Strategies for Exploiting the Immune System to Achieve Prevention and Improve

Therapy of Cancer

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Department of Microbiology and Immunology

University of Saskatchewan, Saskatoon Canada

By Duane H. Hamilton

Abstract

It has steadily become more recognized that even patients with progressively growing tumors are often mounting substantial immune responses against their tumor. The reasons why this immunity is unable to control the outgrowth of the tumor must be understood if we are to develop immunotherapeutic and preventative strategies against cancer. Experimental observations since the 1960s have suggested that cellular immunity generated against tumor antigens is protective, while some studies have led me to believe that humoral immunity may be associated with disease progression. This possibility has led me to test the hypothesis that the cause of immune failure is immune-deviation.

The experimental system I chose employs the P815 mastocytoma and L5178Y lymphoma tumors, both of which are of DBA origin. I have demonstrated that primary resistance to tumors correlates with Th1 responses, while primary progressive tumor growth is associated with a mixed Th1/Th2 immune response generated against tumor antigens. Such correlates were defined directly by assessing tumor-dependent cytokine secretion by T cells, and indirectly by assessing the relative abundance of tumor-specific IgG2a and IgG1 antibodies by western blot and enzyme-linked immunoassays. Moreover, I have demonstrated, utilizing these assays, that low doses of gamma irradiation, which have previously been shown to induce immune-mediated regression of established tumors, is associated with a phenotypic 'switch' in the anti-tumor immune response from a

mixed Th1/Th2 to a predominant Th1 response. The simplicity and reliability of using IgG isotypes to indirectly assess the Th1/Th2 nature of the anti-tumor immune response gives me hope that this work, in the long run, will result in a new way of guiding immunotherapy to effectively treat cancer.

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APC	antigen presenting cell
AMIDA	autoantibody-mediated identification of antigens
CPM	counts per minute
CT	cancer testis antigen
CTL	cytotoxic T cell
CT-X	cancer testis antigen encoded on the X chromosome
DTH	delayed type hypersensitivity
EIA	enzyme immunoassay
ELISPOT	enzyme-linked immunospot
HLA	human leukocyte antigen
IEL	intraepithelial lymphocytes
Ig	Immunoglobulin
IL	Interleukin
INFγ	interferon-gamma
iNKT	invariant natural killer T cell
KIR	Killing Inhibitory Receptor
mAb	monoclonal antibody
MACS	magnetic activated cell sorting
MAGE	melanoma associated gene expression

MHC	major histocompatibility complex
NCR	natural cytotoxicity receptor
NK	natural Killer cells
NKT	natural Killer T cell
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PBST	phosphate buffered saline supplemented with tween 20
RDA	representational difference analysis
SEREX	serological identification of antigens by recombinant expression cloning
TCR	T cell receptor
TNF	tumor necrosis factor

1 Introduction

Immunity to tumors has been studied for well over a century. Tumor immunology was born out of the inherent optimism following the successes scientists had in developing vaccines to prevent devastating infectious diseases such as small pox and poliomyelitis. Scientists such as Ehrlich thought that tumors, like infections, must occur at a fairly high frequency. He felt that the immune system must actively repress the growth of various neoplasias (Ehrlich 1909). More recently, Burnet and Thomas suggested that the immune system must possess an active mechanism to protect long-lived organisms from developing cancer (Burnet 1957; Thomas 1959). Using modern immunological techniques it has been clearly demonstrated that there are spontaneous anti-tumor immune responses being generated in both animal models of and in human cancers (Boon, Cerottini et al. 1994; Boon, Coulie et al. 2006).

1.1 Cellular Control of Neoplasia

Theories of immunosurveillance envisage that there are several layers of defense to control against the outgrowth of neoplastic cells. This defense consists of cellular and acellular components of the innate and adaptive immune systems, functioning in a concerted manner to prevent the development of clinical disease.

Failure of the innate and adaptive immune system could result in disease progression. This introduction will focus mainly on a review of: i) the cellular components of innate and adaptive anti-tumor immunity, ii) the characterization of tumor associated antigens and iii) suppressor/regulatory cells thought to inhibit the generation of effective anti-tumor immunity.

1.1.1 Innate Cellular Control of Neoplasia

1.1.1.1 Intraepithelial Lymphocytes

Intraepithelial lymphocytes (IEL) are defined as a unique group of lymphocytes that primarily survey the epithelial tissues for the presence of infection. In the mouse skin, the resident IEL are composed almost entirely of $\gamma\delta$ T cells while in the gut, the population of IEL consists of roughly 50% $\alpha\beta$ and 50% $\gamma\delta$ T cells (Asarnow, Goodman et al. 1989). Unlike classical $\alpha\beta$ T cells, $\gamma\delta$ T cells in the skin tend to have a more restricted TCR diversity (Shin, El-Diwany et al. 2005), while $\gamma\delta$ T cells in the gut often have a much more diverse TCR repertoire (Cron, Gajewski et al. 1989). $\gamma\delta$ T cells are capable of recognizing unprocessed antigens in an MHC-unrestricted manner (Chien, Jores et al. 1996), and some believe they primarily recognize endogenous proteins expressed on the surface of cells after infection or malignant transformation (Havran, Chien et al. 1991). Upon recognizing a neoplastic cell, γ/δ T cells have the capacity to induce strong cytotoxic effector activities using either death receptor/death ligand or cytolytic

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granule pathways, each capable of inducing apoptotic killing of transformed cells, and to produce cytokines such as tumor necrosis factor-α (TNFα), and interferon-γ (INFγ) to promote inflammation (Hayday 2000; Girardi, Oppenheim et al. 2001). Since the skin contains cells with a great potential to undergo transformation that may lead to malignant growth, these γ/δ T cells residing in the epithelia may play a very important role in both identifying, and initiating the killing of transformed cells.

1.1.1.2 Natural Killer Cells

Natural Killer (NK) cells are a unique population that was first identified for their ability to kill target cells against which they had not previously been primed. In 1986, Kärre reported that NK cells could effectively lyse a tumor cell that did not express MHC class I on its surface (RMA-S), but was unable to lyse the same tumor line if it expressed class I MHC on its surface (RMA) (Cerwenka, Baron et al. 2001). This experimental observation led to the theory that NK cells survey the body for cells that no longer present class I restricted antigens to the immune system, a surveillance that can lead to killing of the recognized cell. This idea became dogma until it was discovered that NK cells are able to reject class I bearing target cells provided that the target cells bore ligands recognized by activating receptors of the NK cell (Lanier, Corliss et al. 1997; Cerwenka, Baron et al. 2001; Diefenbach, Jensen et al. 2001). It has now been shown that NK cells express both inhibitory and activating receptors which coordinately regulate their activity.

NK cells have a large number of receptors, whose engagement alone, or in combination, account for their cytotoxicity. The natural cytotoxicity receptor (NCR) family is composed of three receptors, NKp30, NKp44 and NKp46 (Pessino, Sivori et al. 1998; Vitale, Bottino et al. 1998; Pende, Parolini et al. 1999), and their engagement accounts for an important part of NK cell natural cytotoxicity against multiple types of cancer. In addition to the NCR family of receptors, there are the Killing Inhibitory Receptors (KIRs), such as NKG2A, which forms a heterodimers with CD94. This heterodimers bind to non-classical MHC class I molecules called HLA-E (in humans) and Qa1 (in mice) and act to inhibit NK cytotoxicity (Diefenbach, Jamieson et al. 2000). Unlike traditional KIRs, NKG2D acts as an activating receptor, that when triggered induces the killing of the target cell. NKG2D forms a homodimeric receptor and binds one of several cell surface molecules that are only distantly related to MHC class I molecules (Raulet 2003). NKG2D receptor is expressed on the surface of almost all NK cells. Mouse CD8+ T cells gain expression of NKG2D after activation, while human CD8+ T cells constitutively express it on their surface. Roughly 25% of γ/δ T cells in the spleen, and 100% of γ/δ T cells in the mouse epidermis express the NKG2D receptor, and activated macrophages lose expression of the NKG2D receptor (Raulet 2003).

In normal adult cells, these NKG2D ligands are absent, or present at very low concentrations, and are thought to be upregulated on the surface of 'stressed' cells, effectively alerting the innate immune system to cellular stresses including

transformation events (Nomura, Zou et al. 1996; Girardi, Oppenheim et al. 2001). Various NKG2D ligands have been detected on the surface of numerous murine and human tumor isolates (Groh, Rhinehart et al. 1999). Murine tumor lines that have been engineered to express high amounts of NKG2D ligands on their surface have failed to form a tumor *in-vivo* (Cerwenka, Baron et al. 2001; Diefenbach, Jensen et al. 2001). These studies, showing that ectopic high level expression of various NKG2D ligands results in rejection of primary tumors, contrasts with the observation that most human, and mouse tumor cell lines derived from progressively growing tumors, express these ligands on their surface. Why are NK and γδTCR+ T cells unable to control the outgrowth of these transformed cells? It is possible that these ligands play no role in the *in vivo* rejection of endogenous tumors. It is also possible that these tumor cell lines/isolates may have been selected for variants that do not express the ligands at sufficient levels to result in their killing. Alternatively, endogenously growing tumors may directly suppress NKG2D-mediated killing (Groh, Wu et al. 2002).

1.1.1.3 Natural Killer T cells

Natural Killer T (NKT) cells were first identified as a unique subset of T cells sharing qualities commonly associated with traditional NK cells. NKT cells express the NK1.1 (CD161c) marker, usually attributed to NK cells, however NKT cells also express a highly conserved α/β T cell receptor (Makino, Kanno et al. 1995). NKT cells can recognize lipid antigens in the context of the CD1d molecule (Kawano, Cui

et al. 1997). Until recently, the CD1d-restricted ligand to which NKT cells were capable of responding was unknown. Most early studies utilized a pharmacological ligand capable of polyconally activating of NKT cells. Alpha-galactosyl ceramide (α-GalCer) is a lipid from a marine sponge that was first isolated based upon its ability to prevent tumor metastasis of the B16 tumor to the liver of mice (Kobayashi, Motoki et al. 1995). It was later demonstrated that this activity was due to CD1d-mediated presentation of this lipid antigen to NKT cells expressing the invariant Vα14 T cell receptor which have become known as invariant natural killer T cells (iNKT) (Kawano, Cui et al. 1997). The natural ligands recognized by the iNKT cells remained elusive until very recently. Mycobacterial phosphatidylinositol mannoside (Fischer, Scotet et al. 2004), and mycobacterial phosphoethanolamide (Rauch, Gumperz et al. 2003) have been identified as exogenous antigens recognized by iNKT cells. The endogenous lipid isoglobotrihexosylceramide (iGb3) is recognized by both human and mouse iNKT cells. Not only are iNKT cells able to respond to iGb3 in the context of the CD1d molecule, but gene knockout mice that do not produce iGb3 also fail to generate iNKT cells. This strongly suggests that iGb3 is required for iNKT cell selection and/or maintenance in the periphery of mice (Laduron, Deplus et al. 2004)

1.1.1.4 NKT cells and tumor immunity

The *in-vivo* function of NKT cells is not well understood, and there is some debate as to whether they act simply as immunomodulatory cells or if they have any role in host-defense against tumors and microbial infections. The idea that they act against tumors and microbial infections is based upon the observation that one can induce iNKT cells to secrete INFγ in a CD1d-dependent manner by culturing iNKT cells either with dendritic cells (DC) pulsed with *Salmonella typimurium* extract or with DCs and high levels of IL-12. These observations suggest DCs can present both exogenous and endogenously derived ligands recognizable by iNKT cells (Mattner, Debord et al. 2005). At present, it is hypothesized that NKT cells may have no direct effector function with regards to tumor immunity, but rather may have a regulatory function by secreting INFγ or IL-4 and IL-13 in response to tumor antigens presented in the context of the CD1d molecule (Moodycliffe, Nghiem et al. 2000; Terabe, Matsui et al. 2000).

1.2 Adaptive immune responses

1.2.1 Generating Protective Anti-Tumor Immune Responses

The ability to exploit the immune system to treat cancer has been a tantalizing, yet unrealized, goal of immunologists for well over a century. This apparent failure is despite strong evidence, in animal models, of the immune system's ability to mount an effective anti-tumor immune response.

In the 1950s, with the availability of truely syngeneic strains of mice, contemporary immunologists began their study of the generation of anti-tumor immunity. The relative ease by which one could render mice resistant to a normally lethal dose of a syngeneic tumor led to great optimism that one could exploit the immune system to treat human cancers. Vaccination strategies such as excision priming (Foley 1953; Klein 1959), the administration of sub-tumorogenic doses of replicating tumor cells (Klein, Sjogren et al. 1960), or repeated immunization with heavily irradiated tumor cells (Revesz 1960) rendered mice resistant to a tumor challenge known to be lethal in naïve animals. The protection generated by these various treatments was stable, and specific for the selecting tumor (Klein, Sjogren et al. 1960), and could be transferred to a naïve mouse by transferring T cells (Mikulska, Smith et al. 1966). A determination of the mechanisms by which T cells are capable of controlling the outgrowth of these neoplastic cells have been the basis of many experimental studies.

1.2.2 CD4 and CD8 T cell responses against cancer

Today, we recognize that CD4+ and CD8+ T cells are indispensable components of the adaptive immune system. T cells expressing the CD4+ marker recognize antigen presented by MHC class II molecules expressed by professional antigen presenting cells (APC) such as dendritic cells, macrophages and B cells. In contrast, CD8+ T cells recognize antigen presented by MHC class I molecules. Unlike class II, class I MHC is expressed by the vast majority of nucleated cells, allowing CD8+ T

cells to survey the body for virally infected or transformed cells (Abbas and Lichtman 2000). In addition to recognizing antigen in the context of different MHC molecules, CD4+ and CD8+ T cells have different biological functions.

