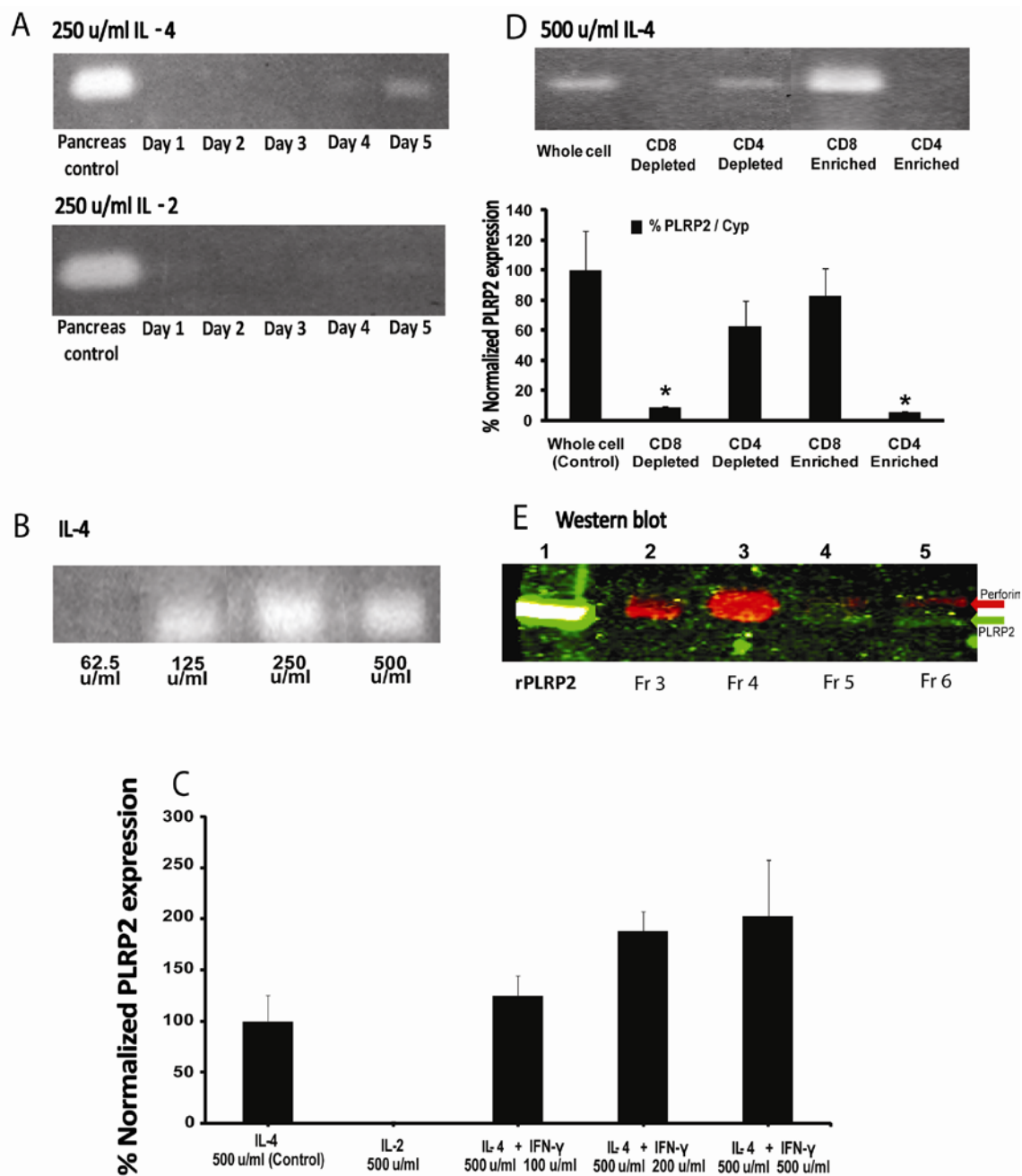


Figure 3.1. Induction of PLRP2 by IL-4. Splenic T cells were activated with conA in media containing differing concentrations of either murine r-IL-2 or r-IL-4. A. Induction of mRNA for PLRP2. A. Time course of induction with IL-4 and lack of induction with IL-2. Over a 5 day time course, cells were cultured with 250 u/ml of cytokine and

collected. PLRP2 expression was determined by RT-PCR. Expression of PLRP2 was first detected on day 4 and increased on day 5 in samples induced with IL-4. PLRP2 expression was barely detectable on day 5 in cells cultured with IL-2. Concurrent amplification of the constitutively expressed gene for cyclophilin indicated that the starting amounts of cDNA were similar (not illustrated).

B. Dose-response with IL-4. Expression of PLRP2 responded to IL-4 in a dose dependent manner observed on day 6. The lowest dose for expression of PLRP2 was 125 u/ml (3.5×10^{-8} M) rIL-4.

C. Lack of effect of IFN- γ on induction of PLRP2. Splenocytes were induced with IL-4 alone or IL-4 plus increasing concentration of IFN- γ . PLRP2 expression was monitored on day 5 using TaqMan^R quantitative RT-PCR. IFN- γ actually appeared to increase the level of PLRP2 expression (by about two-fold) but the differences when compared to PLRP2 expression induced with IL-4 alone were statistically insignificant.

D. CD8⁺ T cells preferentially express PLRP2. To identify the T cell subset expressing PLRP2 we used magnetic bead enrichment and depletion of CD4 and CD8 T cells. The cells were induced with IL-4 and separated on day 5. RNA extracts were tested using both RT-PCR (gel detection) and TaqMan[®] quantitative RT-PCR (bar graph) to determine the levels of PLRP2 expression. PLRP2 mRNA was normalized to cyclophilin to control for variation in sample quality and was detected in the starting cells and in two samples, CD8⁺ cells and cells depleted of CD4⁺ T cells. Data are expressed as the % change from whole cells. When paired T tests were made between any of the 3 positive sets of cells and the CD8 depleted or the CD4-enriched cells, the P values were <0.05, indicated by asterisks. The 100% value was the Q RT-PCR ratio of PLRP2 mRNA to cyclophilin mRNA for the control un-fractionated IL-4 induced WT cells. The actual average cycle

time to PCR threshold (Ct) values for these control un-fractionated IL-4 induced WT cells were 18.0 for cyclophilin and 27.0 for PLRP2, indicating that there was actually 2^9 (512 fold) more cyclophilin mRNA than PLRP2 mRNA in these cells. For perspective, their perforin mRNA was also in low abundance (see Figure 3.6) and the actual average cycle time to PCR threshold (Ct) values were 19.7 for cyclophilin and 28.7 for perforin, indicating that there was 2^9 (512 fold) more cyclophilin mRNA than perforin mRNA. Thus, both perforin and PLRP2 mRNAs are in low abundance in IL-4 induced cells. For the high PLRP2 IL-4 induced long term cell line CT.4R [8] there was 6 fold more PLRP2 mRNA than for the enriched CD8 T cells illustrated here.

E. Detection of PLRP2 protein by immunoblots. Whole cell extracts from day 6 T cells induced with IL-4 were fractionated over a Percoll[®] gradient. The proteins in the different fractions were loaded and separated on a 10% SDS-PAGE gel. The resulting blot was developed with antibodies to perforin (red, 680 nm) and PLRP2 (green, 800 nm). Perforin was enriched in the dense Percoll[®] fraction 4 (lane 3) while PLRP2 was in the lightest fraction 6 (lane 5).

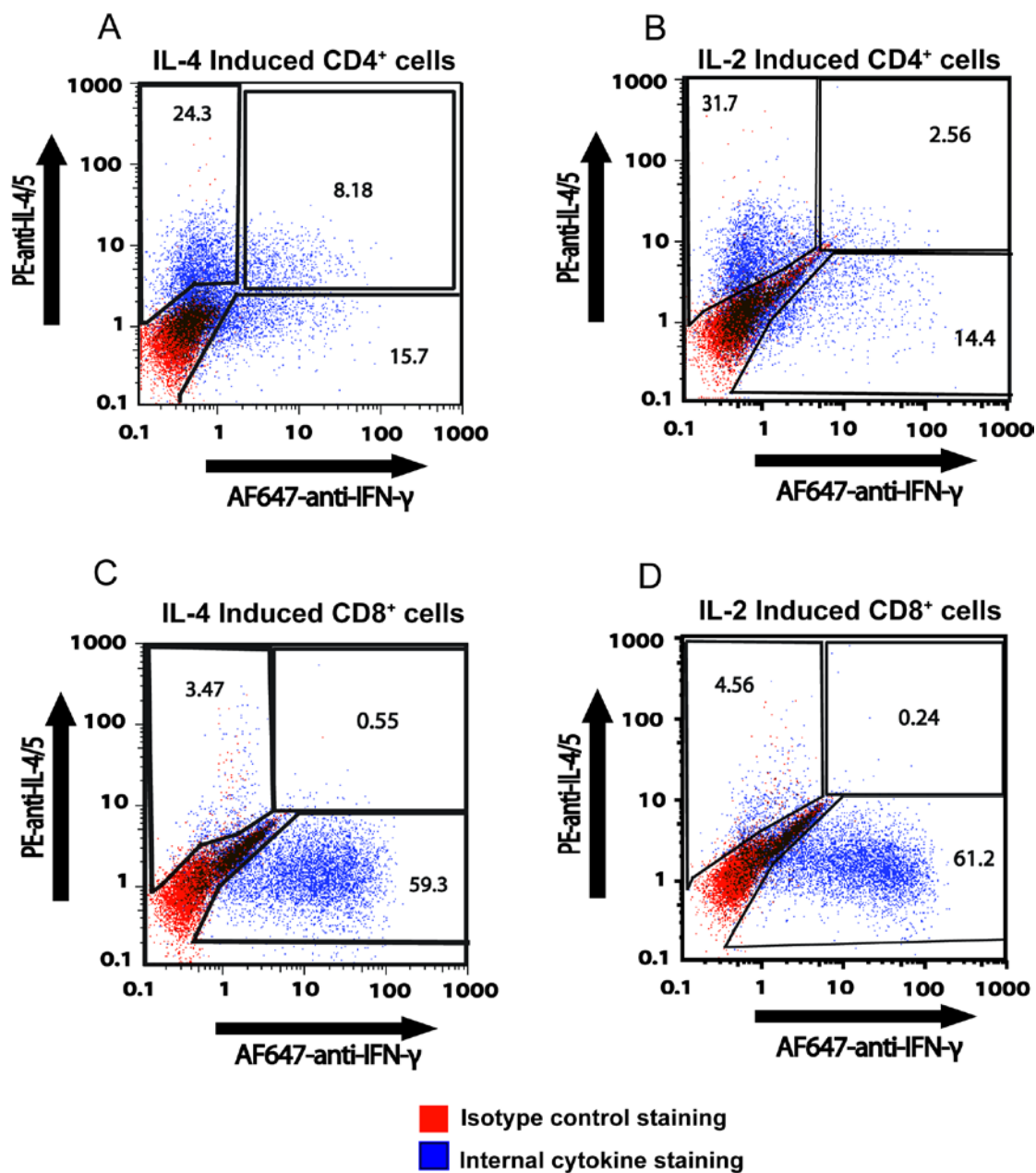


Figure 3.2. IL-4 induced CD8 CTLs contain little IL-4 or IL-5 and thus differ from the phenotype associated with type 2 (Tc2) CTLs. To determine if the IL-4 induced T cells expressed a type 2 phenotype, internal cytokine staining was performed on day 5 after initial induction. Cells were simultaneous stained for ILs 4&5 using PE-conjugated

antibodies to increase potential detection of these type 2 cytokines, while Alexa Fluor 647 antibodies were used to detect IFN- γ . These cells were simultaneously stained with additional antibodies to CD4 and CD8 in the same reaction, so that CD4 and CD8 cells were stained identically for intracellular antigens. A and B. CD4 T cells. The CD4⁺ T cells were gated and are displayed. The gates for PE anti-ILs positive, IFN- γ positive or IL-4/5 + IFN- γ double positive (blue colored dots) were drawn based on cells stained with PE and AF-647 isotype control antibodies (red colored dots). The IL-4 induced culture had ~25% IL-4 and/or IL-5 positive cells that were negative for IFN- γ (Th2 cells) and 20% of the CD4⁺ T cells contained only IFN- γ (Th1 cells). For the IL-2 induced cells, the Th1 and Th2 populations were similar to the IL-4 induced cells. With IL-2, 32% of all CD4⁺ T cells were producers of IL-4 and IL-5, while 14% of the CD4 T cells populations were producers of IFN- γ . The intracellular staining was able to detect type 2 T helper cells and could be expected to detect “type 2 CTLs”. C and D. CD8 T cells. Only about 3% of the CD8 T cells of the IL-4 induced T cells expressed IL-4 or IL-5. In contrast, ~60% of all CD8⁺ T cells expressed high levels of IFN- γ . Staining for intracellular IL-4 & IL-5 and IFN- γ in the IL-2 induced T cell cultures was similar. The intracellular staining could detect Th2 cells but failed to detect Tc2 cells.