For the most part, CD4+ T cells act as helper cells, aiding in the activation, and promotion, of either humoral or cell mediated immunity. There are different theories which attempt to define the mechanisms by which naïve CD4+ T cells are activated in response to antigen (Janeway 1989; Matzinger 1994; Bretscher 1999). A review of these various theories of the activation requirements of naïve CD4+ T cells is beyond the scope of this introduction. Once activated, these effector CD4+ T cells can be divided into at least two populations, based upon their cytokine profiles (Mosmann and Coffman 1989). Cells of Th1 type secrete proinflammatory cytokines such as INFγ (Mosmann and Coffman 1989), and promote the activation of cell mediated immunity which includes cytotoxic T cells (Janssen, Lemmens et al. 2003), macrophages and NK cells (Hoebe, Janssen et al. 2004). These cell types are thought to be more effective at containing the outgrowth of intracellular organisms and tumors (Bretscher, Hamilton et al. 2002). Cells of Th2 type secrete cytokines such as IL-4 and IL-5 in response to antigen (Mosmann and Coffman 1989; Swain, Weinberg et al. 1990), and promote humoral responses. Such Th2 responses are effective at containing extracellular bacteria and parasites (Abbas and Lichtman 2000). Although these two CD4+ T cell subsets are most often distinguished based upon the cytokines they produce, it has been shown in a

number of experimental systems that one can infer the Th1/Th2 nature of an ongoing immune response based upon the relative predominance of antigen-specific Ig isotypes. In mice, it has been shown that exclusive Th1 responses correlate with no antibody production. Predominant Th1 responses correlate with the production of IgG2a antibody, Th1/Th2 mixed responses correlate with production of both IgG2a and IgG1 antibodies while a polarized Th2 response is associated with a predominance of IgG1 and IgE antibodies produced in response to antigen (Pond, Wassom et al. 1989; Menon and Bretscher 1996; Power, Wei et al. 1998). As the Th1 and Th2 nature of the immune response can have profound impacts on the outcome of various microbial infections, it is very important to understand the nature of the decision criterion controlling the Th1/Th2 phenotype of the response.

Several theories attempt to explain what determines the Th1/Th2 nature of an immune response (Constant and Bottomly 1997; Kalinski, Hilkens et al. 1999; Rissoan, Soumelis et al. 1999; Mazzoni and Segal 2004). Many studies in Peter Bretscher's laboratory assess a possible role for T cell cooperation in determining the phenotype of the CD4+ T cell response. This long standing theory proposes that the antigen-mediated cooperation of antigen-specific CD4+ T cells is needed to generate immune responses. Known as the threshold hypothesis, it suggests that low levels of CD4+ T cell cooperation preferentially lead to Th1, while high levels of cooperation lead to Th2 immune responses (Bretscher 1979; Tucker and Bretscher

1982; Bretscher 1983; Bretscher 1986; Ismail and Bretscher 1999; Ismail and Bretscher 2001; Ismail, Basten et al. 2005). Recently, experimental evidence has provided very direct support for this long standing proposition. K.K. McKinstry, a former graduate student in Peter Bretscher's laboratory provided strong evidence that CD4+ T cells can cooperate in the generation of immune responses to linked antigens, and the degree to which the CD4+ T cells are capable of cooperating dictates the Th1/Th2 nature of the immune response (Mckinstry 2005).

In this study, McKinstry transferred a standard number of normal spleen cells into lethally irradiated recipients, and gave an amount of OVA-conjugated xenogeneic red blood cells (XRBC) as antigen ten-fold lower than required to generate a specific immune response. By supplementing this constant number of normal spleen cells with unprimed transgenic CD4+ T cells specific for OVA, he was able to demonstrate that these transgenic cells were able to 'help' in the generation of immune responses against the XRBC to which OVA had been chemically coupled. Furthermore, McKinstry was able to modulate the Th1/Th2 nature of the anti-XRBC response by transferring different numbers of OVA-specific CD4+ T cells. Low numbers of transgenic T cells promoted a Th1 response, while higher numbers promoted a mixed Th1/Th2 immune response against the XRBC. Such modulation was only observed when the two antigens were physically linked (Mckinstry 2005). Such observations critically test the idea that the level of CD4+ T

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cell cooperation influences the Th1/Th2 nature of an antigen-specific immune response.

1.2.2.1 Activation of tumor-reactive CD8+ cytotoxic T cells

It is readily accepted, in most instances, that CD8+ cytotoxic T cells (CTLs) are the major cell type involved in the direct killing of tumor cells; however, understanding the mechanism of how tumor-specific CTLs are activated can be conceptually challenging as many tumors do not express the costimulatory molecules required to fully activate naïve CTLs. Furthermore, as discussed above, many CTL responses require CD4+ T cell help (Mitchison 1983; Guerder and Matzinger 1992; Kirberg, Bruno et al. 1993; Janssen, Lemmens et al. 2003; Shedlock and Shen 2003; Sun and Bevan 2003). As most murine tumor cells do not express MHC class II molecules on their surface, the manner by which this help is delivered has been extensively studied. Professional antigen presenting cells (APCs) have been known for their ability to take up, digest, and present exogenously proteins and present them to the immune system upon MHC class II molecules. This understanding had become dogma until a series of observations led Bevan to propose that professional APCs were capable of presenting exogenous proteins not only on MHC class II, but also on MHC class I molecules. This process has been called cross presentation (Huang, Golumbek et al. 1994; Bennett, Carbone et al. 1997; Albert, Sauter et al. 1998; Heath, Kurts et al. 1998), and has become key in our understanding of how precursor CTLs can become activated in response to neoplastic growth. Presently it

is believed that professional APCs will phagocytose, and present tumor-derived antigens on both MHC class I and II molecules. Effector CD4+ T helper cells, recognizing MHC class II restricted tumor antigens, are thought to then 'license' the APC by inducing the upregulation of costimulatory molecules required to fully activate naïve tumor-specific CD8+ T cells recognizing MHC class I restricted tumor antigens (Bennett, Carbone et al. 1998; Ridge, Di Rosa et al. 1998; Schoenberger, Toes et al. 1998). Once activated, tumor-specific CTLs can directly recognize, and kill transformed cells.

1.2.2.2 Immune-mediated killing of tumor cells

Cytotoxic T cells are the cells whose primary job is to identify, and specifically kill, virally infected or transformed cells (Harty, Tvinnereim et al. 2000). Upon a TCR-mediated specific interaction with a target cell expressing a MHC class I restricted antigen, an activated CTL can utilize one of two distinct pathways to kill the target cell. The first method involves the directed release of granules towards the target cell. These granules contain various proteases along with perforin, which as the name suggests, acts similarly to the complement induced membrane attack complex, and forms pores in the membrane of the target cell (Young, Cohn et al. 1986). The proteases released enter the target cell via the pores created by perforin, leading to a cascade of reactions resulting in the apoptotic death of the target cell (Smyth, O'Connor et al. 1996). The second method of CTL mediated killing involves an interaction between the Fas receptor and the Fas ligand. This

ligand on the CTL, upon interaction with the Fas receptor on the target cell, activates the caspase cascade in the target cell resulting in apoptosis (Nagata 1996).

In addition to the direct killing of tumor cells, CD8+ T cells also release cytokines such as interferon gamma (INFγ), which not only acts as an anti-proliferative agent, but also to increase class I MHC (Stark, Kerr et al. 1998) and TNF receptor expression on tumor cells (Aggarwal, Eessalu et al. 1985; Tsujimoto, Yip et al. 1986), making tumor cells more susceptible to the action of tumor necrosis factor alpha (TNFα) and beta (TNFβ), which can trigger apoptosis of tumor cells via signaling through the TNF receptor (Szatmary 1999).

Other than helping to activate tumor-specific CD8+ T cells, the direct role of CD4+ T cells in anti-tumor immune responses are often underestimated. CD4+ T cells specific for tumor antigens are unable to directly recognize transformed cells because many tumors do not express MHC class II molecules. Instead, CD4+ T cells recognize, and respond to tumor antigens as presented by professional APCs. Despite this limitation, CD4+ T cells are very important components of the anti-tumor immune response. Tumor-specific effector CD4+ T cells are often required for the activation of tumor specific CD8+ cytotoxic T lymphocytes (CTLs) (Janssen, Lemmens et al. 2003), but also promote inflammation, which recruits, and promotes both innate and adaptive effector cells to exhibit their anti-tumor effector functions (Mumberg, Monach et al. 1999).

The demonstrated specificity by which CD4+ and CD8+ T cells can be induced in response to tumors allows one to infer that each tumor expresses unique antigens that can be utilized by the immune system to induce clearance of the tumor (Boyse, Old et al. 1962). It was not until the early 1990s that scientists began to uncover the antigens expressed by tumors that allow the immune system to recognize, and distinguish between normal and neoplastic tissues (Chen, Gure et al. 1998; Chomez, De Backer et al. 2001).

1.2.2.3 Coherence of the humoral and cellular immune responses to linked antigens

It has been appreciated since the early 1970s that the activation of anti-hapten B cells, upon interaction with a hapten-carrier conjugate, is aided by the presence of carrier-specific T cells (Mitchison 1971). Utilizing these systems it was observed that the subclass of antibody generated to the hapten is the same as those generated in response to the carrier to which it is linked (Mckinstry, Ismail et al. 2005). This observed coherence in the isotypes of antibodies generated in response to linked antigens is explainable because the cytokines produced by antigen-specific T cells greatly influence the decision of a B cell to switch to a particular subclass of antibody. It has been demonstrated that CD4+ T helper cells of the Th1, Th1/Th2 mixed or Th2 phenotype promote specific B cells to switch to IgG2a, IgG1 or IgE antibody isotypes respectively (Coffman and Carty 1986; Bond, Shrader et al. 1987; Coffman, Shrader et al. 1987; Murray, McKenzie et al. 1987;

Snapper and Paul 1987; Lebman and Coffman 1988; Snapper, Finkelman et al. 1988). These observations explain the coherence between the phenotypes of the B and T cell responses, and suggest that one may be able to indirectly infer the Th1/Th2 nature of a immune response by analyzing the relative abundance of antigen-specific antibody isotypes (Mckinstry, Ismail et al. 2005). The importance and utility of such inferences will become apparent in subsequent sections.

1.3   The search for tumor antigens

1.3.1   Tumor antigens recognized by cytotoxic T cells

It has been appreciated for quite some time that protective immunity to the vast majority of syngeneic tumors is mediated by T cells. However, the identity of the antigens against which this immunity is generated remained elusive until the early 1990s. Theirry Boon pioneered the technique of cloning cytotoxic T cells (CTLs) from mice/patients suffering from cancer, and determining the antigen specificity of these tumor-specific CTLs. This technique involved isolating and cloning CD8+ T cells from animals capable of lysing the tumor cell in-vitro. As individual CTL cell clones are cultured with the tumor, it places a strong selective pressure upon the tumor, and ultimately results in the selection of a set of tumor variants against which the selecting CTL cell line is no longer effective. The resulting tumor variants selected to be resistant against different CTL clones could be arranged into different groups of variants. Members of the same group showed a similar

susceptibility or resistance to all the CTL clones, which were also grouped according to their ability to lyse each of the respective tumor variants. (Van den Eynde, Lethe et al. 1991).

A cDNA library of the original tumor cell line was generated, and transfected into a cell line expressing syngeneic MHC restriction elements, but against which none of the established CTL cell lines show endogenous cytotoxicity. The resulting transfectants were screened based upon the ability of each of the CTL cell lines to lyse these targets. One can thus isolate the gene encoding the neoantigen being expressed by the transfectants and which rendered them sensitive to particular CTL clone, and identify the proteins to which the immune system is mounting a cytotoxic immune response. Using similar techniques, Theirry Boon's group has identified a large number of tumor antigens in both animal and human cancers against which a CTL response is generated (Boon, Coulie et al. 2006).

1.3.2 The search for tumor antigens recognized by antibodies

In the 1960s, using techniques that had proven useful in identifying bacterial antigens, researchers began immunizing rabbits and other animals with human tumors. Investigators attempted to use this hetero-immune serum to identify antigens uniquely presented on the surface of tumor cells against which immunity is generated (Day 1965). At the time, it was felt the primary insurmountable challenge with these protocols was in the difficulty of removing antibodies within

the hetero-immune sera reactive with normal cellular proteins present in the immunizing tumor. These studies proved to offer very little to the understanding of cancer immunity; however, such studies allowed for the identification of many cell surface differentiation antigens (Old and Stockert 1977) which have proven indispensable to modern immunology. In the 1980s, many researchers attempted to overcome the difficulties associated with hetero-immune typing by using a patient's own sera to define antigens presented on the surface of tumor cells against which they had developed antibodies (Ueda, Shiku et al. 1979). Like hetero-immune typing, these attempts at autologous typing of tumor antigens were also plagued with many technical problems and were, for the most part, unsuccessful. It was not until the discovery and true appreciation of MHC restriction (Zinkernagel and Doherty 1974), that it became apparent that the majority of tumor antigens, against which immunity is generated, are intracellular proteins not expressed on the surface of tumor cells. The technique of Serological Identification of Antigens by Recombinant Expression cloning (SEREX) allowed researchers to identify, and appreciate the intracellular nature, of the proteins recognized by antibodies in autologous sera (Sahin, Tureci et al. 1995).

The SEREX protocol involves collecting fresh tumor samples from patients, and generating a cDNA library from their mRNA. This library is cloned into a λ bacteriophage expression vector, and plated onto a lawn of competent *E. coli* cells. The resulting plaques containing the recombinant proteins are blotted onto

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nitrocellulose. A patient's serum is pre-adsorbed with *E. coli* and λ bacteriophage antigens in an attempt to remove any naturally existing bacterial/viral reactive antibodies in the patient's serum, and the membranes are incubated with this pre-adsorbed patient's serum. Positive clones were identified using an alkaline phosphatase-conjugated goat anti-human IgG secondary antibody, and were visualized using an appropriate detection substrate. Positive clones are isolated by exhaustive rounds of immuno-screening. The cDNAs from positive clones were sequenced and their identity determined (Sahin, Tureci et al. 1995).

Although the SEREX protocol is a modern technique, it still poses a lot of potential problems. The SEREX technique, as with all techniques using cDNA libraries, leads to an over representation of tumor antigens that are expressed at high levels. Although increased expression may be important, they may not be related to either tumorogenesis or tumor immunogenicity. The SEREX technique may underestimate the number of tumor antigens being identified as being recognized by the humoral immune system. This likelihood is based in part on the use of a prokaryotic expression system. It is very possible that many tumor antigens to which the patient has mounted an antibody response are not readily produced by transfected prokaryotic cells, as tumor antigens may be normal antigens modified and made antigenic by secondary processing events, such as glycosylation (Skipper, Hendrickson et al. 1996). It is therefore likely, that the protocol does not support the production of all antigenically relevant proteins. In

an attempt to at least partially overcome these problems, SEREX protocols should be developed using mammalian cell expression systems. Another problem that consistently plagues the SEREX protocol is the large number of false positives. A source of many of the false positives is the presence of B cells within the tumor sample. The cDNA libraries generated from tumor samples that are contaminated with B cells will contain many sequences encoding IgG proteins. These contaminating immunoglobulin proteins will be detected by the secondary antibodies, and result in many false positives (often greater than 90%) (Tureci, Sahin et al. 1996). The SEREX protocol has nevertheless proven very effective at identifying tumor-associated antigens and, despite being over a decade old, most current alternate protocols are simply minor modifications of the original SEREX protocol.