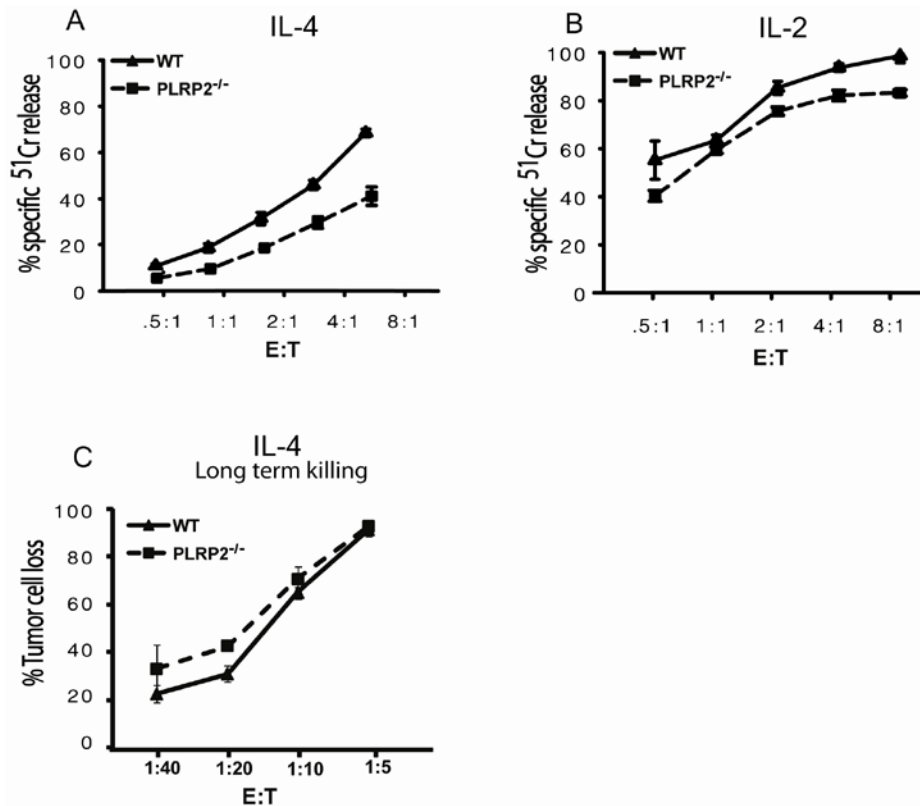


Figure 3.3. CTLs from WT PLRP2^{+/+} mice have more cytotoxic activity compared to PLRP2^{-/-} CTLs in short term killing assays, but similar cytotoxic activity in long term assays for apoptosis. A-B. ⁵¹Cr release assays. The killing potentials of IL-4 and IL-2 induced PLRP2^{-/-} or WT CTLs were tested using short term 4 hour ⁵¹Cr release by redirected lysis of P815 target cells. A. The cytotoxic activity of IL-4 induced CTLs. Cytotoxicity mediated by PLRP⁺ WT mice was 3.5 fold the activity of PLRP2^{-/-} CTLs, based on lytic units, which compare the numbers of different CTLs required to mediate equivalent levels of killing. These data are representative of 5 experiments. B. The cytotoxic activity of IL-2 induced CTLs. The cytotoxicity from wild-type mice was 1.6 fold the cytolytic activity of CTLs generated from PLRP2^{-/-}, as indicated by the increased number of knockout cells needed to generate equivalent cytotoxicity, based on lytic units. C. Tumor cell survival after 48 hours. IL-4 induced PLRP2^{+/+} and PLRP2^{-/-} CTLs

displayed similar cytotoxic activity in eGFP-P815 long term 48 hour killing assays, monitoring redirected lysis at very low E:T ratios. (Note that the lowest E:T is one effector to 40 targets in this assay.) These data are representative of 2 experiments.

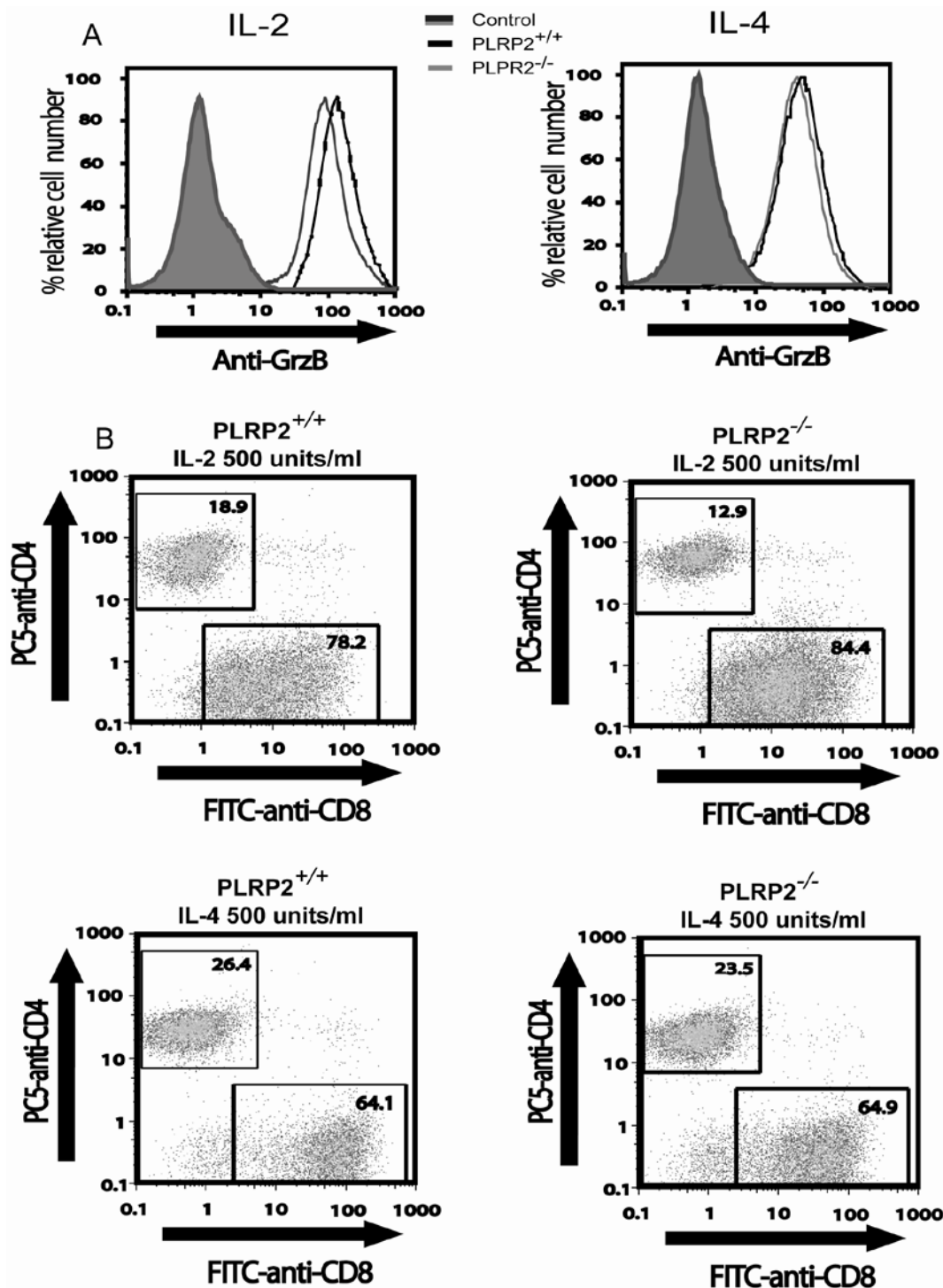


Figure 3.4. The reduced cytotoxic activity of PLRP2^{-/-} CTLs is independent of Grz B production and CD8 CTL frequency. IL-2 and IL-4 induced CTLs from PLRP2^{+/+} and PLRP2^{-/-} mice were examined for their production of granzyme B and the relative

distribution of CD4⁺ and CD8⁺ T cells after 5 days of tissue culture. A. Granzyme B production was similar. IL-2 induced CTLs generated from WT mice slightly more Grz B than from PLRP2^{-/-} mice in this experiment, represented by a less than 2-fold increase in mean fluorescent index (MFI) values (160 for WT and 108 for KO), which can happen with identical samples subjected to intracellular staining for granzyme B. IL-4 induced CTLs, WT and PLRP2^{-/-} also had indistinguishable levels of Grz B. These levels were lower than in the IL-2 induced cells, and had MFI values of 54 and 64. (PLRP2^{+/+}: solid black line, PLRP2^{-/-}: solid gray line. Isotype control: Shaded graph). B. The distributions of CD8 and CD4 cells were unaffected by the deficiency in PLRP2. The cells were gated on CD3⁺ T cells and then analyzed for cells reacting with anti-CD4 or anti-CD8 monoclonal antibodies. The CD8 distributions from IL-2 induced PLRP2^{+/+} vs. PLRP2^{-/-} were 68.5% vs. 77.8% and for CD4 the distributions were 18.9% vs. 12.9%, respectively. The IL-4 induced PLRP2^{+/+} vs. PLRP2^{-/-} exhibited CD8 distributions of 64.1% vs. 64.9% and for CD4 the distributions were 26.4% vs. 23.5%, respectively.