1.3.3 The cancer Immunome

Scientists, utilizing the above mentioned techniques, have catalogued tumor antigens of human cancers that are recognized by the immune system. The resulting database, known as the cancer immunome, has allowed scientists to compare the relative abundance of different tumor associated antigens among a large population of cancer patients. Currently, over 2690 SEREX defined antigens have been put into the cancer immunome database (http://www2.licr.org/CancerImmunomeDB). There are many potential benefits that this information may provide. Firstly, it is very possible that by combining

SEREX-defined antigens common to a specific type of cancer, or to cancer cells in general, one could effectively generate a protocol by which you could potentially diagnose cancer in patients using a simple blood test that assays for the presence of a particular type or various combinations of tumor-specific antibodies. These SEREX antigens could provide a panel of tumor associated antigens that could be used in vaccination trials that attempt to prevent tumors from arising or in trials aimed at the treatment of established tumors (Jaffee and Pardoll 1996).

There have been some comparisons in the literature of the tumor antigens recognized by antibodies, CD4+ and/or CD8+ T cells. Results from these studies have shown a fairly good correlation between SEREX defined, and antigens recognized by CD4 and CD8 T cells (Jager, Jager et al. 2000; Ayyoub, Stevanovic et al. 2002). It is not all that surprising that the immune system is mounting a very concerted anti-tumor immune response, considering that tumor-specific CD4+ T cells are required to activate both tumor-specific B and CD8+ T cells. In this way, it may be possible to infer which antigens T cells are responding to by identifying SEREX-defined antigens.

1.3.4 Common tumor antigens

The realization that some of the tumor antigens against which patients respond are common among a wide variety of unrelated cancers was an unexpected, but a welcome, observation leading to the suggestion that a vaccine capable of protecting people from a wide variety of cancers may be realizable. The question

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that arises is how can a wide array of neoplasms, arising in different tissues, as a result of different genetic insults, share a common antigenic profile? The answer may lie in the rather generic nature of the oncogenic process. Transformed cells carry a number of genetic and epigenetic changes. Not all such alterations are required to generate, but are merely associated with, a transformed phenotype.

1.4 De-novo origins of many tumor antigens

The genetic instability associated with tumor cells results in many genetic and epigenetic changes which can effectively alter the panel of proteins produced by transformed cells. This altered antigenic profile can be identified and exploited by the immune system to enable a discriminatory attack on the neoplasm. In a relatively broad stroke, one can effectively define a tumor antigen as any difference between tumor and normal cells which allows the immune system to distinguish them.

In most cases, the neoplastic process results from a multi-step process (Fearon and Vogelstein 1990), by which a normal cell or group of cells gain a cancerous phenotype. With the exception of mutated self- (Disis and Cheever 1996), or viral-oncogenes (Plata, Langlade-Demoyen et al. 1987), the vast majority of the proteins expressed by these altered cells are not unique to the tumor. Instead the immune system identifies and kills tumor cells based on their expression of "tumor-associated antigens".

Many tumor associated antigens are normal cellular proteins having an altered pattern of expression in tumor cells as compared to normal cells (Van den Eynde, Lethe et al. 1991). It is a common trait for cancer cells to have aberrant expression of genes and thus allow immune recognition of transformed cells. It has become increasingly clear that the generation of such neo-antigens does not often require any mutation in the genetic information encoded within the primary DNA sequence. Transcription from the incorrect DNA strand to produce an anti-sense protein or translation 'out of frame' (Van Den Eynde, Gaugler et al. 1999), or even utilization of cryptic promoters (Guilloux, Lucas et al. 1996) may result in an immunogenic variant of a gene from a wild-type DNA sequence. Deficiencies with regards to post-transcriptional processing and modifications can also effectively introduce *de-novo* epitopes into a 'normal' protein (Skipper, Hendrickson et al. 1996). Hyperactivation or altered patterns of expression of normal cellular proteins has proven itself to be responsible for the generation of many antigenic targets against which an anti-tumor immune response is generated.

Hypomethylation of DNA is a very common occurrence in cancers, and is thought to be responsible for many epigenetic changes often seen in transformed cells (De Smet, De Backer et al. 1996; Nguyen, Weisenberger et al. 2002). The CpG islands are regions in the genome greater than 500 bp in size, having a GC content greater than 55%. Quite often the C^5 position of the cytosine residues in these CpG islands are methylated (Holliday and Pugh 1975; Riggs 1975; Bird 1986). This

methylation affects the chromatin structure, and when it is present in the promoter region of any particular gene prevents the recruitment of transactivating elements. Aberrant hyper- or hypo-methylation of these CpG islands are often found in human cancers (Feinberg and Vogelstein 1983; Gama-Sosa, Slagel et al. 1983; Bedford and van Helden 1987; Baylin, Belinsky et al. 2000; Baylin and Herman 2000; Jones 2002; Florl, Steinhoff et al. 2004). Hypermethylation of CpG islands is associated with aberrant gene silencing, while hypomethlyation of promoter sequences is associated with aberrant gene activation. This hypomethlyation of CpG correlates with the *de-novo* expression of normally silent genes in transformed cells (De Smet, De Backer et al. 1996), (Sigalotti, Fratta et al. 2004), and is thought to be responsible for the expression of a unique panel of tumor-associated antigens shared among a wide variety of tumors, the most common of which have been termed the cancer testis (CT) antigen.

The CT antigens represent a class of tumor associated proteins whose expression is normally restricted to the testis. All CT genes identified to date have associated methylated CpG islands in normal somatic tissues, while in cells undergoing spermatogenesis, or in neoplastic tissues, these CpG islands are unmethylated (Kimmins and Sassone-Corsi 2005). Initially, CT antigens were identified based upon detectable immunity against these antigens; however, the term CT antigen no longer refers only to gene products to which immune responses are generated, but has come to include any gene whose expression

profile is normally restricted to the testes and cancer cells. In this light, there are 44 CT antigen families (Scanlan, Simpson et al. 2004) CT antigens are further categorized as those encoded on the X-chromosome (CT-X antigens), and those that are not (CT-non-X antigens). It has been estimated that roughly 10% of the genes on the X chromosome belong to the CT-X family (Ross, Grafham et al. 2005; Simpson, Caballero et al. 2005). Genes belonging to the CT-non-X family are distributed throughout the genome, and do not generally form gene families (Grizzi, Chiriva-Internati et al. 2003; Tapparel, Reymond et al. 2003; Xu, Yuan et al. 2004). These CT antigens are of particular interest to researchers as it is thought they constitute promising targets against which vaccines might be developed.

The question of why many patients uniquely generate immune responses to these CT, but not to other tumor associated antigens, is not yet fully appreciated. The process of generating immune responses is a function of both the availability of the antigen and the prevalence of precursor T cells specific for each particular antigen. More than 40% of mammalian genes are potentially regulated by methylation of the cis acting CpG promoter elements, yet the upregulation of these genes does not induce detectable immune responses. The uniquely immunogenic characteristic of CT antigens is most likely due to their normal expression profile. The vast majority of CT antigens are expressed only in the testis which is traditionally characterized as an immunoprivileged site (Barker and Billingham 1977; Streilein 1995). Normally, cells of the immune system do not

survey the testes. Moreover, many nucleated cells in the testes express very little, if any, class I MHC molecules on their surface (Hotta, Nagata et al. 2000). In this light, it is likely the immune system is unable to maintain effective tolerance to these CT proteins, and precursor T cells specific to these proteins are able to accumulate in the periphery of normal animals (Valmori, Souleimanian et al. 2005). When tumor cells develop, CT proteins become expressed outside traditional, immunoprivileged sites, and the cells of the immune system in the periphery are capable of both recognizing, and mounting an immune response to these aberrantly expressed proteins.

Although the expression of tumor-associated antigens may vary considerably between different cancer patients, in the same type of cancer, and even among the cellular population of a single tumor, Thierry Boon and others have shown the presence of tumor antigens that are shared by a number of different tumors and patients (Boon, Cerottini et al. 1994; Van den Eynde and van der Bruggen 1997). These observations lead to the suggestion that most tumor-associated antigens result from generic changes that are common among different cancers. These observations support the hope that a universal anti-cancer vaccine can be developed.

1.5 Potential functions of tumor-associated antigens

Expression of tumor associated antigens may merely be associated with transformed cells, and simply act as a target for immune attack, or be required for the oncogenic state. If one proposes to use particular CT antigens as potential targets for vaccine development, it would be advantageous if the proposed targets were required to maintain the neoplastic phenotype. This may decrease the plasticity of their expression profile in the tumor, and increase the effectiveness of the vaccine.

Recent data suggest that CT antigens, such as melanoma associated gene expression (MAGE) proteins, may play an active role in tumor development. MAGE is a family of CT antigens consisting of more than 25 genes characterized by the presence of a large central region termed the MAGE homology domain (MHD) (Chomez, De Backer et al. 2001). This MHD does not contain any regions of significant homology with other known proteins (Taniura, Kobayashi et al. 2005).

Currently, the only CT-X gene product whose protein binding partner has been found is MAGEA1. MAGEA1 has been shown to interact with the transcriptional regulator SK1-interacting protein (SKIP) (Laduron, Deplus et al. 2004). SKIP connects DNA-binding proteins to other proteins that either activate or repress transcription, and participates in many signaling pathways. It has been proposed that MAGE1A, through its interaction with SKIP, may actively repress the expression of genes required for differentiation by disrupting SKIP-mediated

NOTCH-1 signal transduction by binding SKIP and recruiting histone deacetylase (Laduron, Deplus et al. 2004), which may act to disregulate cell proliferation and promote the survival of transformed cells (Wharton, Johansen et al. 1985; Laduron, Deplus et al. 2004).

It has been proposed that other CT antigens may render tumor cells more resistant to immune-mediated killing. For example, members of the GAGE family, such as GAGE7C or GAGE7B, may increase resistance to apoptosis induced by either INFγ or by the death receptor FAAS (Cilensek, Yehiely et al. 2002). It has also been found that expression of at least one of MAGEA1, MAGEA2, or MAGEA3 proteins appear to render cells more resistant to TNF-mediated cytotoxicity (Park, Kong et al. 2002). Such observations have led LJ Old to propose his theory that a 'Gametogenic Program' is induced in cancer cells. According to this theory, these germline genes confer a range of phenotypic traits that are essential for the survival and function of gametes, and such traits would prove advantageous for tumor cells (Old 2001).

1.6 Failure of the immune system to control cancer

As discussed in the above sections, protective anti-tumor immunity is relatively simple to establish in many/most experimental systems, and studies in both experimental animals and humans have shown that both cellular and humoral immune responses are generated in the presence of a progressively growing tumor. The question that arises is why this immunity fails to offer protection

against the tumor. Many theories are presented in the literature that attempt to explain the mechanisms by which the immune system fails cancer patients.

1.6.1 Immune ignorance

The tumor associated antigens recognized by the immune system could be considered as being slightly altered self antigens. Proponents of Janeway's (Janeway 1989) 'infectious-nonself' and Matzinger's (Matzinger 1994) 'danger' models of CD4+ T cell activation suggests a developing tumor fails to produce appropriate inflammatory or 'danger' signals that are envisaged to be essential in the activation of the naïve T cells specific for tumor antigens. These theories of immune ignorance have inspired many studies using various dendritic cells, and viral vectors expressing tumor antigens, in an attempt to overcome the unresponsiveness to tumor antigens envisaged to result in the failure to generate protective immunity (Schuler, Schuler-Thurner et al. 2003). A vast majority of these therapies has not had any appreciable efficacy as assessed in clinical trials. Furthermore, studies in tumor bearing animals which were treated with tumor cells transfected to express the immunocostimulatory B7 molecule on the cell surface had a favorable affect in inducing tumor regression only if given while the primary tumor was very small (Chen, Linsley et al. 1993). Such results, coupled with the striking observation that even in patients with progressively growing tumors, T cells capable of killing tumor cells *in-vitro* can often be easily identified from both the blood (Letsch, Keilholz et al. 2000) and tumor infiltrates (Lurquin, Lethe et al.

2005), have led others to suggest that the failure of the immune system is most likely not at the level of initiating the priming of T cells.

1.6.2 Tumor variants

Antigenic variability in tumors was first shown experimentally in 1977, when it was observed that tumor cells isolated from an animal after partial rejection of the tumor were much less sensitive to lysis by the CD8+ T cells isolated from these animals than were the original tumor cells (Biddison and Palmer 1977). In the early 1980s, Boon *et al.* demonstrated at late time points during tumor progression that animals were able to clear *in-vitro* passaged clones from which the progressively growing tumor was derived, but not cells from the established, progressively growing, tumor (Uyttenhove, Maryanski et al. 1983). These observations have given rise to the idea that the immune system itself places a very heavy selection pressure on the tumor, whose antigenic profile is inherently very plastic, continually selecting tumors expressing an altered antigenic profile not recognized by the pool of tumor-specific effector cells. This theory proposes the immune system is simply unable to keep pace with the continual generation of these tumor variants ultimately resulting in immune failure. Opponents of this theory often point to the simple fact that for the vast majority of murine syngeneic tumors, researchers have devised relatively simple strategies to render mice resistant to a normally lethal dose of the selecting tumor (Foley EJ, 1953; Klein G, 1959; Klein G, 1960). These findings suggest that the immune system, if significant numbers of

variants do arise, is capable of killing all these variants. Furthermore, the immune

system usually responds to several epitopes derived from several different

polypeptides on a tumor, and for a tumor variant to escape killing by the immune

system, and predominate, it would have to simultaneously mutate, or lose the

expression of, several proteins at one time. In fact, it has been reported by Boon

and colleagues that in more than ninety percent of cases, metastatic tumor cells

isolated from patients during the course of disease are capable of restimulating

and being lysed by autologous T cells isolated from patients early in the course of

tumor progression (Boon, Coulie et al. 2006). Such considerations suggest there

may be other reasons for the failure of the immune system to effectively contain

tumors.

1.6.2.1 *Concomitant Immunity*

Concomitant immunity is defined as being present at a period of time, after

implantation with a lethal dose of isogenic tumor cells, during which the animal is

capable of preventing the outgrowth of another normally lethal dose of the same

tumor at a second injection site. The presence of such immunity generally peaks

early during the course of tumor growth, and quickly decays over time (North and

Bursuker 1984). Lymphocytes harvested from tumor-draining lymph nodes two or

three days after implantation of a syngeneic sarcoma were able to inhibit the

growth of the selecting tumor cells *in-vitro* (Rosenau and Moon 1966). Spleen cells

harvested from an animal bearing a progressively growing tumor during this period

of concomitant immunity were able to, upon transfer, protect a naïve mouse from a normally lethal challenge with the selecting tumor (Greene 1980). Such observations strongly suggest that even in the presence of a progressively growing tumor there is an immune response capable of efficiently killing tumor cells (Takei, Levy et al. 1976).