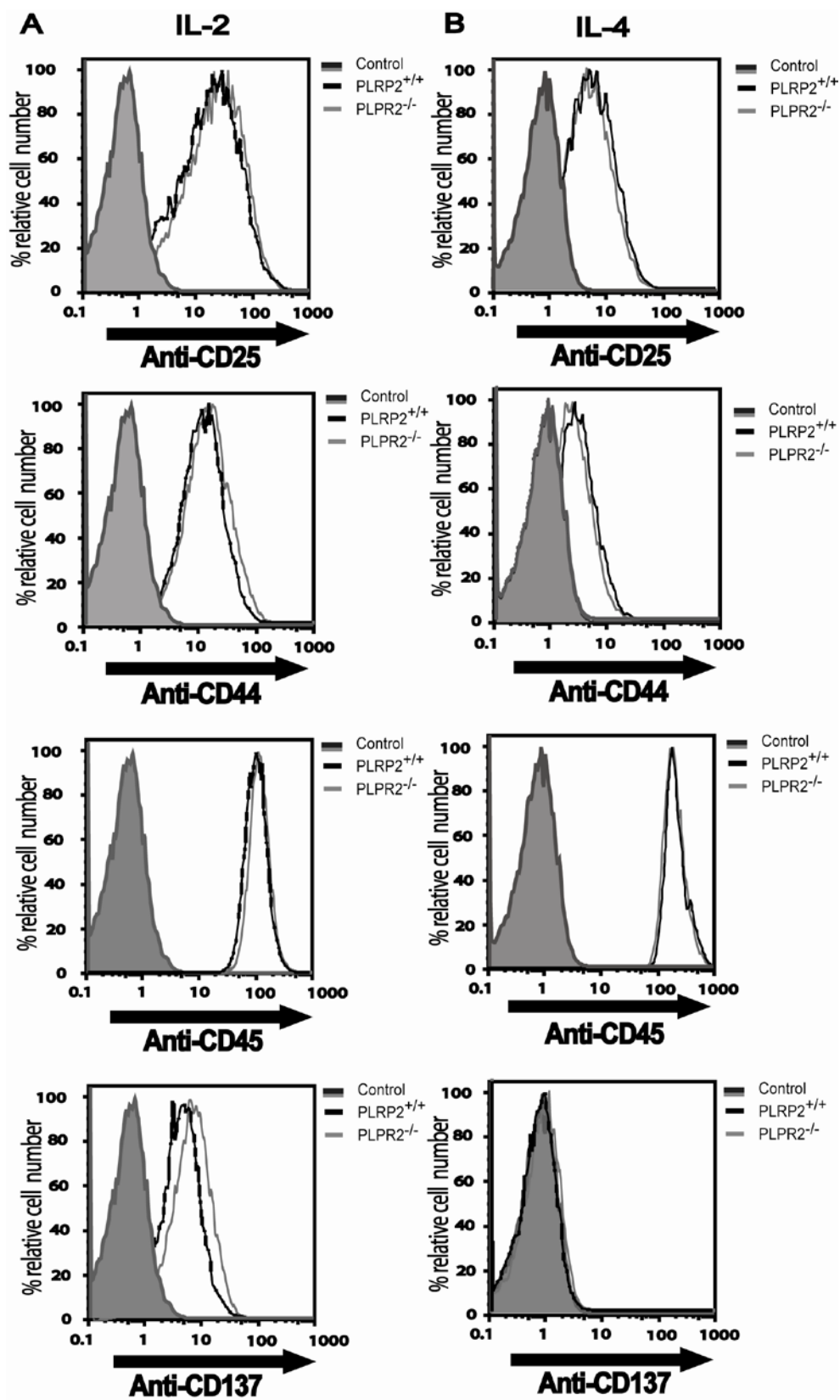


Figure 3.5. PLRP2^{+/+} and PLRP2^{-/-} CTLs are phenotypically indistinguishable by cell surface markers. Splenocytes from PLRP2^{+/+} and PLRP2^{-/-} CTLs were induced with either IL-2 or IL-4 for six days. Surface expression of CD25, CD44, CD45 and CD137 was examined using flow cytometry to monitor ability to up-regulate cell surface proteins that have diverse functions in CTLs. The cells displayed were CD3⁺CD8⁺ cells T cells. We monitored receptors (*e.g.*, CD25 for IL-2), adhesion molecules involved in killing (CD44), a tyrosine phosphatase involved in TCR signal transduction (CD45) and a co-stimulatory molecule, CD137 (4-1 BB). In all cases, the KO had similar values to the WT, by frequencies and by MFIs. PLRP2^{+/+}: solid black line, PLRP2^{-/-}: solid gray line. Unstained controls: shaded peak.

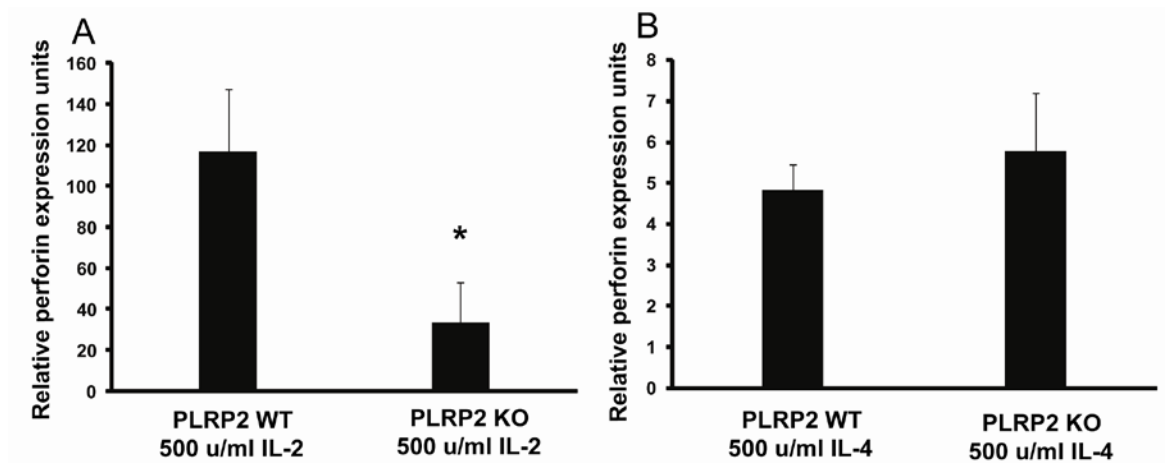


Figure 3.6. mRNA encoding perforin was unaffected by PLRP2 KO in IL-4 induced CTLs. Perforin and cyclophilin mRNAs production were measured using TaqMan^R quantitative RT-PCR and then ratio-ed to compare relative levels of perforin among the different cells. The ratio of perforin to cyclophilin mRNAs in the cells used to standardize the assay was arbitrarily set to a perforin expression unit of 1 (even though there were ~500 fold more cyclophilin mRNAs than perforin mRNAs within these standard cells.) A. Perforin mRNA expression was (unexpectedly) reduced in PLRP2^{-/-} IL-2s induced CTLs, compared to the mRNA expressed in the WT CTLs. The P value for the difference was <0.05. B. Similar perforin mRNA expression was found in PLRP2^{+/+} and PLRP2^{-/-} of CTLs induced with IL-4. Note that the ordinate scale of A is 0-160 while that of B is 0-8 for the ratios of Pfn to cyclophilin mRNAs. As expected based on a previous report [45], perforin mRNA in the presence of IL-4 was depressed.

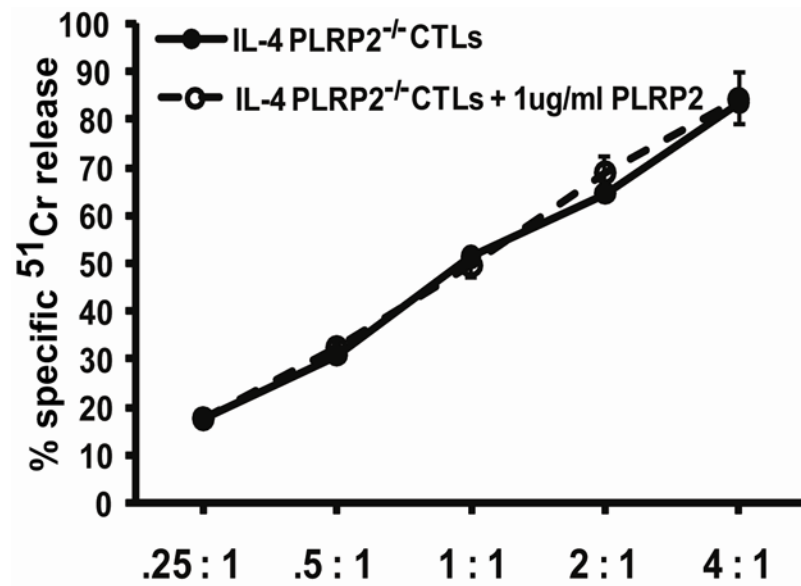


Figure 3.7. The cytotoxic activity of IL-4 induced PLRP2^{-/-} CTLs remained unchanged after rPLRP2 addition. The effects of recombinant PLRP2 addition to CTLs was tested using the 4 hour ⁵¹Cr redirected lysis assay of P815s in which PLRP2 deficiency affected lysis. IL-4 induced PLRP2^{-/-} CTLs were assayed using redirected anti-CD3 toward ⁵¹Cr labeled P815s in the presence or absence of 1 ug/ml rPLRP2. The cytotoxic activity was actually decreased by the addition of rPLRP2. In other experiments, we demonstrated that r-PLRP2 was stable in the presence of granzymes from cytotoxic granule extracts (not illustrated).

Chapter IV

**Triglyceride lipids can enhance the cytotoxicity of induced cytotoxic T lymphocytes
(CTLs)**

ABSTRACT

IL-4 induces a pancreatic lipase, pancreatic lipase related protein 2 (PLRP2), in cytotoxic T lymphocytes (CTLs). Because PLRP2 in semen mediates lipid-dependent toxicity to sperm, we questioned whether CTL-derived PLRP2 could support similar cytotoxicity towards tumor cells. First we tested recombinant PLRP2. Microgram/ml concentrations were toxic to P815 tumor cells in 48 hour assays when lipase, lipid and co-lipase were present. Next, we found that CTLs secrete triglyceride lipase. Despite the released lipase, IL-4 induced CTLs were unable to mediate lipid-dependent cytotoxicity. A single lot of IL-4 was the exception and reproducibly induced CTLs with lipid-dependent cytotoxicity. The triglyceride-enhanced cytotoxicity induced by this lot of IL-4 was perforin-independent. In efforts to induce the lipid-dependent CTL killing again, attempts were made to block type 1-polarizing cytokines which might counteract the IL-4 (type 2) induction of T cells. These attempts included: biasing the T cells cultures with more IL-4 or with IL-12 blocking antibodies, and using T cells that genetically lacked IFN- γ receptors. We also activated the cells with anti-CD3/CD28 (instead of concanavalin A). These attempts were unsuccessful at inducing CTLs with triglyceride-enhanced cytotoxicity. We found that while CTLs induced with IL-4 consistently had mRNA for PLRP2, they lacked mRNA for co-lipase. Therefore, we added exogenous colipase to the CTL assays. Cytotoxicity was unaltered by the addition of colipase. We conclude (1) that lipid-dependent cytotoxicity, promoted by PLRP2, will kill tumor cells but requires colipase and (2) that more than PLRP2 is required for lipid-dependent cytotoxicity mediated by CTLs.

INTRODUCTION

Lipases are mainly enzymes of the pancreas with extensively characterized roles in the digestion of dietary fats [1,2]. Several immune cells also express lipases [3-6], causing us to query what their roles might be. Macrophages produce and secrete lipoprotein lipase that will digest lipids associated with serum proteins [7-10] and IL-4 induced T lymphocytes (T cells) produce a pancreatic lipase, pancreatic lipase related protein 2 (PLRP2) [3-5]. Lymphocytes grown in the presence of triglycerides hydrolyzed fatty acids from the triglycerides, providing further evidence for a T cell derived lipase [11].

Support for lipases with immunologic function came from studies of PLRP2^{-/-} cytotoxic T lymphocytes (CTLs) [5]. T cells taken *ex vivo* displayed reduced cytotoxicity compared to T cells taken from wild type littermates. In addition to a potential direct role in T cell-mediated cytotoxicity, PLRP2 exhibited non-immune indirect toxic effects on sperm samples [12,13]. In these studies, PLRP2 was identified as the enzyme responsible for the reduction in viability of goat semen during long term storage. The loss of sperm viability was caused by the generation of fatty acids by PLRP2 from milk triglycerides added to the storage solution.

Studies have shown that unsaturated fatty acids (UFAs) such as arachidonic, as well as linoleic and oleic acids at elevated concentrations can cause apoptotic as well as necrotic cellular death of tumor cells [14-19]. Tumor cells grown in UFAs have increased DNA fragmentation as well as increased caspase activity [14-16,20]. We wanted to determine if the IL-4 induced T cell lipase, PLRP2, or another similar lipase found within

cytotoxic T lymphocytes may mediate killing indirectly by generation of fatty acids from triglycerides found in the fluids of the body.

Lymphatic and serum fluids contain triglycerides in the form of chylomicrons and very low density lipoproteins (VLDLs) that could serve as substrates to support lipase-dependent cytotoxicity. Triglycerides are customarily viewed as energy storage molecules found within the body and are circulated through both the lymphatic and vascular systems. Each triglyceride is composed of a glycerol backbone with three fatty acid chains attached. During the breakdown of triglycerides by extracellular lipases, fatty acid chains are released from the glycerol, bound to albumin, and transported into cells that oxidize the fatty acid chains to produce ATP.

Using recombinant human PLRP2 (rPLRP2) or native pancreatic triglyceride lipase (PTL), we found that both of these lipases can indirectly mediate cellular death of P815 tumor cells in the presence of triglyceride lipids. Direct treatment of tumor target cells with either PTL or rPLRP2 was ineffective at inducing tumor cell death of the targets. We investigated whether the addition of the triglycerides to IL-4 induced, PLRP2-positive CTLs would enhance the CTL-mediated killing of tumor targets. Long term anti-CD3 ϵ redirected lysis assays of P815 cells and allogeneic killing assays in the presence of triglycerides were used. The cytotoxic activity of IL-4 induced CTLs toward P815 tumor cells was unchanged by the addition of triglycerides, except in one exceptional case. CTLs induced with one particular lot of IL-4 (BD lot 60506) displayed increased cytotoxicity in the presence of lipids toward tumor cells that was reproducible. The triglyceride-enhanced cytotoxicity was absent in assays using PLRP2- IL-2 induced CTLs. Additionally, the triglyceride-enhanced cytotoxicity was determined to be

perforin independent using perforin^{-/-} CTLs. We also determined that IL-4 induced T cells generated a soluble toxic factor from the triglycerides.