1.6.3 Suppressor cells

For over forty years, it has been appreciated that there is a population of T cells within animals carrying a late stage progressively growing tumor which are capable of specifically suppressing the stable generation of an effective anti-tumor immune response upon transfer into naïve animals (Eggers and Wunderlich 1975; Fujimoto, Greene et al. 1976; Small and Trainin 1976; Reinisch, Andrew et al. 1977). The existence of such a suppressor population has been demonstrated both *in-vitro* and *in-vivo* using different tumor models (Gershon and Kondo 1971; Takei, Levy et al. 1977; North 1984; North and Bursuker 1984). Cells isolated from the draining lymph nodes two or three days after implantation of a syngeneic sarcoma were able to inhibit the growth of the selecting tumor cells *in-vitro* (Rosenau and Moon 1966). Spleen cells from mice with an established P815 tumor had T cells capable of specifically suppressing the *in-vitro* generation of specific cytotoxicity against P815 (Takei, Levy et al. 1976; Takei, Levy et al. 1977). The suppressor function of these cells are not restricted to either the K or D MHC loci, so they are probably CD4+ T cells (Levy, Maier et al. 1979).

Many investigators have examined the development of these suppressor cells in relation to the observations of concomitant immunity. Greene was able to demonstrate that progression of the primary tumor was associated with the disappearance of detectable concomitant immunity (Greene 1980). RJ North and colleagues subsequently demonstrated that the observed loss of concomitant immunity was due to a progressive increase in the numbers of the so called 'suppressor cells' which he demonstrated bear the Ly1+,2- marker (CD4+ T cells) (Bursuker and North 1984; North and Bursuker 1984). These observations mirror those by D.G. Kilburn and colleagues who demonstrated that spleen cells of mice with an established P815 tumor had T cells capable of specifically suppressing the *in-vitro* generation of specific cytotoxicity against P815 (Takei, Levy et al. 1976; Takei, Levy et al. 1977). If these CD4+ suppressor T cells are responsible for immune failure, then an understanding of their generation and mode of action is central to the development of effective anti-tumor immunotherapeutic strategies.

1.6.4 Suppressor cells in a modern world

The idea of suppressor T cells as a mechanism of immune failure with regards to cancer fell out of vogue in the mid-eighties and had remained essentially an afterthought in the literature until the late 1990s. At that time, essentially two revolutionary ideas helped to put suppressor cells back onto the immunological map. Firstly, as discussed above, T. Boon and L.J. Old's pioneering work in the development of the cancer immunome (Lee, Obata et al. 2003; Boon, Coulie et al.

2006) had unexpectedly added a new depth of analysis by demonstrating that the

vast majority of tumor antigens are normal cellular proteins. The second dramatic

finding was the existence of what are now known as CD4+ CD25+ Foxp3+ T

regulatory cells (Sakaguchi, Sakaguchi et al. 2001; Sakaguchi 2005). Many groups

have reincarnated suppressor cells in the guise of CD4+ CD25+ Foxp3+ T regulatory

cells.

1.6.4.1   *Suppressor T cells as CD4+ CD25+ Foxp3+ regulatory T cells*

Over thirty years ago, it was observed, in certain strains of mice, that thymectomy

three days after birth resulted in the subsequent development of organ-specific

autoimmunity (Nishizuka and Sakakura 1969). This autoimmunity induced by day

three thymectomy could be prevented by transferring CD4+ T cells from the

spleens of adult or thymocytes from day ten mice to recipients shortly after

thymectomy (Penhale, Irvine et al. 1976; Sakaguchi, Takahashi et al. 1982;

Sakaguchi, Fukuma et al. 1985). Such observations strongly suggest the existence

of a population of T cells capable of controlling the development of autoimmunity.

Further studies have characterized these cells as having constitutive high level

expression of the alpha chain of the IL-2 receptor (CD25) (Sakaguchi, Sakaguchi et

al. 1995) and the transcription factor foxp3 (Hori, Nomura et al. 2003). These cells

have been called regulatory T cells, and have been shown to actively inhibit the

function of a wide variety of immune cells usually in a cell-contact dependent

manner (Murakami, Sakamoto et al. 2002; Azuma, Takahashi et al. 2003;

Trzonkowski, Szmit et al. 2004; Lim, Hillsamer et al. 2005; Romagnani, Della Chiesa et al. 2005; Chen 2006). The recent literature has become inundated with reports of CD4+ T regulatory cells which regulate the generation or function of other T cells. Initially, it was thought that these natural CD4+CD25+ regulatory T cells which are found in the thymus, might be selected to have a specificity repertoire focused on self antigens, and hence have a major role in the maintenance of peripheral tolerance through a modified mechanism of 'infectious tolerance' (Piccirillo and Shevach 2004). More recently, it has been observed that these regulatory T cells also play a role in many models of infectious disease, and that they have specificity for foreign pathogens (Belkaid and Rouse 2005).

The evolution of the cancer immunome has quickly forced scientists to realize that the vast majority of tumor-associated antigens, to which patients are mounting immune responses, are in fact non-mutated, normal cellular proteins. As such, many theories of immune failure against tumors involve the proposal that such 'self' tumor-associated antigens induce a particular class of T cell, namely CD4+CD25+ natural T regulatory cells, which dampens the ability of the immune system to generate an effective anti-tumor immune response (Murakami, Sakamoto et al. 2002; Shevach 2002; Azuma, Takahashi et al. 2003; Dunn, Old et al. 2004; Trzonkowski, Szmit et al. 2004; Lim, Hillsamer et al. 2005; Romagnani, Della Chiesa et al. 2005; Sakaguchi 2005; Chen 2006). In fact, treatments to reduce the number of CD25+ T cells *in-vivo* have shown positive effects in some tumor models

(Onizuka, Tawara et al. 1999; Shimizu, Yamazaki et al. 1999; Steitz, Bruck et al. 2001; Sutmuller, van Duivenvoorde et al. 2001; Golgher, Jones et al. 2002; Casares, Arribillaga et al. 2003; Ko, Yamazaki et al. 2005; Prasad, Farrand et al. 2005).

The observations described above suggest a potentially powerful role for CD4+CD25+ T regulatory cells in the inhibition of protective anti-tumor immune responses. However, one must be aware that the reinterpretation of suppressor cells as T regulatory cells contravenes many classical observations made in both tumor and non-tumor systems.

1.6.4.2 An argument against T regulatory cells as mediators of immune failure

On the whole, there are relatively few *in-vivo* murine studies which demonstrate that depletion of CD25+ T cells induces tumor regression (Zou 2006). In most models, one must deplete CD25+ T cells before, or very soon after, tumor implantation to have any measurable effect on tumor growth (Onizuka, Tawara et al. 1999). Such observations are surprising when put into the context of concomitant immunity. If CD25+ T cells are the prime cause that an effective anti-tumor immune response fails to be generated, one would predict that depletion of CD25+ cells at any point before concomitant immunity begins to wane should result in the generation of protective immunity. Furthermore, the implication of a preexisting regulatory T cell population specific for tumor antigens in naïve animals is not easily reconciled with the simple observation that, in most experimental

models of cancer, it is very simple to generate protective anti-tumor immune responses (Arca, Krauss et al. 1996; Ashley, Faiola et al. 1997; DeMatos, Abdel-Wahab et al. 1998; Schreurs, de Boer et al. 1998; Tuting, Steitz et al. 1999). One must reconcile such observations by postulating that there are selective and differential requirements for the activation of T regulatory cells as compared to effector T cells; however, no one has proposed such a model that accounts for the generation and decay of concomitant immunity.

Robert North and colleagues observed that in some tumor models, one could preferentially inhibit the generation of suppressor cells by treatment with cyclophospamide (Awwad and North 1989) or with low levels of irradiation (North 1986). These observations led to the thought that suppressor cells were more sensitive to anti-proliferative treatments than protective cells. It has subsequently been extrapolated that CD4+CD25+ regulatory T cells must also be exceptionally sensitive to anti-proliferative treatments (Thornton, Piccirillo et al. 2004; Ercolini, Ladle et al. 2005; Motoyoshi, Kaminoda et al. 2006). There have been calls to utilize low doses of cyclophosphamide to inhibit the effects of T regulatory cells in all immunotherapeutic tumor treatment strategies (Ghiringhelli, Larmonier et al. 2004; Turk, Guevara-Patino et al. 2004). It is somewhat surprising that a hyporesponsive regulatory cell be more sensitive to such treatments compared to the rapidly dividing T cells that differentiate into effector T cells. Furthermore, Takei et al., demonstrated in-vitro that their suppressor cells, when diluted,

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effectively become T helpers (Takei, Levy et al. 1977). One could interpret such observations as having their effect by simple virtue of limiting T cell cooperation.

As described in previous sections, there is evidence suggesting that the degree to which specific CD4+ T cells are able to cooperate can determine the Th1/Th2 nature of an immune response. This degree of cooperation can be modulated by altering the number of specific, and interacting CD4+ T cells or by altering the dose of antigen. In support of such a model, experimental evidence has demonstrated that decreasing either the number of CD4+ T (Ismail and Bretscher 2001; Ismail, Basten et al. 2005; Mckinstry 2005), or the antigen load (Bretscher, Wei et al. 1992; Menon and Bretscher 1998; Power, Wei et al. 1998; Hailu, Menon et al. 2001; Ismail and Bretscher 2001; Ismail, Basten et al. 2005), can modulate a predominant Th2 or mixed Th1/Th2 response towards the Th1 pole. In the subsequent sections we will discuss how several successful attempts at modulating tumor-specific responses can be interpreted as a consequence of limiting the degree of CD4+ T cell cooperation, which we hypothesize to promote the generation of Th1 responses, which are more effective than Th1/Th2 mixed responses at containing the outgrowth of neoplastic cells.

1.6.4.3 Possible effects of CD25 monoclonal antibody treatment on T cell cooperation

The vast majority of studies examining the effects of *in-vivo* depletion of CD25+ cells utilize the PC61 B cell hybridoma (Shimizu, Yamazaki et al. 1999; Imai, Saio et

al. 2007) which has been shown to produce a monoclonal antibody recognizing the alpha-chain of the IL-2 receptor. Binding of this antibody to CD25 on the surface of T cells prevents subsequent IL-2 binding (Lowenthal, Corthesy et al. 1985). IL-2 is the most important T cell growth factor and it is therefore possible, by blocking the function of IL-2, that this anti-CD25 treatment may have many powerful phenotypic consequences other than simply depleting a small population of CD4+CD25+ T cells. Perhaps the effects one observes could also be due to limiting the degree of T cell cooperation in the generation of the anti-tumor immune response.

Ultimately, one must concede that the role of CD4+CD25+ regulatory T cells in a normal physiological state and their role in promotion of disease remain to be fully understood. Until one understands this population of cells better, it is perhaps wise to remain cautious in promoting the general assumption that T regulatory cells are in fact the sole purveyors of the phenotypic 'tolerance' in cancer patients to their progressively growing tumor. It is very likely these cells may simply be one cog in the wheel of immune failures associated with progressive tumor growth.

1.6.5 Classical suppressor T cells

It has been appreciated for many years that humoral and cell mediated immunity tend to be mutually exclusive of one another (Parish 1972). Early observations supported the idea that the generation of each class of immunity was also associated with the generation of antigen-specific T cells that acted to suppress the

generation of the opposing class of immunity. Experimentally it has been shown that generation of a humoral immune response against a particular antigen results in the generation of a population of CD4+ (Ly1+Ly2-) T cells which, upon transfer, renders naïve, recipient animals refractory for the induction of cell mediated immunity, in the form of delayed type hypersensitivity, to the same antigen (Lagrange, Mackaness et al. 1974; Ramshaw, Bretscher et al. 1976; Ramshaw, McKenzie et al. 1977). The specificity of suppression is a primary facet of both the suppressor cells reported by North and colleagues (Bursuker and North 1984; North and Bursuker 1984), as well as the classical suppressor CD4+ T cells discussed above; however, this specificity of action is not known to be shared by regulatory T cells. It is commonly thought that although regulatory T cells are generated in response to specific antigens, their mode of action is not antigen-specific (Shevach 2002). Such observations, to my mind, do not support the idea that the suppressor T cells described by North in his tumor systems are CD4+ CD25+ natural T regulatory cells. We support the idea that these suppressor cells act to specifically maintain the exclusivity of humoral (Th2) and cell mediated (Th1) immune responses to specific antigens.

1.6.6 *Suppressor T cells and the Th1/Th2 paradigm*

It has been appreciated in some situations that clinical infectious diseases are not due to a lack of specific immunity, but rather due to the generation of immunity ineffective at containing the pathogen (Bretscher, Ismail et al. 2001). It has been

suggested for quite some time, that such a paradigm also exists in tumor immunology. For fifty years it has been suggested that the generation of cell mediated immunity to tumor antigens correlates with protection, while disease progression is associated with humoral immunity to tumor antigens. In 1968, George Klein stated that "It will be most important to establish what variables of…immunization…dosage, route of administration, and timing [of antigen administration] are critical to achieve the objective, which seems to be a stimulation of host cell-mediated rejection with minimum risk of antibody-mediated enhancement" (Klein 1968). In a modern sense, one may propose induced resistance as being a form of Th1- immune deviation. In such a model, Th1 type responses are effective in containing tumors, while Th2 type responses against tumor antigens are ineffective in containing the outgrowth of tumor cells and normally occur during tumor progression.

It has been clearly demonstrated by North that the kinetic development and subsequent extinguishment of concomitant immunity is associated with the development of tumor-specific suppressor CD4+ T cells (North 1984; North and Bursuker 1984). It is possible to envisage that concomitant immunity and its extinction correlate with cell-mediated Th1 immunity and with the evolution of a substantial Th2 component of the immune response. This hypothesis is attractive in part because it explains the parallel nature of the evolution of the Th1/Th2 nature of the immune response with North's findings in tumor systems. The

observations of Salvin (Salvin 1958), are parallel to, and predate by several decades, observations made with replicating antigens. Mice given a sufficiently low number of a slowly replicating microorgansism only generate INFγ producing Th1 cells. Mice given a high number of replicating microorganisms produce an initial wave of specific CD4+ T cells secreting INFγ, corresponding to Th-1 cells, which is subsequently replaced by either a mixed Th1/Th2, or a predominant Th2 immune response, as assessed by specific IL-4 production by CD4+ T cells. (Bretscher, Wei et al. 1992; Power, Wei et al. 1998; Bretscher, Ismail et al. 2001). The idea of suppressor cells being tumor-specific Th2-type cells may offer an additional explanation for many observations in many models of immunological treatment of cancer, as I shall comment upon in the discussion.