All other lots of IL-4 tested supported PLRP2 induction, CTL growth, and activation but were unable to induce the triglyceride-dependent killing. Multiple attempts were made to determine why this single lot of IL-4 was able to induce such impressive effects on CTLs function. Attempts were made to block type 1-polarizing cytokines which might counteract IL-4 (type 2) induction of T cells. These attempts using T cells that were either: induced with 3x more IL-4, treated with IL-12 blocking antibodies and/or T cell that lacked the IFN- γ receptor. We also activated the cells with anti-CD3/CD28 (instead of conA). In other experiments, IFN- γ was also added to test whether increased type 1 cytokine signaling might recreate the triglyceride enhanced cytotoxicity. These methods of CTL induction were unsuccessful at inducing CTLs with triglyceride-enhanced cytotoxicity. The growth rates of the IL-4 induced CTLs were found to be similar between each lot of IL-4 eliminating differences in growth stimulation as a possible explanation. The addition of the lipase cofactor, colipase, which increases PLRP2 activity was unable to reproduce the triglyceride enhanced cytotoxicity previously observed with the original lot of IL-4. At this time we have been unable to identify the factor responsible for the lipid-enhanced killing we observed with IL-4 BD lot 60506.

In summary, we have found that purified PLRP2 together with triglyceride lipid and colipase can mediate indirect cytotoxicity. We also found evidence for triglyceride-enhanced CTL-mediated cytotoxicity to tumor cells but found that PLRP2⁺ CTLs induced with IL-4 alone lack triglyceride-enhanced cytotoxicity.

METHODS AND MATERIALS

Materials: Recombinant (r-) mouse IL-2 was purchased from eBioscience (San Diego, CA, 1×10^7 units/mg; 1.7×10^{11} units/mmol). r-Mouse IL-4 was purchased from BD biosciences (San Jose, CA, 2.5×10^7 units/mg, 3.5×10^{11} units/mmol) or purchased from eBioscience (San Diego, CA, 1×10^7 units/mg, 1.4×10^{11} units/mmol). For the lot numbers see tables 4.1 and 4.2.

Animal Studies. The animal protocols for this study were approved by the University of Nevada, Reno Institutional Animal Care and Use Committee.

Cell Culture. Splenocytes were harvested from the following strains of mice: BALB/c mice wild-type (Jackson Labs, Bar Harbor, ME), BALB/c perforin^{-/-} mice [21] (kindly provided by Dr Thomas Sayers, the National Cancer Institute (NCI, Frederick, MD)) and PLRP2^{-/-} [5] and their littermate controls (kindly provided by Dr. Mark Lowe, University of Pittsburgh). T cells were cultured at 6.5×10^5 cells/ml in T-75 flasks (Starstedt) with complete tissue culture media containing RPMI-1640 media (Sigma Chemical Co, St. Louis, MO), 10% fetal calf serum (HyClone, Logan, UT), 10 mM HEPES buffer (Fisher Scientific, Waltham, MA), 1% Pen-Strep (Sigma), 24 mM sodium bicarbonate (Fisher), 25 μ M 2-mercaptoethanol (Sigma), 2.5 μ g/ml concanavalin A (conA) (Sigma) and 500 units per ml of either IL-2 or IL-4 to induce the splenocytes. Following an initial 3 days of culture with conA and cytokines, the T cells were washed to remove the mitogen conA and cultured in complete tissue culture medium lacking conA with either IL-4 or IL-2 for

an additional 2 days before use in assays. In experiments where plate bound anti-CD3 and anti-CD28 were substituted for conA stimulation, 10 $\mu\text{g/ml}$ anti-CD3 and 20 $\mu\text{g/ml}$ anti-CD28 were used to initially coat plates. eGFP-P815 mastocytoma cells [22] were maintained in Dulbecco's Modified Eagle Medium, DMEM, (Gibco, Grand Island, New York) with 180 mM sodium bicarbonate (Fisher), 1 $\mu\text{g/ml}$ puromycin (Sigma) and 10% FBS (HyClone). All cell cultures were incubated at 37°C and 5% CO₂.

Measurement of lipase activity released by CTLs: Lipase activity released by CTLs was determined by measuring the amount of ³H-fatty acid released from ³H labeled triolein during anti-CD3 induced exocytosis. To create media with lipid micelles, 0.312 mM unlabeled triolein (Sigma) was added to complete RPMI-1640 media followed by the addition of 1.14x10⁻⁷ mM ³H-triolein (spec. act. 91 Ci/mmol, Perkin Elmer, Waltham, Massachusetts). The lipid containing media was vortexed for 3 minutes to create micelles. The 135 μl s of micelle-containing media were plated out into each well of a 96 wells plate. Following the micelles, 135 μl s of media containing CTLs and anti-CD3 (clone 4-2C11) (eBiosciences) coated beads was added to each well. The assay was incubated at 37°C with 5% humidity for 4 hours. Following this incubation, 200 μl s cells and media were collected and the released fatty acids were extracted using 800 μl s of a chloroform / methanol / heptanes solution (14.5 parts / 12 parts /10 parts). The resulting extraction was made basic with the addition of 250 μl s from a 50 mM carbonate (Sigma) solution, pH 10. The phase separation of the fatty acids and lipid layers was created by centrifugation at 2600 rpm (1000 g) for 5 minutes at room temperature. From the top phase containing fatty acids, 200 μl s was collected, and mixed with 500 μl s of

scintillation cocktail, (MicroScint-20, Perkin Elmer) and counted in a Beckman scintillation counter (LS 6000IC Fullerton, CA).

⁵¹Cr redirected lysis assays: IL-2 or IL-4 induced CTLs cytotoxicity was tested using 8 hour redirected lysis assays with eGFP-P815 mastocytoma cells as radio-labeled targets. eGFP-P815 target cells were labeled with Na⁵¹CrO₄ (Perkin Elmer) [23] for 1 hour. T cells were redirected to the IgG Fc receptor-bearing P815 cells with 1 µg/ml anti-mouse CD3 mAb (clone 4-2C11) (eBiosciences, San Diego, CA). For control conditions, T cells were incubated with ⁵¹Cr labeled P815s in the presence of golden Syrian hamster IgG (14-4914-85, eBiosciences, San Diego, CA). Assays were run in quadruplicate with 10,000 radio-labeled cells per well for 8 hours with and without 1.5 mM trilinolein (Sigma). Trilinolein (Sigma) was dissolved in ethanol and added to the CTL: P815 mixtures. The final ethanol concentration in all assays did not exceed 0.07%, which we found to lack detectable toxicity toward CTL or P815s (data not shown).

Specific release was calculated by the formula:

$$\% \text{ Specific Release} = [(Experimental \text{ CPM}) - (Spontaneous \text{ Release})] / [(SDS \text{ Total Release}) - (Spontaneous \text{ Release})] \times 100.$$

Cytometric tumor outgrowth assay: On day 5, CTLs were plated out with eGFP-P815s at specified effector to target ratios in the presence of 1 µg/ml anti-CD3 (clone 4-2C11) (eBioscience) or with 1 µg/ml of golden Syrian hamster IgG (eBioscience, San Diego, CA).

Trilinolein (Sigma) was dissolved in ethanol and added to the CTL: P815 mixtures as above. The assay was incubated at 37°C with 5% humidity for 48 hours. Upon completion of the incubation, cells were harvested and measured with a Beckman Coulter XL/MCL Flow Cytometer. Live and dead eGFP-P815s were distinguished from one another based on GFP fluorescence and forward scatter properties. To avoid counting errors caused by cell doublets and debris from dead cells, counting beads (Polymer Labs, Amherst, MA) were added to each sample and used to normalize the viable P815 cell number within each sample. The data were analyzed with FlowJo software (Tree Star, Inc., Ashland, OR). In some experiments, JURKAT cells (ATCC) were evaluated as alternate targets, monitoring live cells by forward and side light scatter properties.

Lipid product toxicity assay: Day 5 IL-2 or IL-4 induced T cells were harvested and plated out with anti-CD3 (clone 145-2C11) (eBioscience) coated streptavidin beads (Polysciences, Inc., Warrington, PA) at varying bead to CTL ratios in the presence of 1.5 mM trilinolein. The assay was incubated at 37°C with 5% humidity for 48 hours. Upon completion of the incubation, cells and media were collected and spun in 1.5 ml Eppendorf tubes at 10,000 rpm (10,200 g) for 10 minutes. The cell-free supernatant was collected and was added 1 to 1 to media containing eGFP-P815s at a concentration of 200,000 cells per ml. The assays were incubated at 37°C with 5% humidity for another 48 hours. Viable P815 cells were measured by flow cytometry as above.

Quantitative RT-PCR. TaqmanR quantitative RT-PCR was performed with TaqMan® FAM-dye labeled probes for PLRP2, co-lipase, and cyclophilin mRNAs per the

manufacturer's directions. Cyclophilin was used as an internal calibration control to adjust for differences in the amounts of mRNA in different samples. Purified pancreatic total RNA (Biochain) was used as a positive control for PLRP2 and colipase expression.

Statistics: Results are expressed as mean +/- standard error of the mean of the collected data. Statistical evaluations were performed with Excel and Sigma plot software applying Student's T-test. Differences were considered significant if the P value was <0.05.

Results

Triglyceride lipases, including PLRP2, together with lipid substrates, mediated indirect toxicity toward tumor cells

We questioned if the IL-4 inducible lipase, PLRP2, was directly or indirectly toxic towards tumor cells. We incubated recombinant PLRP2 with P815 cells, with or without triglyceride lipids, and in the presence or absence of the lipase cofactor, colipase over a 48 hour period. Native purified pancreatic triglyceride lipase (PTL) was used as a control because it is a similar enzyme and is commercially available in large quantities. P815 viability was reduced when these lipases and colipase were combined with the triglyceride, trilinolein (Figure 4.1A). Without the lipid substrate, there was no cytotoxicity, indicating that the lipases, even with their co-factor, were unable to directly mediate cytotoxicity. PLRP2 was much more toxic with colipase (Figure 4.1A). Similar concentrations of PLRP2 without colipase mediated little indirect cytotoxicity when provided triglycerides (Figure 4.1A). JURKAT cells were also tested as tumor cell targets for direct and indirect cytotoxicity by PLRP2 or by PTL. They were also invulnerable to direct attack (in the absence of exogenous lipids) and susceptible to indirect toxic effects by both lipases when combined with lipid substrate (Figure 4.1B). PLRP2 alone at 10 $\mu\text{g/ml}$ mediated modest indirect cytotoxicity toward JURKAT cells when provided triglycerides (Figure 4.1B). These lipases will release 2 or 3 molecules of linoleic acid from each molecule of trilinolein. The toxicity generated by each lipase in the presence of triglycerides was similar to the toxicity observed when P815 cells were incubated with high concentrations of linoleic acid for 48 hours (Figure 4.1C). The 1.5 mM trilinolein substrate in the assays and the activities of the lipases would be sufficient

to produce the 100+ micromolar concentrations of fatty acids needed. Furthermore, addition of delipidated bovine serum albumin blocked the toxic effects (not illustrated). The BSA was comparable to albumin concentrations found in serum that can bind as much as 3.6 mM fatty acid (calculated at a buffering capacity of 7 molecules of fatty acid per molecule of albumin [24]). These findings suggest that the CTL-associated lipase PLRP2 could generate toxic fatty acids from triglycerides if the concentrations of PLRP2 were sufficient.