2 *Research Objectives*

The mechanisms by which the immune system fails to protect against cancer are not well understood. The primary goal of this research is to attempt to understand the types of immunity generated in mice suffering from progressive tumor growth as compared to mice rendered resistant to a normally lethal challenge of the same tumor. Such understanding, in the future, will enable one to develop strategies to exploit the endogenous immunity generated against cancer to develop better treatments.

Utilizing experimental animal models by which one can render mice resistant to a normally lethal challenge with either the P815 or L5178Y tumors, I sought to develop assays to test the hypothesis that the Th1/Th2 nature of the anti-tumor immune response is correlated with tumor resistance/susceptibility. I also sought to exploit any correlates found to design and test strategies to induce tumor regression.

3 Materials and Methods

3.1 Mice

DBA/2J mice were either obtained from Jackson Laboratories (Bar Harbor, Maine, USA) or from the University of Saskatchewan animal colony. CBA/J mice were obtained from the University of Saskatchewan animal colony. Mice were housed under specific-pathogen free conditions, and were routinely screened to ensure they were free of subclinical viral and bacterial infections. Male and female mice used in experiments were between the ages of 6-12 weeks. All animals were maintained according to the guidelines of the Canadian Council of Animal Care.

3.2 Cell lines

3.2.1 Tumor cell lines

P815 was derived from a male DBA/2 mouse by painting its skin with 0.2% methylcholanthrene in ether three times a week for seven weeks (Dunn and Potter 1957), and was obtained as TIB-64, Lot#2310374, from the American Type Culture Collection, Rockville, Maryland, USA. L5178Y is a thymoma induced by

methylcholanthrene in ether, kindly provided by Dr. RJ. North, Trudeau Institute, Saranac Lake, NY, USA.

3.2.2 B cell hybridomas

11B11 (HB-188) is a rat hybridoma secreting monoclonal IgG1 antibodies capable of neutralizing murine IL-4 (America Type Culture Collection) (Ohara and Paul 1985). GK1.5 is a rat hybridoma secreting monoclonal IgG2b antibodies capable of binding to the mouse CD4 antigen. This hybridoma was obtained from the American Type Tissue Culture Collection (Dialynas, Quan et al. 1983).

3.3 Media

RPMI 1640 media supplemented with L-glutamine and Leibovitzovitz media were prepared from powdered stocks per the manufacturer's instructions (Gibco Laboratories, Grand Island, NY, USA). Media was sterilized by passage through a 0.22 μm filter (Millipore, Molsheim, France). RPMI 1640 media was further supplemented with 10% fetal bovine sera (Hyclone, Logan, UT, USA), 100 U/ml penicillin/streptomycin (Gibco), 0.8 mM sodium pyruvate, and $5x10^{-5}$ M β-mercaptoethanol to make RPMI complete media that was used for all *in-vitro* cultures. When required, *Leishmania major* parasites were grown in RPMI 1640 media at a 1:1 ratio with 199 media (Gibco) supplemented with 20% fetal bovine sera (Hyclone).

Calcium and magnesium-free phosphate-buffered saline (PBS) was prepared as a 10x stock solution (pH ~7.0) containing 80.0 g/L NaCl, 2.0 g/L KCl, 11.5g/L Na$_2$HPO$_4$·12H$_2$O, and 2.0g/L KH$_2$PO$_4$ in 1L distilled deionized H$_2$O. PBS was used as a 1x solution, and was autoclaved as required. 1x PBS was supplemented with 0.075% Tween 20 to make PBST.

3.3.1 Parasites

Leishmania major strain, MHOM (WHO MHOM/IL/80/Friedlin) were used to infect mice subcutaneously into the hind foot pad with stationary phase promastigotes in a volume of 20μL RPMI 1640:199 serum-free media.

3.4 Preparation of single cell suspensions

3.4.1 Spleen cells

Mice were killed by cervical dislocation. The spleen was aseptically removed, and was placed into 10 ml of ice cold Leibovitz media. The spleen was cut up into small fragments, and a single cell suspension was obtained by gently pressing these small fragments through a 60 gauge stainless steel wire mesh. The resulting suspension was transferred to a 15 ml tube, and was left for approximately 2 minutes to allow debris to settle. Nine ml of the supernatant was collected, and spun by centrifugation at 500 x g for 10 minutes at 4° C. Cell viability was determined using trypan blue exclusion using a hemocytometer slide.

3.4.2 Peripheral blood leukocytes

Mice were warmed under a heating lamp for approximately 10 minutes, and 10-12 drops of blood (roughly 250 μl) was collected from their tail into a sterile tube containing roughly 20 units of heparin. When necessary, the blood was spun at 500 x g for 10 minutes and the sera was collected and stored at -20º C until use. Blood was mixed with 1 ml of cold Leibovitz media, and red blood cells were removed by density gradient centrifugation for 20 minutes at 890 X g on 1 ml of Ficoll ® 400 (Pharmacia Biotech AB, Uppsala, Sweden). Cells at the interface were harvested, placed into 10 ml of cold Leibovitz medium, washed once, and resuspended into a final volume of 1.5 ml RPMI complete medium. Cell viability was determined using trypan blue exclusion, and cells were counted using a hemocytometer slide.

3.5 Nylon wool purification of spleen cells

Nylon wool fiber (Polysciences Inc. Warrington, PA, USA) was washed and autoclaved in saline a minimum of three times prior to use. Columns were prepared by placing roughly 6ml of lightly packed nylon wool into a 10 ml syringe with a 23 gauge needle attached. The column was washed by running 20 ml of Leibovitz medium through the column. The column was blocked by running 10 ml of warm Leibovitz media supplemented with 10% fetal bovine serum through the column, and placing it in a 37° C incubator for a minimum of 2 hours before use.

One spleen in a volume of three ml warm Leibovitz medium supplemented with 10% FBS was layered on the top of the column, and allowed to run until the entire suspension entered the nylon wool. The column was incubated at 37° C for one hour, and non-adherent cells were eluted by running 40 ml of warm Leibovitz medium supplemented with 10% FBS through the column. This eluted fraction was collected, and the fractionated cells were counted. Percent recoveries ranged between 20-35%.

3.6 Magnetic activated cell sorting (MACS) fractionation of spleen cells

Red blood cells were removed from spleen cells by resuspending spleen cells in 3 ml of Leibovitz medium per spleen. 5 ml of filter-sterilized ACK lysing buffer (0.15M NH_4Cl, 1mM $KHCO_3$, 0.1mM Na_2EDTA, pH 7.2) was added, and left at room temperature for 3 minutes. Cells were filtered through a 30 μm nylon mesh filter to remove cellular debris, and the remaining cells were washed 3 times in Leibovitz medium. Cells were resuspended in MACS buffer (5% fetal calf serum in PBS) at a concentration of 1.1×10^8 cells/ml. 10 μL of appropriate magnetically labeled antibodies (anti-CD4, CD8 and Thy1.2) (Milteni Biotec, Bergisch Gladbach, Germany) were added per 10^7 cells (90μL of cell suspension). After mixing, the mixture was left to incubate for 15 minutes at 4°C. Cells were washed in, and then resuspended in 500μL of MACS buffer. Depletion (LD) columns were placed in the magnet, washed with 3 ml of MACS buffer, and the cell suspension was applied to the column. The unlabeled fraction was collected by washing the column three times with 3 ml MACS buffer. Positively labeled cells were collected by removing the column from the

magnetic field, and flushing the column with 5 ml MACS buffer. Purity of each fraction was confirmed by flow cytometry.

3.7 Flow cytometry

Spleen cells or cells harvested after *in-vitro* culture were washed twice, filtered through a 30 μm nylon mesh filter to remove any clumps, and cells were resuspended at a final concentration of 10^7 cells per ml PBS supplemented with 5% FCS (FACS buffer). 100 μl of the suspension was labeled with 7-10 μL of FITC or PE-labeled antibodies (anti-CD4 (YTS 191.1.2 clonotype), anti-CD8a (YTS 169 AG 101 HL clonotype), anti-CD19 (6D5 clonotype), anti-CD45R (RA3-6B2 clonotype), anti-CD90 (Thy 1.2; 5a8 clonotype), anti-α/β TCR (H57-597 clonotype), anti-I-A^d (34-5-3S clonotype) (Cedarlane Laboratories Limited, Hornby, Ontario, Canada) anti-H-2D^d (34-2-12 clonotype) (BD Biosciences, Franklin Lakes, NJ, USA). Cells were incubated on ice for 30 minutes, washed, and resuspended in one ml FACS buffer. Labeled cells were examined using a Beckman Coulter Epics Flow cytometer, and were analyzed using the Expo 32 v1.2 software package (Coulter, Mississauga, ON, Canada).

3.8 ELISPOT assay

3.8.1 Preparation of ELISPOT plates

96-well Unifilter 350 nitrocellulose bottom plates (Whatman-Polyfiltronics, Clifton, NJ, USA) were coated with either 0.1 μg purified anti-IL-4 (11B11) or anti-INFγ (R4-6A2) monoclonal antibodies (Pharmingen, San Diego, CA, USA) per well in 1M bicarbonate buffer at a pH of 9.6. Plates were left at 4°C overnight. Plates were

washed once with RPMI, and subsequently blocked for at least 2 hours with complete-RPMI before use.

3.8.2 ELISPOT assay for the detection of P815-Dependent INFγ and IL-4 secretion by spleen cells

Spleen cells harvested from mice exposed to the P815 tumor were prepared, and purified on nylon wool columns. This enriches for T cells, and eliminates the majority of contaminating tumor cells. These primed spleen cells are plated at various dilutions with the addition of spleen cells from normal mice to obtain a total of 2×10^6 spleen cells/well. This complementation with normal spleen ensures the number of APC is not limiting the detection of antigen-specific T cells, making the assay highly sensitive and valid for enumerating effector T cells. 5×10^5 gamma-irradiated P815 cells (~20 000 Rads) were used as antigen. Plates were allowed to incubate at 37° C in a 5% CO_2 atmosphere for 8 hours.

3.8.3 ELISPOT assay for the detection of L5178Y-dependent cytokine secretion by spleen cells

Spleen cells harvested from mice exposed to the L5178Y tumor were prepared, and plated at various dilutions with the addition of spleen cells from normal, non-immunized mice to obtain a total of 1×10^6 spleen cells per well. 1×10^5 gamma-irradiated L5178Y cells (~20 000 Rads) were used as antigen. Plates were allowed to incubate at 37° C in a 5% CO_2 atmosphere for 8 hours.

3.8.4 ELISPOT assay for the detection of Leishmania major-dependent cytokine secretion by spleen cells

Spleen cells harvested from L. major infected mice were prepared and plated at various dilutions with the addition of spleen cells from normal, nom-immunized mice to obtain a total of 1×10^6 spleen cells per well. 30 μg/ml of freeze-thawed sonicated *L. major* was added as antigen to appropriate wells (Power, Grand et al. 1999; Uzonna and Bretscher 2001). Total protein content of antigen was determined by the bicinchoninic assay protein reagent (Pierce, Rockford, IL). Plates were allowed to incubate at 37° C in a 5% CO_2 atmosphere for 8 hours.

3.8.5 Development of Spots in the ELISPOT assay

Cells were removed from the plate by washing twice with PBST. Any remaining cells were lysed by washing the plate with ddH_2O. Residual cellular debris was removed by washing the plates an additional four times with PBST. 1.0 μg/ml PBST of biotinylated anti-INFγ (XMG1.2) or anti-IL-4 (BVD6-24G2) (BD PharMingen, Mississauga, ON, Canada) was added to appropriate wells. The plates were incubated at 4°C overnight. Plates were then washed eight times with PBST to remove any unbound antibodies, and 100 μL of alkaline-phosphatase strepavidin (Jackson Immuno Research Laboratories Inc., West Grove, PA, USA) at a concentration of 0.2 μg/ml in PBST was added to each well, and allowed to incubate at room temperature for 1.5 hours. Plates were then washed eight times with distilled water, and 100 μL of NBT/BCIP (Roche, Basel, Switzerland) diluted

1:50 in substrate buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, and 0.05 M MgCl$_2$). Spots were allowed to develop in the dark, and the color reaction was stopped by washing the plates three times with distilled water. Plates were allowed to dry, and spots were enumerated using a dissecting microscope.

3.9 Western blot to detect P815-specific antibodies

3.9.1 P815 antigen

P815 cells were harvested from *in-vitro* culture, washed 4 times in sterile PBS and resuspended at 10^{8}cells/ml. The cell suspension was freeze/thawed, and sonicated for 24 cycles on ice using a Branson Sonifer 450 (Branson, Danbury, CT, USA) set at 50% duty cycle. When required, the soluble and insoluble fractions were separated by spinning the resulting suspension for 30 minutes at at 20 000 x g. The soluble fraction was collected, and the insoluble fraction was re-suspended in an equivalent volume of fresh PBS (sonicated 3-4 times to resuspend the pellet). The P815 antigen was stored at -20°C until use.

3.9.2 Western blot

P815 antigen samples were diluted 1:1 with Laemmli Sample Buffer (Bio-Rad Laboratories, CA, USA) and boiled for 4 minutes. 200 μL of sample was run on a 4% stacking, and 10% separating sodium doecyl sulphate polyacrylamide gel (29:1 ratio of acrylamide/bis) using a mini-Protean II Electrophoresis Cell (Bio-Rad

Laboratories) at 200 Volts for approximately 45 minutes. The separated proteins were transferred onto a 0.45μm nitrocellulose membrane (Bio-Rad Laboratories) at 20 Volts for 20 minutes using a Trans-Blot Semi-Dry Transfer Cell (Bio-Rad Laboratories). The Membrane was blocked for 1-2 hours at room temperature on a rocker with a 5% blotting grade non-fat dry milk blocker (Bio-Rad Laboratories) in PBST. After blocking, the membrane was assembled into a mini protean II multi-screen apparatus (Bio-Rad Laboratories). Mouse serum was diluted as indicated, in our 5% milk in PBST solution, and 400 μL of the sample dilution were run in each lane. The membrane was incubated overnight at 4° C on a rocker. The membrane was washed six times for five minutes with PBST. The membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse IgG1 or IgG2a (Southern Biotechnology Associates Inc., AL, USA) at a dilution of 1:5000 in our 5% milk in PBST blocking solution for 1 hour at room temperature with constant rocking. The membrane was removed from the multi-screen apparatus and washed four times for fifteen minutes with PBST. The membrane was developed using Immun-Star™ HRP (horseradish peroxidase) (Bio-Rad Laboratories) chemiluminescence kit as per the manufacturer's instructions. The membrane was wrapped in clear plastic and exposed to BioMax Light Film (Kodak). Note: The procedure for western blots using fluorescence detection method is the same as those used for detection using chemiluminescence with a few minor exceptions. To decrease background blotted nitrocellulose membranes are blocked in PBS + 5% non-fat dry milk (Bio-Rad)

without the addition of tween. Furthermore we utilize secondary antibodies conjugated with either IRDye 800™ conjugated goat anti-mouse IgG1 (Rockland Immunochemicals, Gilbertsville, PA, USA) and Alexa-Fluor® 680 conjuncated goat anti-mouse IgG2a (Invitrogen) antibodies. Bands were visualized using the Licor Odyssey Imaging System (Licor, Lincoin, NE, USA).

3.10 An enzyme-linked immunoassay for the detection of IgG1 and IgG2a antibodies specific for the L5178Y tumor P815 antigen

3.10.1 L5178Y antigen

L5178Y cells were harvested from *in-vitro* culture, washed 4 times in sterile PBS and resuspended at 3.3×10^7 cells/ml. The cell suspension was freeze/thawed, and sonicated for 24 cycles on ice using a Branson Sonifer 450 (Branson, Danbury, CT, USA) set at 50% duty cycle. When required, the soluble and insoluble fractions were separated by spinning the resulting suspension for 30 minutes at a 20 000 RCF. The soluble fraction was collected, and the insoluble fraction was re-suspended in an equivalent volume of fresh PBS (sonicated 3-4 times to resuspend the pellet). The L5178Y antigen was stored at -20°C until use.

3.10.2 Enzyme-linked immunosorbant assay

96 well high protein binding polystyrene Immuno-Maxisorp Plates (NUNC, Denmark) were coated overnight at 4° C with an equivalent of 3.33×10^6 freeze-

thawed L5178Y cells per well in PBS. Plates were washed twice with 200 μL PBST

per well, and blocked for 2 hours at 37° C with 200 μL of PBS supplemented with

10% heat inactivated calf serum (Gibco Laboratories, Grand Island, NY, USA). Plates

were washed twice with 200 μL PBST per well. Serum samples were diluted in 1/50

in PBS supplemented with 10% calf serum, and two-fold serial dilutions of the sera

were performed in a 96 well low protein binding polyethylene plates (NUNC). Sera

dilutions were transferred to the Immuno-Maxisorp test plate, and was incubated

for 2 hours at 37° C. Test plates were washed six times with PBST, and 100 μL of

horseradish peroxidase-conjugated goat anti-mouse IgG1 or IgG2a (Southern

Biotechnology Associates Inc., AL, USA) was added in each well of the test plate at

a dilution of 1:5000 in PBS supplemented with 10% calf sera. The plate was

incubated at 37° C for one hour then washed 6 times with PBST. 100 μL ATBS (2,2`-

azino-di (3-ethyl-benzthiazoline-6-sulfonate)) 1-component microwell peroxidase

substrate (Kirkegaard & Perry Laboratories, Inc, Gaithersburg, MD, USA) was added

to each well. The test plate was incubated for 20 minutes at room temperature in

the dark. The test plate was read using an E-max microplate reader (Molecular

Devices Corporation, Sunnyvale, CA, USA) at a wavelength of 405 nm, and resulting

data was collected and analyzed using the Softmax Professional version 2.2.2

(Molecular Devices Corporation) computer program. The signal from serum from

naïve mice is never above the values obtained from our conjugate blank control

wells (an adsorbance of 0.065). I assigned the serum titer using a cutoff value of

this assay as being an adsorbance two times above the value obtained when using sera from a non-immunized animal.

3.11 Monoclonal antibody production, and purification

Antibodies were generated as ascities in mice. Mice greater than 12 weeks of age were injected with 0.5 ml of either pristine (Sigma, St Louis, MO, USA), or Incomplete Freund's Adjuvant (Difco Laboratories, Detroit, MI, USA) into their peritoneum. One to two weeks later, mice were given 1500 Rads of gamma irradiation and were implanted with 10^7 hybridoma cells into their peritoneum in 0.5 ml sterile PBS. Roughly 7 days later the resulting ascities was collected by aspirating their peritoneum. The antibodies were purified using a T-Gel^{TM} Purification Kit (Pierce Biotechnology Inc., Rockford, IL, USA), and antibody concentration was determined using a BCA^{TM} Protein Assay Kit (Pierce Biotechnology Inc.) as per the instructions of the respective manufacturers.

3.12 Cell proliferation assays

To determine if the presence of neutralizing anti-IL-4, or control antibodies decreased the rate of *in-vitro* growth of P815 cells, we plated 10^4 P815 cells/well of a 96 well plate, and incubated the cells with varying concentrations of antibodies (11B11 or GK1.5 clonotypes) for 24 hours. Cells were labeled with 0.5 μCi ^{3}H thymidine for the last four hours of culture. Radiolabeled DNA was harvested using a PHD^{TM} Cell Harvester (Cambridge Technology INC, Lexington, MA, USA note: this

product is currently produced by Brandel INC, Gaithersburg, MD, USA) onto glass fiber filter strips (Brandel Inc) which are placed into 6 ml liquid scintillation vials (Wheaton Science Products, Millville, NJ, USA). The samples were air-dried overnight, and 4 ml of syntillation fluid, Touline supplemented with 4 g/L omniflour (Packard Instrument Company INC, Meriden, CT, USA), was added to each tube. Radioactivity of the incorporated radio-isotope was assessed using a Beckman LS5000TD syntillation counter(Beckman Instruments, Fullerton, CA, USA).

3.13 Statistical analysis

Unless otherwise indicated the error bars on all graphs are representative of the standard deviation of the number of spots counted in three assay wells in the ELISPOT assay.

4 Results

4.1 Generation of a protective anti-P815 Immune Response

I have utilized a priming strategy by which we can consistently render DBA/2J mice resistant to a normally lethal challenge of the P815 tumor. Mice are primed by injecting 10^5 P815 cells subcutaneously into their right hind footpad. These mice can resist a subsequent, normally lethal, challenge of 10^5 to 2×10^6 P815 cells given intradermally into their belly, given a minimum of two weeks post priming (**Figure 1**). This priming strategy has been performed on more than 200 mice, and consistently renders over 80% of animals resistant to a normally lethal challenge of the P815 tumor. The efficacy of protection generated by this protocol remains stable for one year.
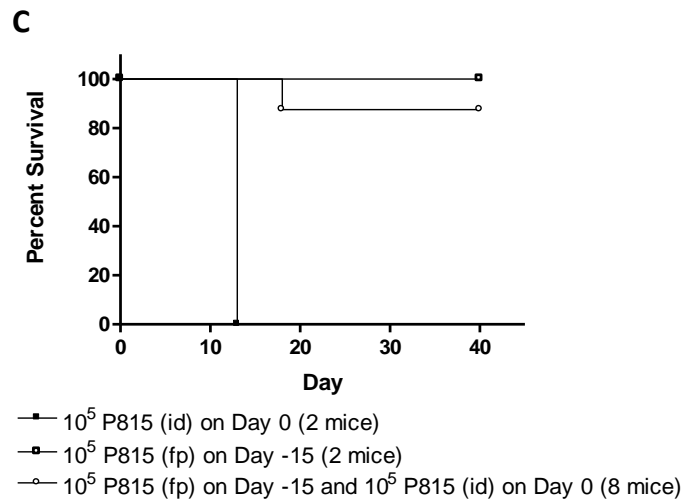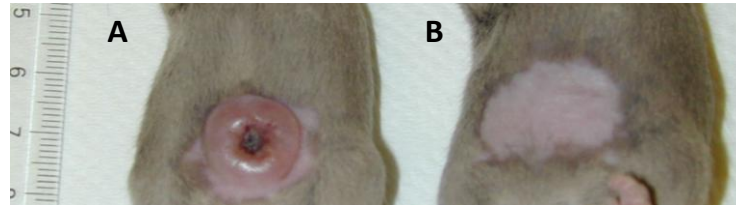
Figure 1: Rendering mice resistant to a normally lethal P815 challenge.

A) The mouse was given no pre-treatment, while the mouse shown in **B)** was given 10^5 P815 cells subcutaneously into its right hind footpad 14 days prior to both mice being challenged with 10^5 P815 cells intradermally into their abdomen. This photo was taken 19 days post challenge. **C)** DBA/2J mice were injected with 10^5 P815 cells subcutaneously into their right hind footpad (fp). 15 days later, appropriate mice were implanted with 10^5 P815 cells injected intradermally (id) into their abdomen. Percent survival was assessed over a period of 40 days. Seven of the eight mice primed with the P815 resisted the challenge.

4.2 Standardization of the ELISPOT assay for the detection of P815-dependent INFγ and IL-4 secreting cells

A protocol was established to characterize the Th1/Th2 nature of the anti-tumor immune response being mounted against the P815 cells by enumerating P815-dependent cytokine secreting spleen cells in a modified ELISPOT assay. Several interdependent difficulties were encountered in my attempts to detect P815-dependent cytokine secreting cells. The first major difficulty is that P815 rapidly metastasizes to the spleen of mice. To assess the antigen-dependent cytokine production by splenocytes one must find a protocol to remove the majority of P815 cells contaminating the spleen preparations. Fortunately, I found one can remove P815 cells which have metastasized to the spleen by running our cell preparations through a nylon wool column. Another difficulty I encountered was determining an appropriate antigen preparation to utilize in the P815 ELISPOT assay. I began by using freeze-thawed P815 cells as the antigenic source; however, it became clear that such an antigen preparation failed to induce any antigen dependent cytokine secretion while gamma-irradiated or mytomycin-C treated antigen preparations were able to elicit antigen-dependent cytokine secretion by spleen cells from P815-primed animals (**Figure 2**).
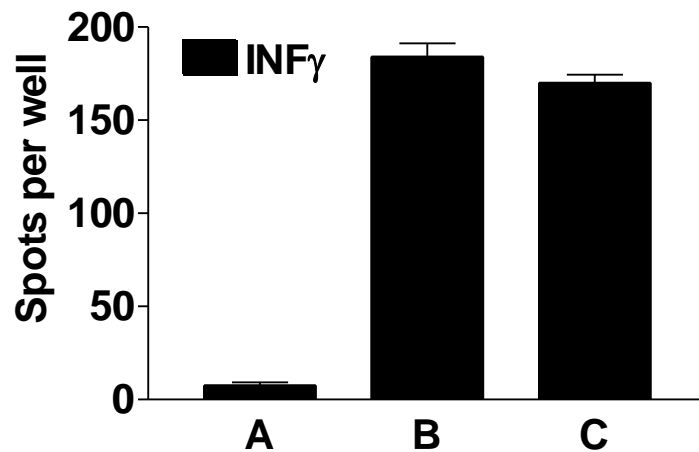
Figure 2: Intact P815 antigen is required for optimal detection of tumor-dependent INFγ secretion by primed spleen cells.

DBA/2J mice were primed ten days prior with 10^5 P815 cells intradermally into their abdomen. Spleen cells were purified on nylon wool columns to remove any P815 cells that have metastasized to the spleen. The non-adherent effluent cells were plated in the ELISPOT assay at an equivalent of 1.5×10^6 cells per well. Splenocytes from a naïve DBA/2J mouse were added as APC to give an equivalent of 2×10^6 total spleen cells per well. An equivalent of 5×10^5 P815 cells were used as antigen in each well. Number of spots detected using **A)** freeze-thawed antigen, **B)** gamma irradiated (approximately 20,000 Rads) P815 cells, **C)** P815 cells treated with mitomycin C (50μg MitoC/10^6 tumor cells) at 37° C for one hour prior to utilization as antigen in our ELISPOT assay. Data is graphed as tumor dependent INFγ production per 2×10^6 primed spleen cells.

Furthermore, I was able to demonstrate this freeze-thawed antigen not only failed to induce, but also inhibited the cytokine production by primed spleen cells when co-cultured with gamma-irradiated P815 cells (**Figure 3**). It is very likely that disrupting the membrane of P815 cells, which is a mastocytoma, releases a number of bioactive molecules toxic to cells in culture. Based upon these observations, I began to use whole tumor cell as antigen in the ELISPOT assay.

It was observed that a relatively small proportion of P815 cells (0.04-0.14%) secrete IL-4 during the eight hour restimulation process of the ELISPOT assay. Although relatively infrequent, this IL-4 secretion by P815 cells used as antigen can effectively mask the detection of P815-dependent IL-4 secretion by primed spleen cells. Fortunately, I was able to show that treating P815 cells with either Mitomycin C, or gamma-irradiation (~20 000 Rads) prevented IL-4 secretion by P815 cells, allowing me to use them as the antigenic source in the ELISPOT assay (**Figure 4**).

To determine the optimal antigen concentration for the detection of P815-dependent IL-4 and INFγ secreting cells a range of gamma-irradiated P815 antigen were given per well together with a constant number of primed spleen cells. As shown in **Figure 5**, 5×10^5 gamma-irradiated P815 cells proved to be optimal number for the detection of tumor-dependent cytokine secretion by spleen cells.

The chart shows the following categories on the y-axis with IL-4 values:
- 10^7 Freeze-Thawed P815
- 10^6 Freeze-Thawed P815
- 10^5 Freeze-Thawed P815
- 10^7 Freeze-Thawed P815 + 5×10^5 γ-irradiated P815
- 10^6 Freeze-Thawed P815 + 5×10^5 γ-irradiated P815
- 10^5 Freeze-Thawed P815 + 5×10^5 γ-irradiated P815
- 5×10^5 γ-irradiated P815

X-axis: P815-Dependent Cytokine Secreting Cells per 2×10^6 Spleen Cells (0 to 50)

Legend: IL-4
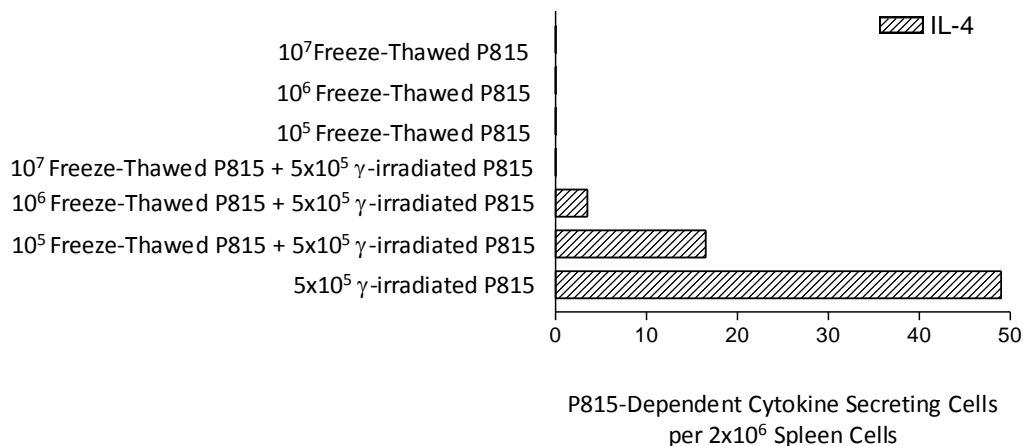
Figure 3: The presence of freeze-thawed P815 antigen inhibits the generation of spots from primed spleen cells stimulated with gamma-irradiated P815 cells.