Triglyceride-hydrolyzing lipases are secreted by IL-4-induced CTLs

The possibilities for PLRP2 (and other lipase) release include constitutive secretion, a burst of exocytosis and/or prolonged secretion that are stimulated after T cells encounter their antigens. To simulate antigen recognition, we treated the CTLs with either phorbol myristate acetate (PMA) combined with an ionophore (ionomycin) [25] or with solid phase anti-CD3 [26]. IL-4 induced T cells were stimulated with PMA and ionomycin or with DMSO as an unstimulated control for 4 hours in media with ^3H -triolein in lipid micelles. The cell-free supernatant was collected and tested for the release of radio-labeled fatty acids from ^3H -triolein. Supernatants from DMSO treated CTLs displayed triglyceride lipase activity (Figure 4.2). Fetal calf serum in the media contributed lipase activity adding to the background (Figure 4.2). The CTL-mediated constitutive secretion of lipase compared to the media had a $P > 0.05$ in two of three experiments, including the one illustrated. Thus the activity was low but detectable. Stimulation with PMA and ionomycin or anti-CD3 (data not illustrated) increased lipase

release over constitutive secretion in two of three experiments, but was not statistically significantly greater than DMSO stimulated controls (Figure 4.2). CTLs grown in IL-2 also had constitutively secreted triglyceride lipase(s) but there was no evidence of stimulated release of lipase (data not illustrated). The background hydrolysis of ^3H -triolein by the media alone handicapped detection of the cellular lipases; however, it was necessary to retain fetal calf serum in these experiments in order to reduce the death of PMA-stimulated T cells. Calibration with PLRP2 as an internal standard in the assays indicated that there would be less than 30 ng of PLRP2 secreted per 10^6 IL-4 CTLs, assuming that all the secreted lipase activity was mediated by only PLRP2.

Triglyceride lipids increased the cytotoxic activity of CTLs, but only CTLs induced with one unusual lot of IL-4

To determine if the addition of the triglyceride, trilinolein, would affect CTL cytotoxic activity, we performed two types of cytotoxic killing assays, 8 hour ^{51}Cr -release and 48 hour tumor viability assays. CTLs from perforin KO mice induced with IL-4 (BD lot 60506) demonstrated low but statistically significant lipid-enhanced cytotoxicity at 8 hours (Figure 4.3A). Similarly induced CTLs had little lipid enhanced cytotoxicity at 4 hours (not shown). The lipid-dependent effects in the 8 hour assays were later found to be non-reproducible with other lots of IL-4 (Figure 4.3B). All of these lots did induce PLRP2 (data not illustrated). These results are particularly disappointing since PLRP2 in semen (with lipid) was toxic to sperm within 60 minutes. For reference, lipid was also without effect on the cytotoxicity of perforin KO CTLs induced with IL-2 (not

illustrated). Thus we obtained a tantalizing indication of lipid-enhanced cytotoxicity with CTLs induced in the presence of one lot of IL-4 (which contained IL-4 and probably an additional factor). At the time we first discovered this phenomenon, we continued our investigations with the same lot of IL-4, using assays that favor detection of apoptosis.

Longer term assays, that are almost equally effective with WT and perforin KO CTLs, were evaluated next. Trilinolein was added to 48 hour anti-CD3 redirected lysis of eGFP-P815 cells using IL-2 and IL-4 induced CTLs. For reference, the viability of P815 cells was unaffected when the P815s were incubated with lipid alone. Viable P815 cells were reduced in the triglyceride-treated assays when compared to the assays containing IL-4 induced CTLs without lipids (Figure 4.4A). This triglyceride-enhanced cytotoxicity was absent in the long term killing assays using IL-2 induced CTLs (Figure 4.4B). Triglyceride-enhanced lysis of P815 was also tested in allogeneic killing assays in which the anti-CD3 antibody was omitted (Figure 4.4C). The BALB/c effectors and the P815 cells are both H-2^d but differ at minor histocompatibility loci [27]. Again, IL-4 induced cells displayed higher cytotoxic activity toward P815s when lipid was present (Figure 4.4C). In summary, CTLs induced with IL-4 plus a probable contaminant demonstrated a novel method of mediating tumor cell death that utilizes triglyceride lipids.

Triglyceride-enhanced cytotoxicity by the induced CTLs was perforin independent.

Perforin is a primary mediator of the cytotoxic activity utilized by CTLs. Perforin deficient CTLs have dramatically less cytotoxic capacity than their wild type counterparts [28]. We questioned if the triglyceride-enhanced cytotoxic activity was perforin dependent or independent using the long term killing assays with IL-2 or IL-4 induced

CTLs from perforin^{-/-} mice. The lytic activity of the IL-4 perforin^{-/-} CTL was enhanced with the addition of triglyceride in the long term assay (Figure 4.5A). As was seen with the WT IL-2 induced CTLs, perforin knockout IL-2 induced CTLs showed little to no enhancement upon the addition of triglycerides (Figure 4.5B). The triglyceride-enhanced cytotoxicity was also easier to detect with the perforin KO CTLs than the WT CTLs (compare Figure 4.4A with Figure 4.5A). The lipid-enhanced cytotoxicity was consistent in 3 experiments with WT CTLs and 3 experiments with perforin KO CTLs, using BD lot 60506 IL-4 (Table 4.1).

The induced CTLs generated toxic products in the presence of lipid substrate

At this time we were unaware of the dependence of the lipid-enhanced killing on a single lot of IL-4, and proceeded with further experiments. To determine if the lipid-enhanced killing was mediated by toxic products produced from the lipid, IL-2 and IL-4 induced CTLs were stimulated to undergo exocytosis in the presence or absence of triglycerides and cell-free supernatants were evaluated for toxicity. Exocytosis from CTLs was stimulated with anti-CD3 coated beads for 48 hours. Following exocytosis, cell-free supernatants were collected and added to eGFP-P815 cells and the cells were incubated for 48 hours. Lipid was present either during the initial 48 hours of CTL exocytosis (to test for lipase activity upon the lipid) or the lipid was present only during the incubation of the CTL supernatant with eGFP-P815s (to test for a released lipase that might remain active in the cell-free supernatants and later be able to convert lipids into toxic products). As another experimental condition, lipid was present during both the 48 hours of CTL exocytosis as well as during the incubation of the supernatants with the

P815s. P815 cell viability was reduced by the cell free supernatants that were initially generated from CTLs undergoing exocytosis in the presence of triglycerides (Figure 4.6). It is unfortunate that we consumed all of lot 60506 IL-4 before we realized its unique qualities. These findings concerning the CTLs and their cell-free supernatants indicate that lipid-enhanced killing *can* be generated by CTLs in the presence of IL-4. It is unresolved if the IL-4 was necessary and if the supernatants contained fatty acids as potential mediators.

Efforts to reproduce the triglyceride-enhanced cytotoxicity

To our dismay, the triglyceride-enhanced cytotoxicity as well as the production of the soluble toxic factor was missing when new lots of IL-4 were used to induce these cells (see Table 4.1). We initially suspected that the new IL-4 lots were less potent than BD lot 60506 and could not induce PLRP2. Studies of IL-4 induced T cells using the lots of IL-4 following the exhaustion of lot 60506 expressed mRNA for PLRP2 (see chapter 3 and data not illustrated). IL-4 is a growth factor for T cells, if later lots of IL-4 were less potent then T cell growth would be reduced. However, the T cells growth rates from day 0 to day 3 were similar with each lot of IL-4, suggesting a separate unidentified factor is responsible for the triglyceride-enhanced killing (Figure 4.7). The growth rates from day 3 (when the conA was removed) to day 5 were even more similar (not illustrated). Attempts were also made to reproduce the triglyceride-enhanced toxicity by increasing the IL-4 concentration, without effect. As for the factor being the result of increased lipopolysaccharide (LPS), lot 60506 actually had less LPS than the other IL-4 lots as reported by the vendors, eliminating LPS as the unknown factor.

Following these results and while gathering information about other lots of IL-4, we questioned whether lack of the triglyceride enhanced toxicity could be caused by increased type 1 responses in potentially modified cultures. Both IL-12 and IFN- γ are the dominant cytokines associated with the generation of a type 1 response in naïve T cells [29,30]. To reduce these type 1 polarizing cytokines, IFN- γ receptor^{-/-} splenocytes were induced with IL-4 in the presence of 20 μ g/ml IL-12 blocking antibodies. Again, these conditions were unsuccessful at reproducing the triglyceride enhanced killing. We also tested for effects of type 1 polarization by adding high concentrations of recombinant mouse IFN- γ plus IL-4 during splenocyte activation; however, these experiments were performed with ineffective lots of IL-4 and as might be expected, the triglyceride-enhanced killing was still absent for CTLs induced with standard IL-4 plus IFN- γ (Table 4.2). To test if different activating stimuli could reproduce triglyceride-enhanced cytotoxicity in IL-4 induced CTLs we used plate bound anti-CD3/CD28 to activate splenocytes instead of conA activation. This induction method was also unsuccessful at reproducing the lipid-enhanced toxicity originally observed in the IL-4 induced CTLs (Table 4.2).

Another possible explanation for the triglyceride-enhanced killing would be the coincident CTL expression of the cofactor that increases the activity pancreatic lipases, a small protein termed colipase. Colipase increases both PTL and PLRP2 activity toward triglycerides [31-33]. We tested for the presence of colipase in lymphocytes by Taqman® RT-PCR, using pancreatic mRNA for calibration, and were unable to detect any mRNA for co-lipase in lymphocytes. We wondered if the unknown factor could induce colipase. Since lot 60506 was consumed, direct information was impossible. We

tested if the addition of purified colipase to CTLs induced with standard lots of IL-4 would support lipid-enhanced cytotoxicity by increasing the activity of a released lipase. The colipase increased the triglyceride-enhanced cytotoxicity of purified PTL but was without effect on the IL-4 induced CTLs. At this time, we can confidently report that PLRP2-positive CTLs induced with standard lots of IL-4 are unable to mediate the triglyceride-dependent cytotoxicity that was observed for PLRP2 in semen. However, the results associated with IL-4 lot 60506 hints at the potential existence of a CTLs-mediated triglyceride-dependent cytotoxicity.

DISCUSSION

Here we discuss and provide perspectives on three aspects of our work. First, we believe we are the first to report that lipases generate products in the presence of triglycerides that are toxic to tumor cells. Next, we examine the ability of CTLs to support triglyceride-dependent killing of tumor cells. Essentially, CTLs with the potentially toxic lipase PLRP2 are unable to mediate triglyceride-dependent killing. However, there is potential for CTLs to acquire this unique mechanism. Lastly, we offer future approaches that could guide researchers willing to pursue a novel cytotoxic mechanism.

The most positive results are the discovery that lipases can cause toxicity to tumor cells. Two lipases, PTL, a digestive lipase of the pancreas, and PLRP2, found in the pancreas, lymphocytes, and testes, were toxic to tumor cells if appropriate triglyceride lipid substrates were provided.

Without lipid, the lipases were nontoxic, indicating that toxicity was indirect and mediated by triglyceride byproducts. We found that two tumor lines, murine P815s that are somewhat resistant to apoptosis and the apoptosis-sensitive human JURKAT cells, could be killed when incubated in the presence of lipase and triglyceride lipids. The products generated from the lipid, trilinolein, during lipase hydrolysis include the fatty acid, linoleic acid with di- and mono-glycerides. Research considering the effects of fatty acids on tumor cells has demonstrated that at high concentrations, unsaturated fatty acids have toxic effects on different tumor cells [14,16,34,35]. Normally most fatty acids are either conjugated to albumin or are bound to glycerol to form triglycerides. Lipases, such as those used here, are employed by the body to release fatty acids from triglycerides.

Our experiments using linoleic acid revealed similar levels of toxicity toward the tumor cells can be caused by 48 hour exposure to concentration of 100 μ M linoleic acid and above. From the enzymatic activities of these two lipases in the tissue culture media with radioactive triolein as a substrate, fatty acids in excess of toxic concentrations of 100 μ M could be generated within hours. Thus, the levels of released fatty acids were generated in potentially toxic concentrations. Furthermore, assays carried out in media containing physiological concentrations of albumin (which binds 7 fatty acid molecules per molecule of albumin) blocked the toxicity suggesting that released fatty acids are responsible for the observed toxicity.

Additionally, death from fatty acids requires more than 24 hours. This corresponds to the time required for the death observed in tumor cells incubated with the combination of lipase, lipid, and cofactor. *In vivo* efficacy would require lipid substrate, which occurs in millimolar concentrations in serum very light density lipoproteins (VLDLs) and in chylomicrons [36]. Considerations for practical tumor therapy include the physiological clearance of the toxic products via blood and lymph and the buffering of fatty acids by serum albumin. Both of these mechanisms could reduce fatty acids to nontoxic levels. However, for perspective, tumor micro-environments may include regions with low circulation and low serum albumin, where CTLs could be invasive effector cells.