Mice were implanted with 10^5 P815 intradermally into their belly. On Day 10 of tumor growth spleens were harvested, and prepared as previously described. Primed spleen cells were plated in the ELISPOT assay with normal spleen as a source of APC (see legend to Figure 3), and various amounts of freeze-thawed P815 cells were added to a constant number of gamma-irradiated (20 000 Rads) P815 cells. P815-dependent IL-4 secretion was enumerated.

63
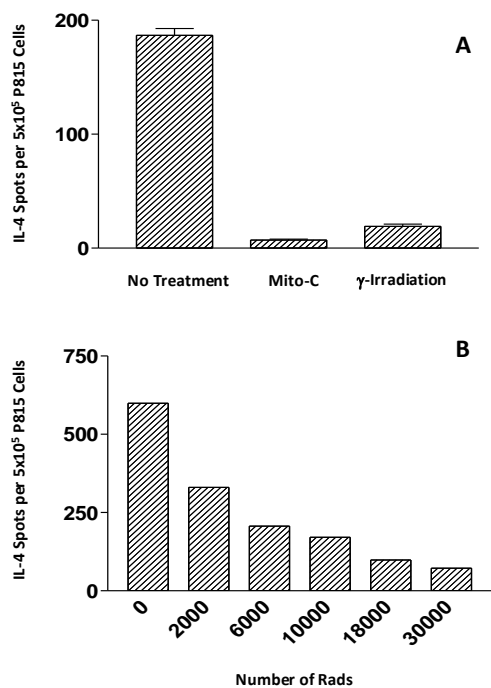
Figure 4: Inhibition of endogenous IL-4 secretion by P815 cells.

A) The effect of different treatments of P815 cells on IL-4 spot formation. P815 cells were treated with either mitomycin C (50µg Mito-C/10^6 tumor cells for one hour at 37°) or given 20000 Rads of gamma irradiation on their ability to produce spots corresponding to IL-4 producing cells. **B)** Determination of the dose of gamma irradiation required to inhibit secretion of IL-4 by P815 cells. Non-irradiated or P815 cells given different doses of gamma-irradiation were plated in our ELISPOT assay at $5x10^5$ cells/well to determine their ability to secrete IL-4 during our standard 8 hour ELISPOT incubation.
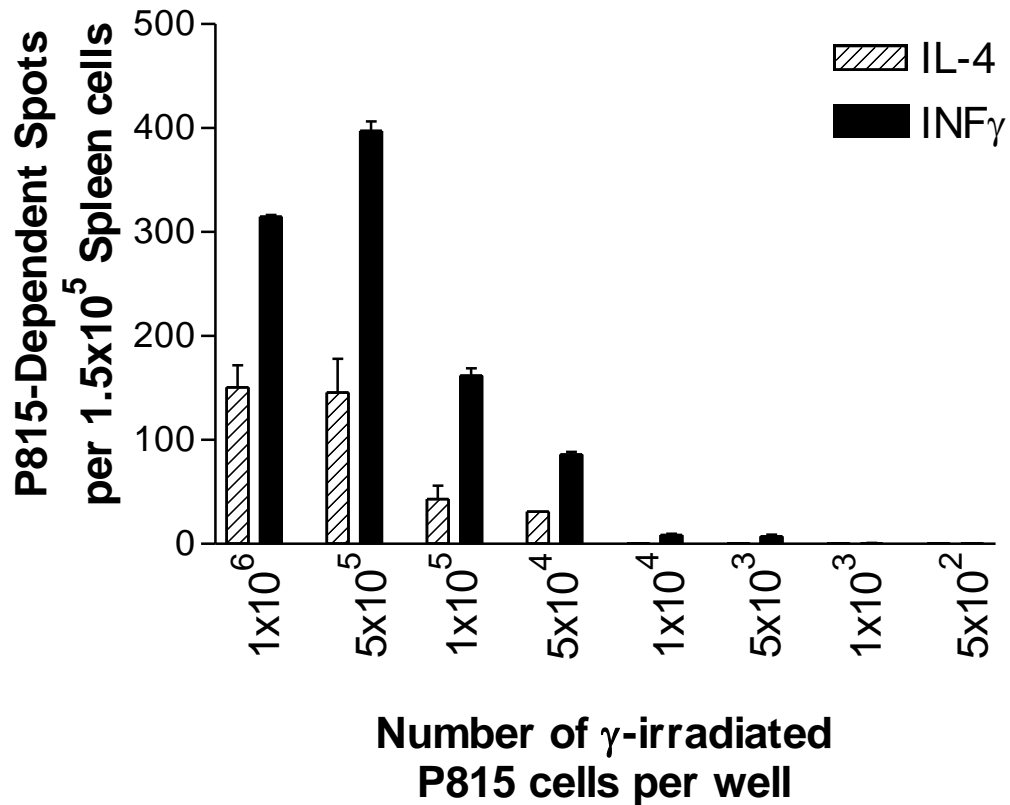
Figure 5: Assessing the optimal antigen concentration for the detection of P815-dependent INFγ and IL-4 producing cells by the ELISPOT assay.

Mice were primed in the hind footpad with 10^5 P815 40 days prior to challenge with $2x10^6$ P815 intradermally into their belly. ELISPOT was performed 20 days after challenge. Spleen cells from three mice were pooled and tumor-dependent cytokine production was determined using the ELISPOT assay as previously described. Irradiated P815 cells (20000 Rads) were used as a source of antigen and were plated at varying dilutions. Antigen dependent INFγ and IL-4 production is depicted as antigen dependent spots per well.

Linearity of the spots being detected by the ELISPOT assay is essential to ensure the tumor-dependent spots being generated are due only to the primed cells added to our assay. To assess whether the assay had this desirable feature, I diluted the primed cells, while keeping all other variables in our assay constant by adding feeder cells from non-immunized mice. As shown in **Figure 6**, both the number of IL-4 and INFγ producing cells detected by the assay depend on the number of primed spleen cells plated. Based on these experimental findings, we can conclude that the ELISPOT assay has been standardized and optimized for the detection of P815-dependent IL-4 and INFγ secreting cells.
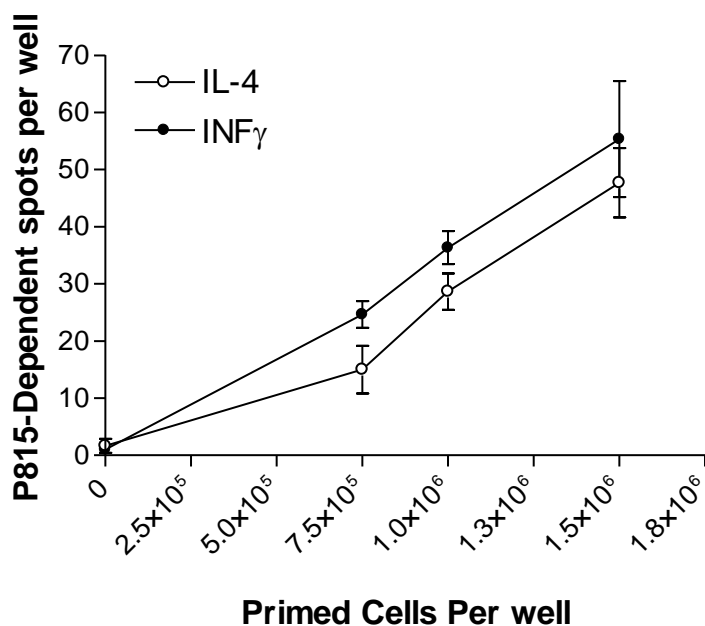
Figure 6: Linearity of the detection of P815-dependent INFγ and IL-4 producing cells is directly related to the number of primed spleen cells plated in the ELISPOT assay.

DBA/2J mice were injected with 10^5 P815 cells intradermally into their abdomen. 16 days after inplantation, spleens from 2 DBA/2J mice were pooled and prepared as previously described. Different numbers of primed spleen cells were plated in the ELISPOT assay and complemented with additional spleen cells from normal mice to keep the total cell number constant at $2x10^6$ cells per well. $5x10^5$ gamma-irradiated (20000 rads) P815 cells as antigen were added to appropriate wells. Antigen-dependent INFγ and IL-4 spots were enumerated.

4.3 Standardization of the L5178Y ELISPOT assay

Optimizing the detection of L5178Y-specific cytokine secreting cells in the ELISPOT assay was significantly less complicated than for P815-specific cells. I have observed that L5178Y cells do not endogenously produce either INFγ or IL-4 and, most importantly, this tumor cell line does not readily metastasize to the spleen in mice rendered resistant, eliminating many potential problems of significant antigen carry-over in the ELISPOT assay. I have observed mice bearing large tumors have a significant tumor burden in their spleen (**Figure 7**). This late metastasis to the spleen of mice with progressively growing tumors has not proven itself to be an obstacle in our experimental system.
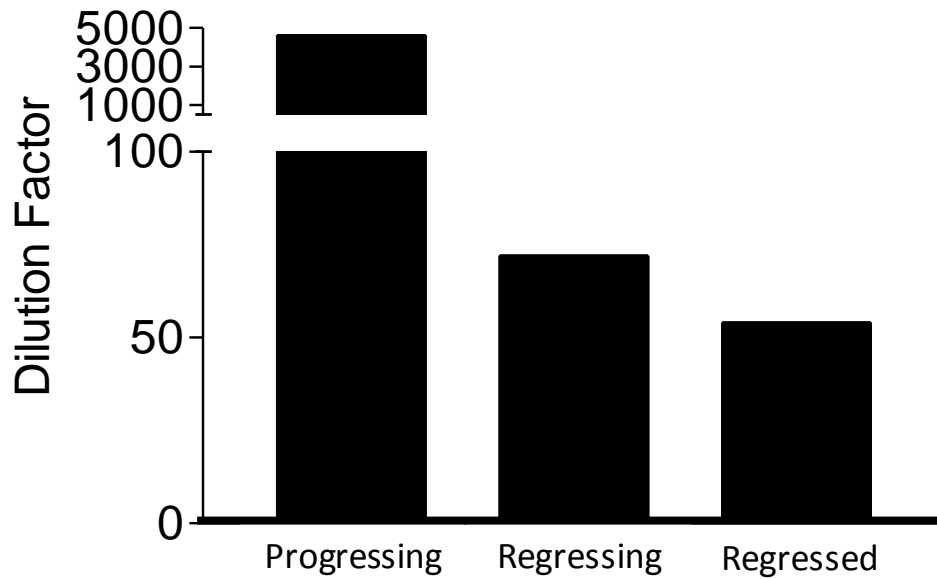
Figure 7: Metastasis of L5178Y cells to the spleen of mice bearing large,

progressively growing tumors.

Spleen cells from mice with tumors that are either progressing, regressing or from

mice having successfully induced complete tumor regression at the site of tumor

challenge were assessed for tumor burden in their spleen. Spleen cells were plated

in a 96 well plate at an original concentration of $2x10^7$ cells per well in triplicate,

and a 1/3 dilution series was performed. The dilution factor plotted corresponds to

the lowest dilution not to support tumor growth.

I wished to explore the use of intact tumor cells as a source of antigen in the ELISPOT assay to enumerate L5178Y-specific cytokine producing cells. To prevent the L5178Y cells from dividing in the ELISPOT assay, they were given a dose of gamma irradiation (5000 rads) I determined would prevent their division. As with our P815 ELISPOT assay, I determined the optimal concentration of gamma-irradiated L5178Y cells for the detection of tumor-dependent cytokine secreting cells by plating a constant number of primed cells in the presence of differing numbers of gamma-irradiated L5178Y cells (**Figure 8**). In this manner, I determined $1x10^5$ gamma-irradiated L5178Y cells was the optimal concentration of antigen for use in our ELISPOT assay. To assess whether the number of tumor-specific spots generated in our ELISPOT assay were linearly dependent on the number of sensitized cells plated, I diluted the primed cells while keeping all other variables in our assay constant. As is shown in **Figure 9**, the assay is linear for INFγ and IL-4 producing cells. Based upon these observations, we consider the ELISPOT assay is optimized for the detection of L5178Y-dependent INFγ and IL-4 secreting cells.
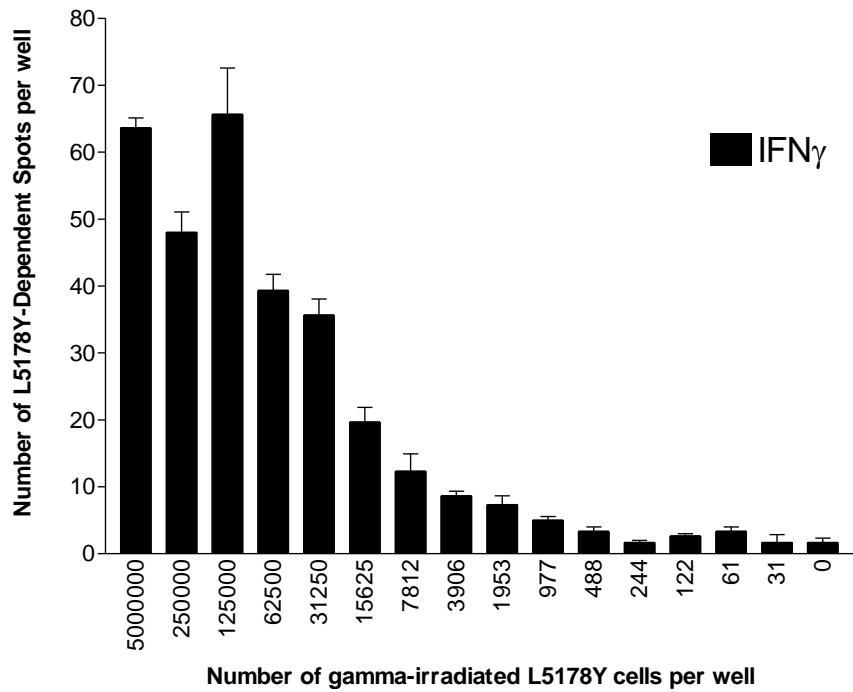
Figure 8: Assessing the optimal number of L5178Y cells required for stimulating tumor primed spleen cells for the detection of tumor-dependent INFγ-producing cells in the ELISPOT assay.