The disappointing results of the paper concern CTL lipid-dependent cytotoxicity. Even though all lots of IL-4 induced PLRP2 in CTLs (manuscript submitted, dissertation chapter 3), the IL-4 induced CTLs lacked lipid-dependent cytotoxicity with one exception. We were hoping to explain the two-three fold greater activity of WT than

PLRP2^{-/-} CTLs in 4 hour ⁵¹Cr assays (manuscript submitted, dissertation chapter 3).

Thus, despite the work here and elsewhere [5], the effect of PLRP2 gene ablation on rapid CTL killing remains unexplained. The exceptional lot of IL-4 indicates that CTLs can mediate lipid-dependent cytotoxicity. It may be prudent to view this phenomenon as evidence that the CTLs can secrete lipase(s), which might be intended for cellular nutrition instead of as a cytotoxic mechanism [11].

We close this discussion with thoughts for the future. The method of preparation of the exceptional lot of IL-4, BD 60506, by expression in insect cells infected with IL-4 encoding baculovirus and antibody affinity chromatography, suggests that stimulation of Toll receptors would be worth investigating. Toll receptors are found on dendritic cells and macrophages, both of which would be included with our splenocyte cultures. Viral nucleic acids or other viral components could stimulate Toll receptors 2 and 9 [37-39]. Unfortunately, the vendor was unable to provide more of this lot of r-IL-4 for characterization. We recommend that future studies consider the use of trilinolein as the lipid substrate, long term tumor viability assays to monitor lipid enhanced toxicity, and use of perforin-KO CTLs from BALB/c mice which are a 'type 2'-responder strain [40].

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Table 4.1. The triglyceride-enhanced cytotoxicity of IL-4 induced CTLs

The triglyceride-enhanced cytotoxicity was supported by only BD lot 60606 IL-4 ^a .				
IL-4 lot ^b	Mouse strains ^c	Lytic units per 10 ⁶ CTLs		Lipid enhancement ^f
		IL-4 CTLs ^d	IL-4 CTLs + lipid ^e	
BD: 60506	Balb/c WT	17902	29574	1.7
BD: 60506	Balb/c WT	20837	29250	1.4
BD: 60506	Balb/c WT	17125	23825	1.4
BD: 60506	Balb/c perforin KO	24041	74989	3.1
BD: 60506	Balb/c perforin KO	10955	38416	3.5
BD: 60506	Balb/c perforin KO	2359	11936	5.1
BD:83653	Balb/c WT	11915	9402	0.8
BD:83653	Balb/c WT	22222	25763	1.2
BD:83653	Balb/c perforin KO	28529	29749	1.0
BD:83653	Balb/c perforin KO	23378	27227	1.2
eBio:E028417	Balb/c WT	9824	8734	0.9
eBio:E028417	Balb/c perforin KO	23820	23433	1.0
eBio:E031047	Balb/c WT	35815	21322	0.6
eBio:E031047	Balb/c perforin KO	30388	37775	1.2
eBio:E031047	C57B6	35815	21322	0.6
eBio:E031047	C57B6 perforin KO	15226	11749	0.8

^aCells were cultured for 5 days with 500 units/ml of IL-4. ^bThe vendors and lots of IL-4.

^cMouse strains and genetic modifications. ^dLytic units per 10M CTLs for IL-4 induced

CTLs without triglycerides. ^eLytic units per 10M CTLs for IL-4 induced CTLs with

triglycerides. ^fFold differences between the lytic activity of CTLs with lipids compared to

CTLs without lipid.

Table 4.2. The alternative methods of CTL induction tested

Different methods of activation were unable to reproduce the lipid-enhanced cytotoxicity induced by IL-4 lot 60506.						
IL-4 lot ^a	Conditions ^b	Activation ^c	Mouse strain ^d	Lytic units per 10 ⁶ CTLs		Lipid enhancement ^g
				IL-4 CTLs ^e	IL-4 CTLs + lipid ^f	
BD:83653	1500 U/ml IL-4	ConA	Balb/c WT	16031	16950	1.1
eBio:E031047	1500 U/ml IL-4	ConA	Balb/c perforin KO	23246	31979	1.4
eBio:E031047	500 U/ml IL-4	ConA	IFN γ receptor KO B6	26246	16407	0.6
eBio:E031047	500 U/ml IL-4 + anti-IL-12	ConA	IFN γ receptor KO B6	46398	16408	0.4
eBio:E031047	500 U/ml IL-4	Anti-CD3/CD28	C57B6	30063	27780	0.9
eBio:E031047	500 U/ml IL-4	Anti-CD3/CD29	C57B6	23088	27931	1.2
eBio:E031047	500U/ml IL-4 + 400U/ml IFN γ	ConA	C57B6	3543	1351	0.4

^aLot of IL-4. ^bConcentration of cytokine during induction of T cells. ^dMouse strain and genetic modifications. ^eLytic units per 10M CTLs for IL-4 induced CTLs without triglycerides. ^fLytic units per 10M CTLs for IL-4 induced CTLs with triglycerides. ^gFold differences between the lytic activity of IL-4 induced CTLs without lipids compared to IL-4 induced CTLs with lipid.

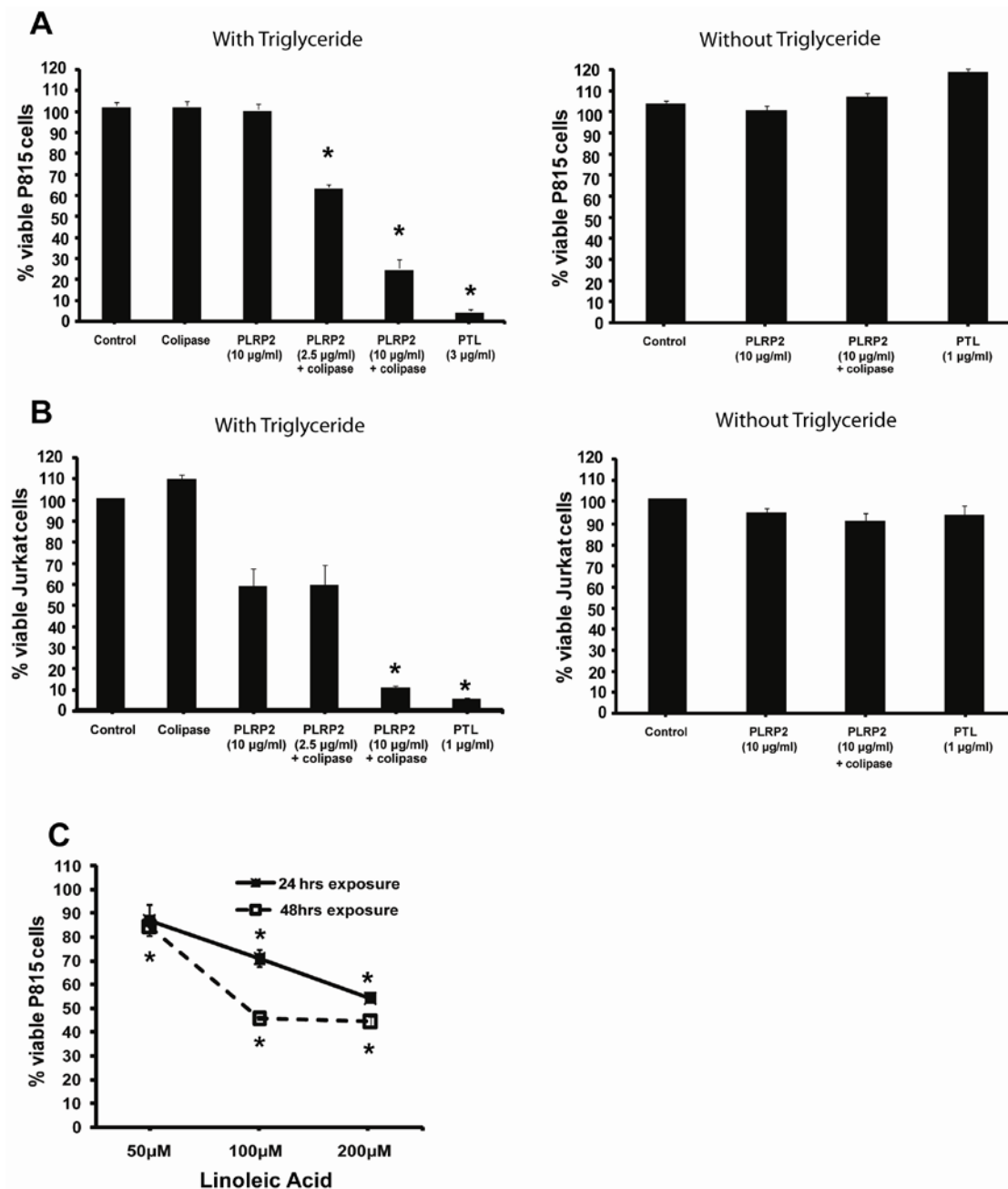


Figure 4.1. PLRP2 with triglyceride lipid substrate mediated indirect toxicity toward tumor cells. eGFP-P815 or JURKAT tumor cells were incubated with varying concentrations of recombinant PLRP2 and a constant concentration of colipase, with and without the triglyceride trilinolein for 48 hours. The fluorescent viable P815s and the

intact JURKATS (determined by forward and side scatter properties) were counted by flow cytometry. Recovery was calculated using marker beads to normalize each sample to a constant volume. Cell debris, gated out in these analyses, indicated that the tumor cells were dying rather than simply arresting their growth. The error bars in this figure and the following figures represent standard errors of the mean. The asterisks indicate P values <0.05 for reduction of viable cells compared to control cells that were cultured in lipid-containing media but without lipases. A and B. PLRP2 together with colipase significantly reduced P815 and JURKAT cell recovery when triglycerides were present. C. eGFP-P815 cells were incubated with varying concentrations of linoleic acid for 48 hours. P815 cells died at 100 micromolar and greater concentrations of linoleic acid.

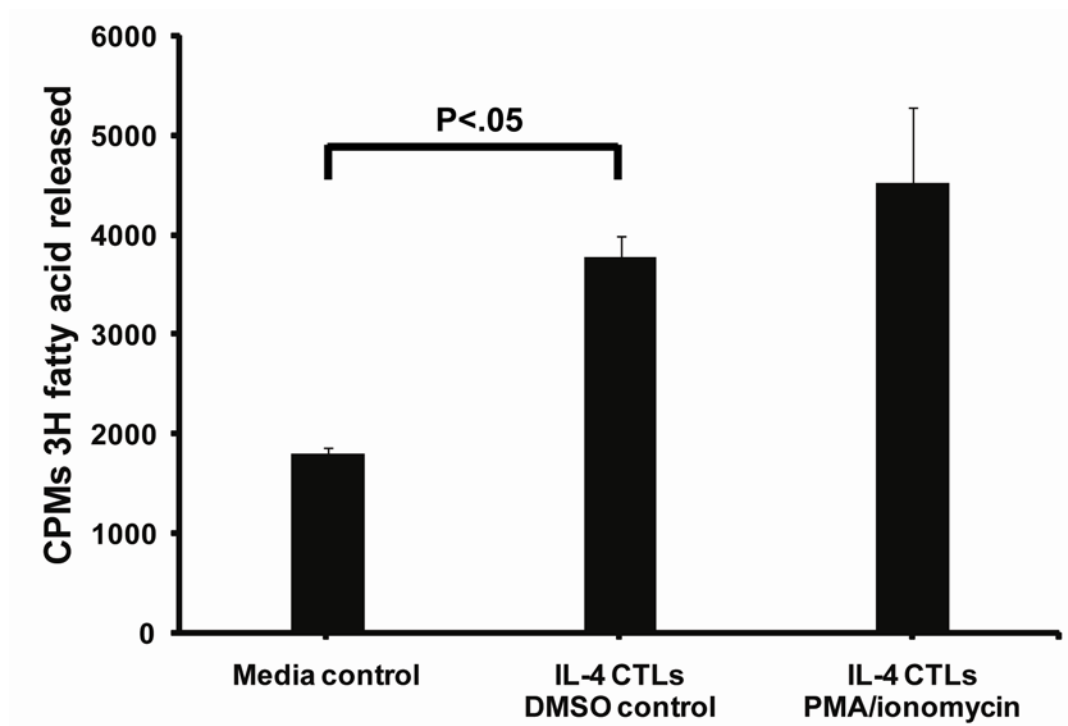


Figure 4.2. CTLs secreted triglyceride lipase(s). CTLs after 5 days of culture with lot 83653 IL-4 were incubated for 4 hours with lipid micelles that contained ^3H -triolein as a substrate to detect lipase activity. PMA and calcium ionophore were used to initiate degranulation and cytokine release from the CTLs. The fatty acids released by lipases into the cell-free supernatants were separated from the substrate lipid by extraction with organic solvents. The lipase present in the media control is due to the addition of fetal calf serum.

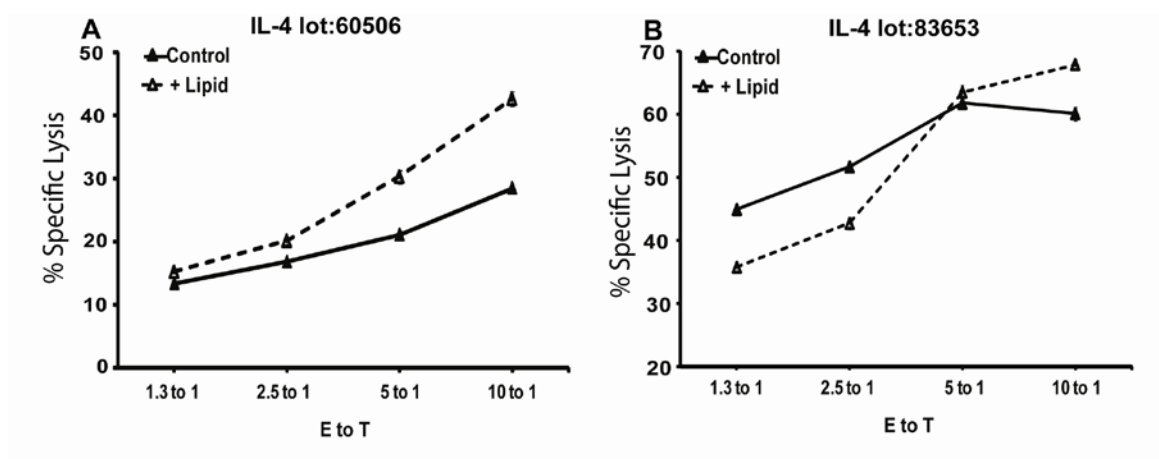


Figure 4.3. Triglyceride lipids enhance the cytotoxic activity of CTLs in 8 hour

51Cr-release assays. CTLs, cultured with different lots of IL-4, were incubated with radio-labeled P815 cells in anti-CD3 redirected assays that were stopped after 8 hours.

The assays were performed with or without trilinolein. A. CTLs cultured with lot 60506 IL-4 displayed lipid-enhanced cytotoxicity by 8 hours. B. CTLs cultured with lot 83653 IL-4 lacked lipid-enhanced cytotoxicity.

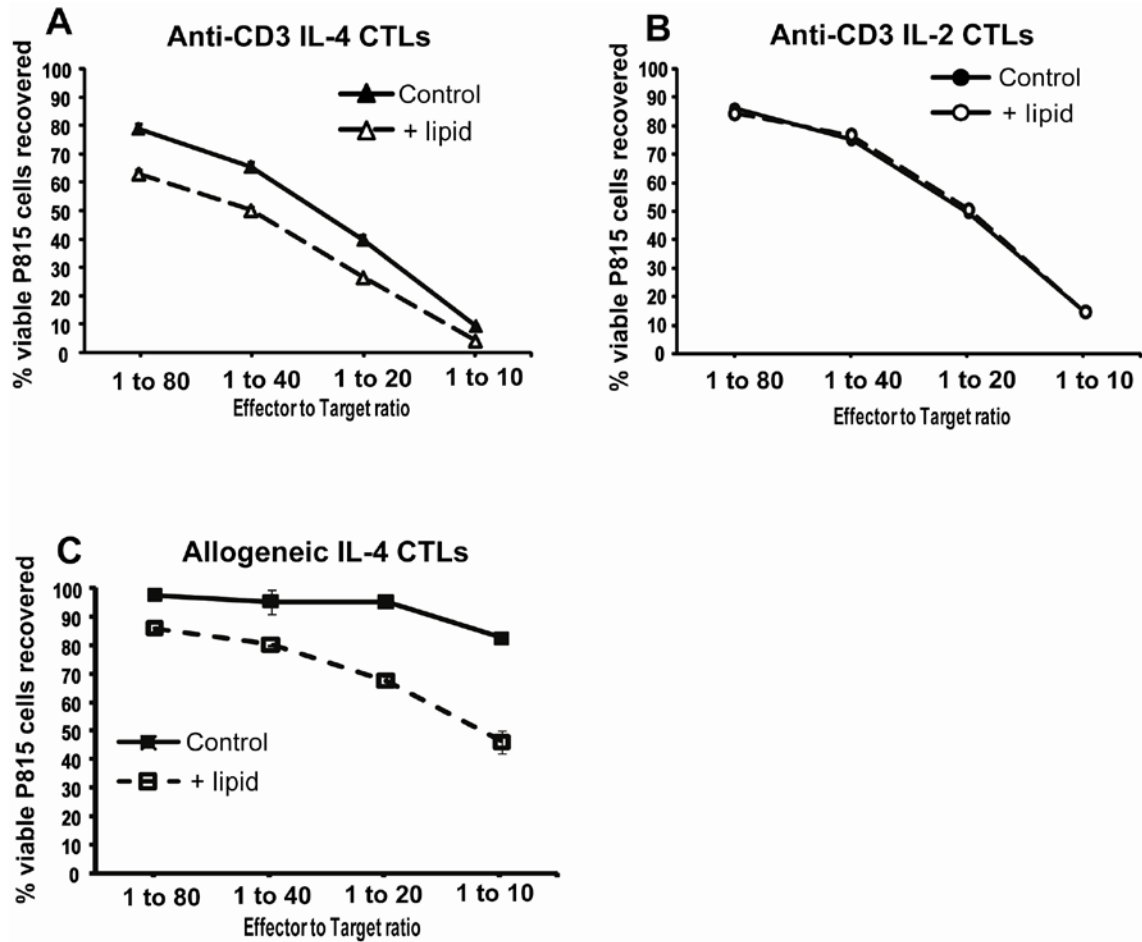


Figure 4.4: Triglyceride lipid enhanced the cytotoxic activity of IL-4 lot 60506 induced CTLs in long term tumor viability. The effects of trilinolein addition to IL-2 and lot 60506 IL-4 induced CTLs were tested in anti-CD3 redirected tumor viability assays. These assays used eGFP-P815s incubated with the CTLs for 48 hours and were assessed by flow cytometry as in Figure 4.1. A. Lot 60506 IL-4 induced CTLs displayed an increase in cytotoxic activity in the presence of triglyceride. B. IL-2 induced CTLs showed no enhancement with the addition of triglycerides. C. Allogeneic long term tumor viability assays were used to evaluate if the lipid-enhanced killing would occur without anti-CD3 redirected CTL engagement of the tumor cells. The lot 60506 IL-4-induced CTLs displayed triglyceride lipid-dependent cytotoxicity.

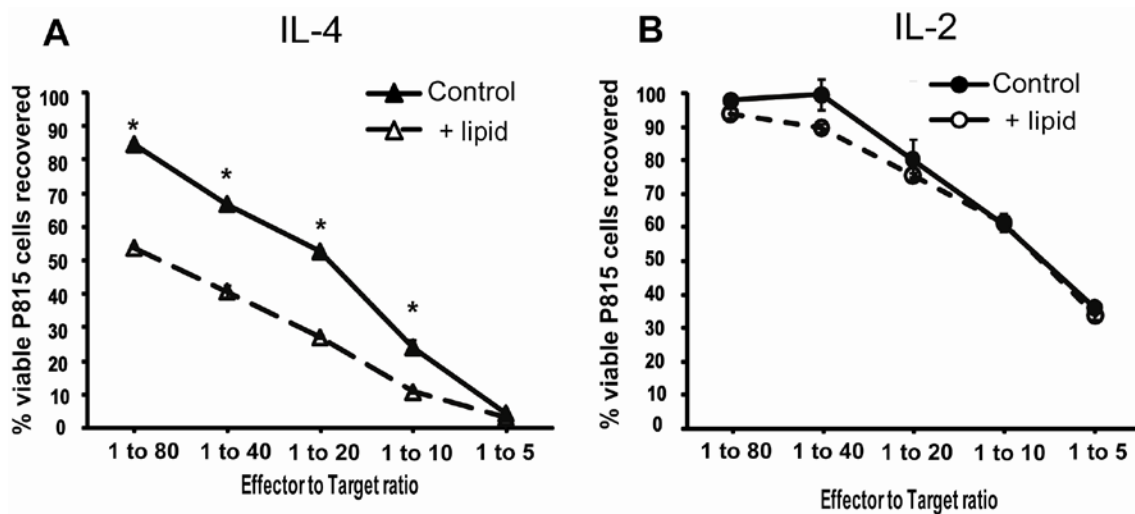


Figure 4.5: Triglyceride enhanced CTL activity was perforin independent. IL-2 and IL-4 induced CTLs were generated from a perforin knockout BALB/c mouse to determine if the triglyceride enhancement was perforin independent. Triglyceride enhancement was observed in the lot 60506 IL-4 induced perforin knockout CTLs. This activity was again absent from IL-2 induced CTLs, suggesting triglyceride-enhanced cytotoxicity is independent of perforin.

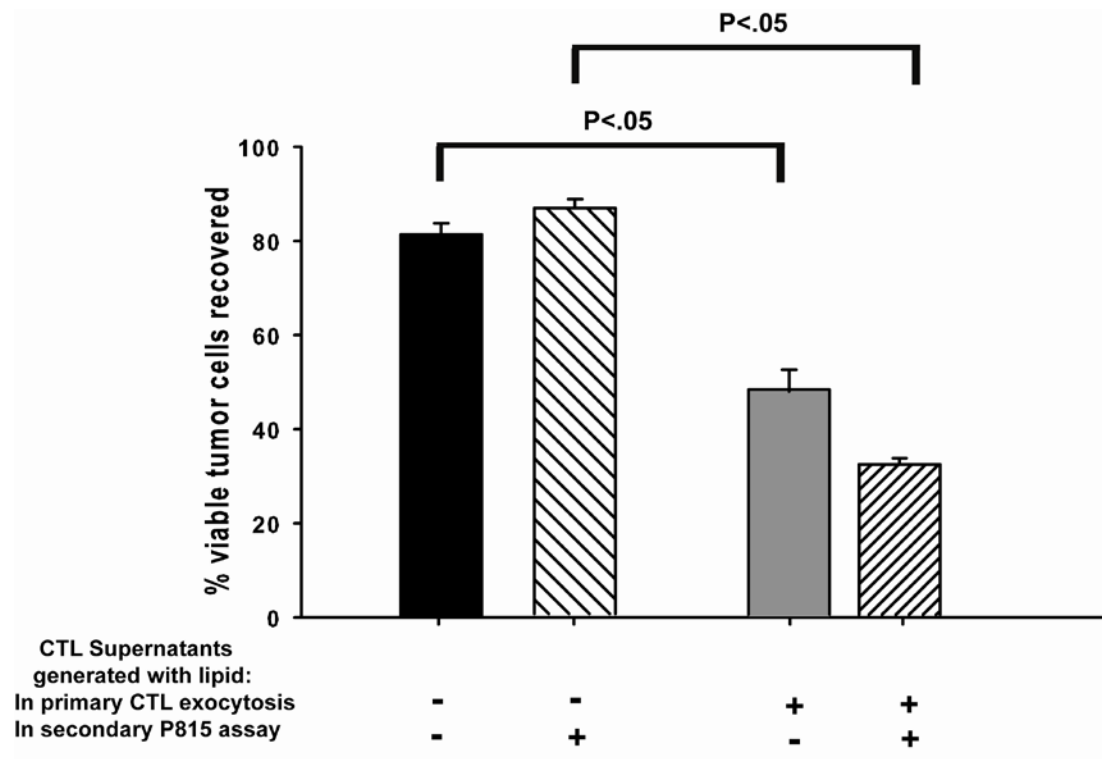


Figure 4.6: Lot 60506 IL-4 induced CTLs produced toxic products in the presence of triglyceride lipids. Lot 60506 IL-4 induced CTLs were stimulated to undergo exocytosis using anti-CD3 coated beads for 48 hours in the presence or absence of triglycerides. Following the initial 48 hour stimulated exocytosis, the cell free supernatant was collected and added to eGFP-P815 cells. The P815 cells were in media with or without fresh triglycerides in the hope of detecting released lipases. Cell free supernatants generated in the presence of triglycerides reduced the number of viable P815s compared to cell free supernatants produced without triglycerides during exocytosis.

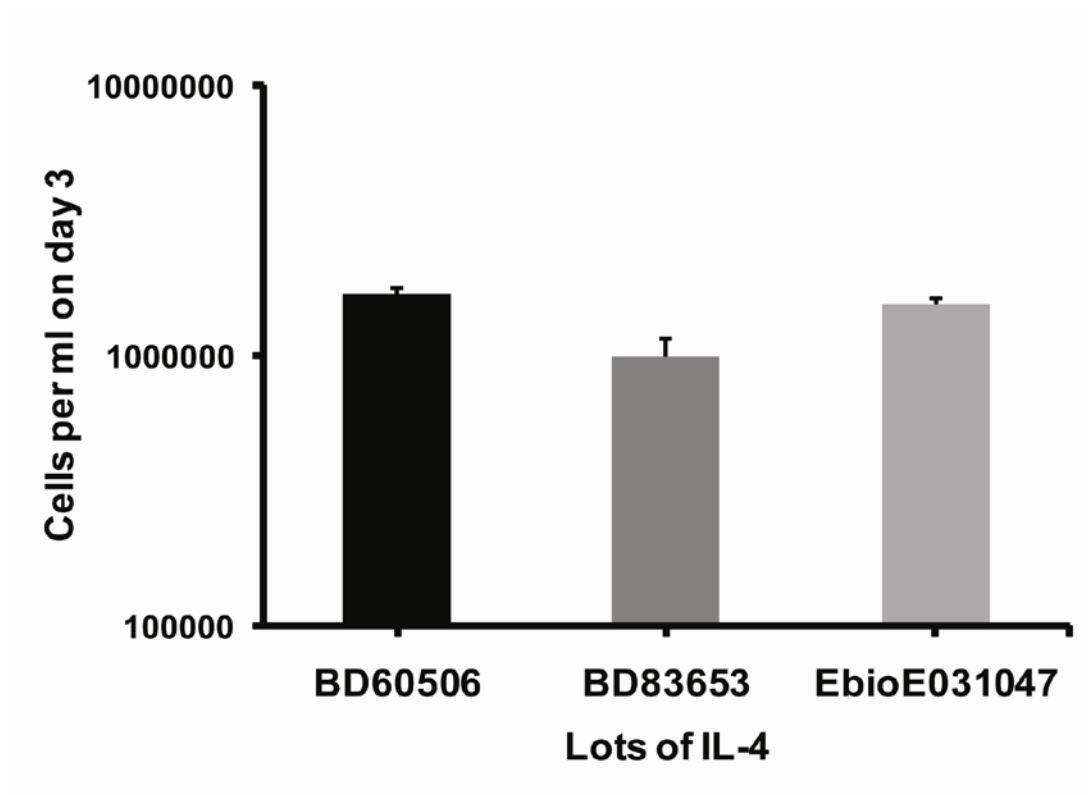


Figure 4.7: The growth of CTLs was similar among different lots of IL-4. Cells were cultured at 650,000 cells per ml with different lots of IL-4 at 500 units/ml. The viable cells recovered were counted by Trypan blue exclusion on day 3.

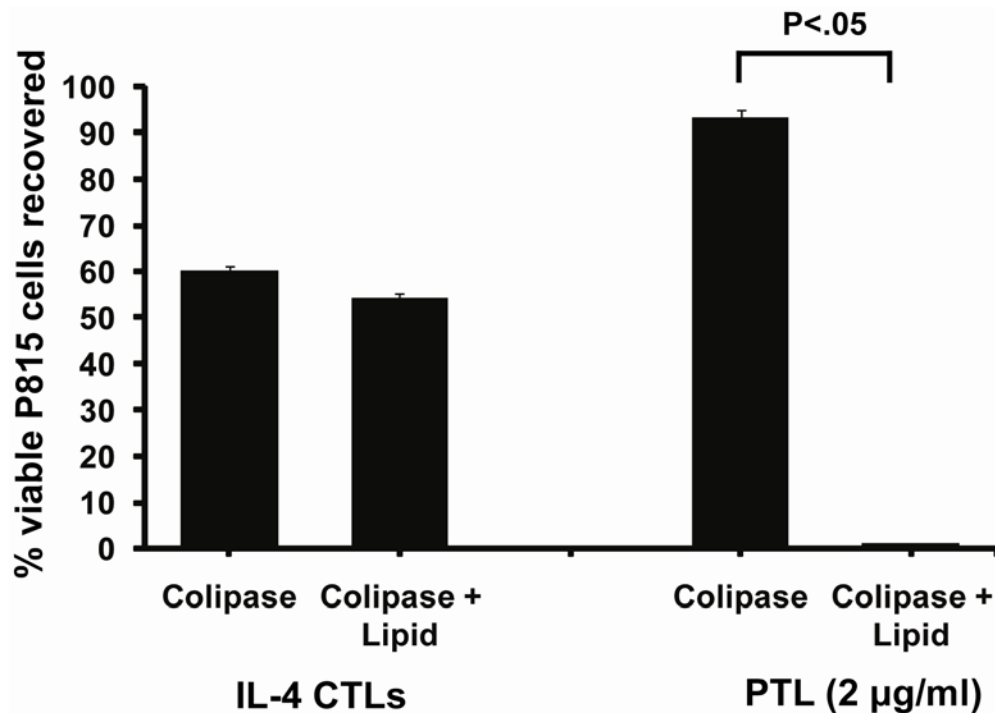


Figure 4.8: Purified colipase increased lipid-dependent cytotoxicity mediated by PTL but was without effect on the activity of IL-4-induced CTLs. The CTLs were induced for 5 days with lot E031047 IL-4 and expressed PLRP2 mRNA (not illustrated). CTLs or PTL were combined with or without colipase and tested in 48 hour tumor viability assays in the presence or absence of trilinolein. Tumor viability was unchanged by the addition of colipase to CTLs incubated in the presence of lipid when compared to assays lacking lipids. In assays containing PTL, tumor viability was significantly reduced with that addition of colipase to lipid containing assays when compared to assays where lipids were absent.

Chapter V
Perspectives and Conclusions

Perspectives and Conclusions

What purpose could a pancreatic lipase possibly serve in cytotoxic T lymphocytes (CTLs)? The known T cell lipase, PLRP2, was first identified as an IL-4 inducible T cell protein in the early 90s. As detailed within this dissertation, studies using PLRP2 knockout mice showed that PLRP2 deficient T cells have reduced lytic capacity when compared to WT T cells, suggesting that PLRP2 may function in T cell mediated killing [1]. Other studies have found that PLRP2 may display indirect toxicity toward cells through the generation of free fatty acids [2,3].

I have focused my research over the past 4 1/2 years on the characterization of PLRP2's expression and the function of PLRP2 and other lipases in T cell mediated killing. I have proven that PLRP2 is a product of T cells during IL-4 induction and that its expression is unaffected by the addition of type 1 cytokines. This result indicates that PLRP2s expression in T cells is independent of either type 1 or type 2 polarizations. CTLs deficient for PLRP2 display reduced cytotoxic activity towards tumor targets in short term assays, but had similar cytotoxic activity in longer killing assays. If so, what cytotoxic function could PLRP2 perform during the short term CTL mediated killing? My studies into T cell lipases discovered that during stimulated exocytosis active lipases are released, but PLRP2 appears to be a minor component of this released lipase activity. Studies into the released lipase activity revealed little contribution to the degradation of tumor cell membranes. Most of the degradation of tumor cell membranes is carried out by endogenous tumor lipases activated after death.

I attempted to identify how the loss of a minor lipase could still result in reduced cytotoxic activity of CTLs. Add back assays involving recombinant PLRP2 were

unsuccessful at improving the cytotoxic activity of PLRP2^{-/-} CTLs. This result suggests that the reduced cytotoxic function of PLRP2^{-/-} CTLs was a result of another deficiency rather than PLRP2s direct effect on tumor cells during CTL mediated killing. Phenotypic evaluation of both PLRP2^{-/-} and PLRP^{+/+} T cells found equal distribution of both CD4 and CD8 T cells as well as nearly identical surface phenotypes. In addition, expression of granzyme B and perforin was similar between IL-4 induced PLRP2^{-/-} and PLRP2^{+/+} CTLs. These results advocate for other explanations other than difference in cellular phenotype or in cytotoxic proteins. I currently question if the reduced cytotoxic activity observed in PLRP2^{-/-} CTL is the result of an immune developmental defect which occurred due to malnourishment during the juvenile, pre-pubescent stages of mouse development.

My final project has focused on the indirect cytotoxic potential associated with PLRP2 in the presence of triglycerides. Goat PLRP2 was discovered to have indirect cytotoxic function toward sperm when provided lipid sources from which PLRP2 released fatty acids to toxic levels [2,3]. I hypothesized that released lipase activity would have similar toxic effect on tumor cells. Using recombinant PLRP2, I found that PLRP2 could cause indirect cytotoxicity over 48 hours to the surrounding tumor cells when provided triglycerides. Similarly, 48 hour tumor survival assays were performed with IL-4 induced CTLs in the presence or absence of triglycerides. In all but one exceptional case, IL-4 induced CTLs displayed unchanged cytotoxic activity when triglycerides were present. One unique lot of IL-4 (BD lot 60506) was able to induced CTLs which exhibited a triglyceride-enhanced form of T cell mediated cytotoxicity. This increased cytotoxic activity was reproducible with this unique lot of IL-4, but all other

lots of IL-4 were found to be insufficient to generate the triglyceride-enhanced cytotoxicity. Our efforts to identify the component responsible the lipid enhanced cytotoxic activity have been unsuccessful to date.

From the research and results discussed in this dissertation, I now conclude that PLRP2 of IL-4 induced T cells does not perform a direct role in T cell mediated killing of tumor targets cells. Although PLRP2 deficient T cells display reduced cytotoxic activity compared to WT T cells it appears to be the result of a broader genetic effect involving genes near PLRP2 or due to a developmental defect of these mice. The manipulation of the mouse genome through the ablation of genes has historically caused many unexpected results. Several times the reported effects of a knockout models have turned out to be the result of how neighboring genes upstream or downstream of the ablated gene have been altered [4-6]. Also, developmental effects on the immune function can occur as a result of malnutrition at critical stages during development [7-10]. It is possible that in PLRP2 deficient mice, which are underweight due to the reduced ability to digest lipids for the first few weeks after birth, the stunted growth could impair T cell immune function during a critical time of T cell development.

My research into indirect toxic effects of lipase through the generation of lipid byproducts has resulted in an interesting conundrum. We have shown that rPLRP2, if provided a triglyceride, can mediate indirect toxicity to surrounding tumor cells. Studies into the function of PLRP2+ IL-4 induced T cells found that T cell mediated cytotoxicity was unaltered by the addition of triglycerides except in one fascinating case. Although my effort to reproduce the triglyceride-enhanced cytotoxicity exhibited by this unique case have been unsuccessful, I believe that this result hints at a unique cytotoxic

mechanism of T cells that has yet to be characterized and warrants further investigation.

As with any long term scientific endeavors, the work is never truly complete, but my research adds significantly to the ever growing body of knowledge surrounding T cell proteins and their function.

Chapter 5 Bibliography

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Appendix

Papers co-authored:

1. Berner, M.D., Sura, M.E., Alves, B.N. and Hunter, K.W., Jr. (2005) IFN-gamma primes macrophages for enhanced TNF-alpha expression in response to stimulatory and non-stimulatory amounts of microparticulate beta-glucan. *Immunol. Lett.*, 98, 115-122.
2. Tamang, D.L., Redelman, D., Alves, B.N., Vollger, L., Bethley, C. and Hudig, D. (2006) Induction of granzyme B and T cell cytotoxic capacity by IL-2 or IL-15 without antigens: Multiclonal responses that are extremely lytic if triggered and short-lived after cytokine withdrawal. *Cytokine.*, 36, 148-159.
3. Tamang, D.L., Alves, B.N., Elliott, V., Fraser, S.A., Redelman, D. and Hudig, D. (2008) Low dose IL-15 induces snap arming of CD44(low) T lymphocytes in the absence of antigen. *Cell Immunol.*, 251, 93-101.