Mice were implanted on day 0 with 10^6 L5178Y cells intradermally into their belly. On day 18, the spleens of three mice, whose tumor had begun to regress, were pooled. Spleen cells were plated at 10^6 cells per well and varying numbers of gamma-irradiated (5000 rads) L5178Y cells were added as a source of antigen, and the number of antigen-dependent spots was assessed.

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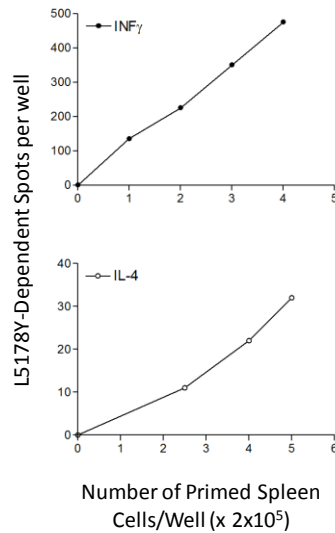
Figure 9: The number of L5178Y-dependent INFγ and IL-4 spot forming cells detected depends linearly on the number of sensitized spleen cells plated in the ELISPOT.

Top panel: Mice that had previously rejected a dose of 10^3 L5178Y tumor cells were challenged with 10^6 L5178Y cells. 20 days after challenge the spleens of two mice were pooled and used as a source of sensitized cells for the experiment. Bottom panel: the spleens of two mice, implanted 20 days prior to assay with 10^6 L5178Y cells, were pooled. Spleen cells were plated into our ELISPOT assay at various dilutions, using spleen cells from naïve animals to keep the total cell number constant at 2×10^6 cells per well 5×10^5 gamma-irradiated (5000 Rads) P815 antigen was added to appropriate wells. Antigen-dependent INFγ and IL-4 spots were enumerated.

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4.4 Immune correlates of tumor progression or regression

4.4.1 Correlates of protection in the P815 mastocytoma system

Even with the optimized P815 ELISPOT assay it has proven to be relatively difficult to consistently detect P815-dependent cytokine secretion. I believe such variability is primarily due to different rates of tumor growth and the associated variable kinetics in the anti-tumor immune response. Observations illustrating these statements will be described in subsequent sections. Despite some variability in detecting immune responses in the optimized ELISPOT assay, its use has allowed me to assess the Th1/Th2 nature of anti-P815 immune response in some mice with progressively growing P815 tumors and in some mice rejecting the tumor. As shown in **Figure 10**, progressive tumor growth is associated with a mixed INFγ/IL-4 response, while protected mice mount a predominant, sustained INFγ response to P815 antigens.
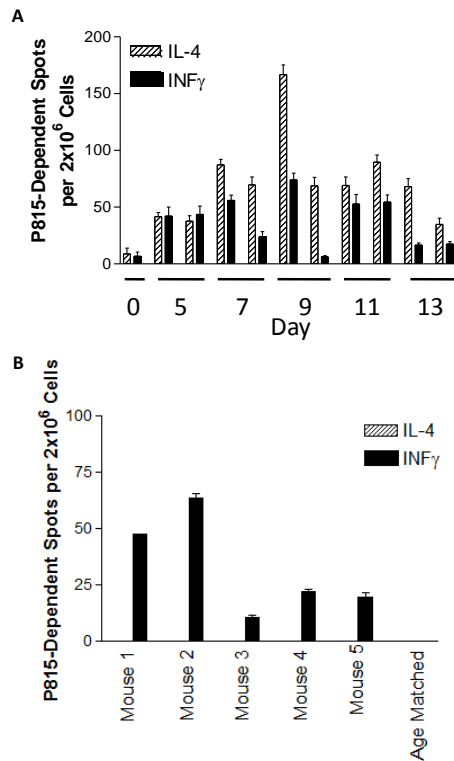
Figure 10: Lymphocytes from mice rendered resistant to P815 secrete INFγ, while mice suffering from progressive tumor growth secrete mixed IL-4 and INFγ cytokines in response to tumor antigens.

A) Mice were implanted with $2\text{x}10^6$ P815 cells intradermally on Day 0. Two mice were assayed at each time point for enumeration of tumor-specific INFγ and IL-4 producing cells by ELISPOT. **B)** Mice were rendered resistant to P815 challenge by priming mice with 10^5 P815 cells into their hind footpad, and challenged 14 days later with 10^5 P815 cells intradermally into their belly. The immune response was assessed 30 days post challenge. Results are presented as tumor-dependent INFγ or IL-4 spots per $2\text{x}10^6$ immune splenocytes.

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4.4.1.1 *Surface marker phenotype of P815-dependent INFγ and IL-4 secreting cells*

To determine the cells responsible for P815-dependent spot formation in the ELISPOT assay, we used MACS sorting to purify Thy1.2+, CD8+ and CD4+ cells. Purity of the respective populations was assessed by flow cytometry, and determined to be greater than 90% (**Figure 11 A**). We observed, using these purified cells in our ELISPOT assay that cytokine-producing cells were predominately Thy1.2 positive cells. IL-4 was secreted by CD4+ cells, while most INFγ secreting cells were CD8+ T cells (**Figure 11 B and C**).

Figure 11: Phenotype of cells producing P815-dependent INFγ and IL-4 spots in the ELISPOT assay.

A) Assessment of the purity of spleen populations obtained utilizing midiMACS as per manufacturer's standard protocol for Thy1.2+, CD8+ and CD4+ T cells. B) Mice were rendered resistant to P815 challenge by first priming 4 months prior with 10^5 P815 cells into their hind footpads, and challenged intradermally with 2×10^6 P815 cells into their belly. Ten days later, three mice were killed and their spleens were harvested, pooled and used to assess the phenotype of cells responsible for spot formation indicating INFγ and IL-4 cytokine producing cells. C) Three mice were given 10^6 P815 cells intradermally into their belly 10 days prior to sacrifice and their spleen cells were harvested and pooled. The spleen populations were fractionated using midi-Macs for Thy1.2+, CD4+ and CD8+ positive and negative ELISPOT assay. Figure is representative of two independent experiments.
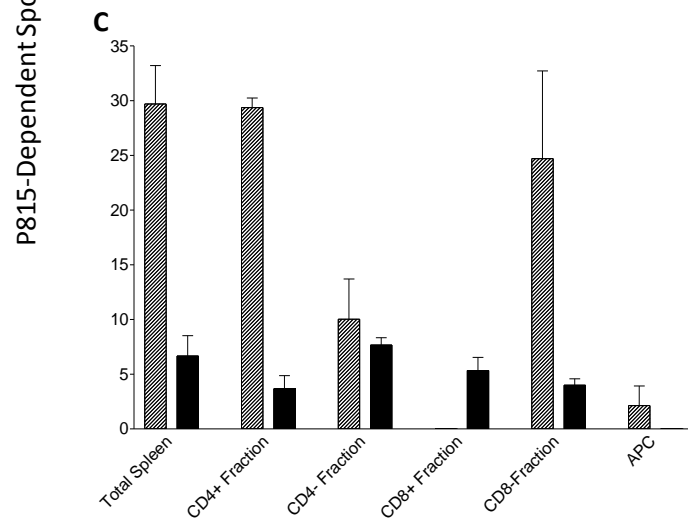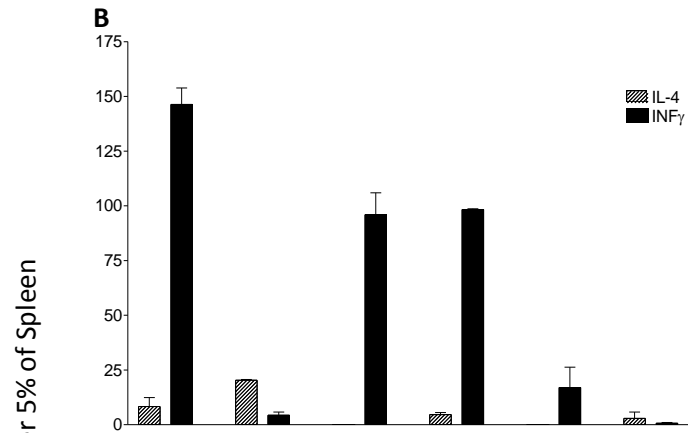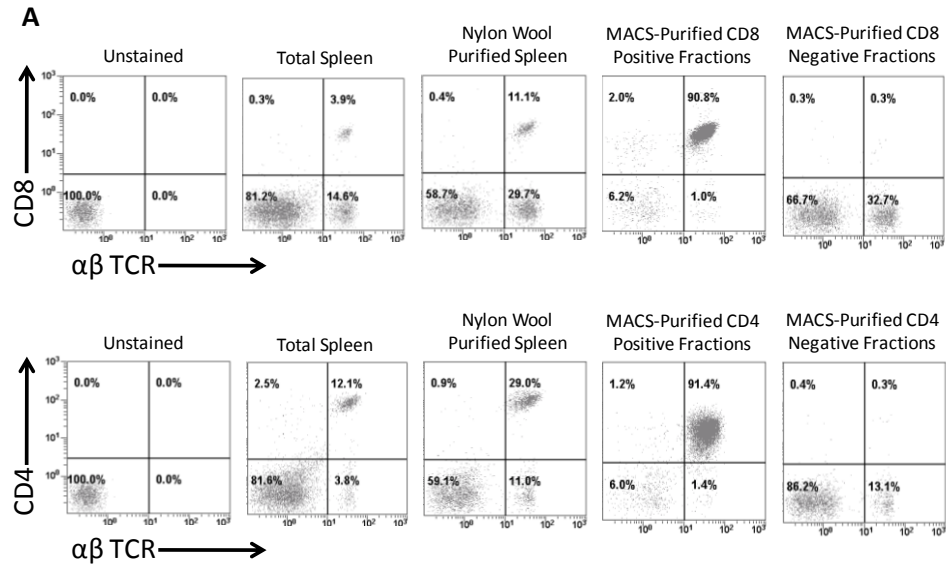
4.4.2 Correlates of protection in the L5178Y lymphoma system

The variability in the detection of P815-dependent cytokine secreting cells are not mirrored in the L5178Y system. When we first received the L5178Y cell line from Dr. R.J. North, we observed some initial difficulties in inducing progressive tumor growth. Initially, the majority of mice given what was reported to be a normally lethal tumor challenge, 10^6 cells implanted i.d., spontaneously resolved their tumors. I was able to increase the tumorogenicity of this cell line by passaging the cells *in-vivo* a number of times. The initial inability to reliably establish progressive tumor growth was a set back, but allowed us to compare the Th1/Th2 nature of the immunity being generated in mice challenged with the same tumor dose but spontaneously rejecting the tumor or suffering from progressive tumor growth. DBA mice were challenged with 10^6 L5178Y tumor cells i.d. thirty-three days after implantation 9 out of 12 mice had undergone complete tumor regression. An ELISPOT assay was performed to compare the immune responses between these two groups of mice. The results essentially mirror those obtained using the P815 tumor. A significant predominance of IL-4 was observed in mice suffering from progressive tumor growth, while INFγ dominates the immune response in mice that had successfully contained the tumor (**Figure 12**). As with the P815 system we assessed the cell populations responsible for the cytokines produced in response to tumor antigens.
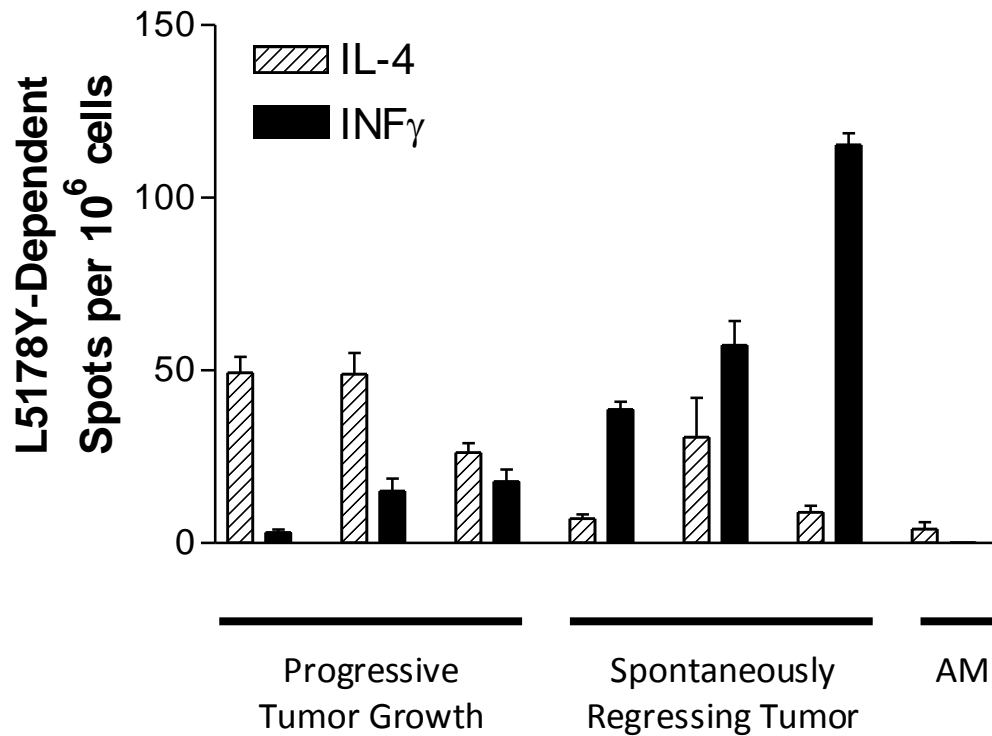
Figure 12: Resistance to the L5178Y tumor is associated with antigen-dependent INFγ production while tumor progression is associated with IL-4 production.

DBA mice were challenged with 10^6 L5178Y tumor cells intradermally into their belly. Three of twenty mice developed progressive tumor growth. We assessed 33 days post-challenge the types of anti-tumor immune responses associated with mice suffering from progressive tumor growth and in those mice that rejected the tumor. AM refers to aged matched control.

As shown in **Figure 13**, IL-4 is produced by CD4+ T cells along with non-T cells, while the INFγ is produced by both CD4+ and CD8+ T cells in response to L5178Y antigens.

4.5   Modulation of MHC Expression on P815 and L5178Y tumor cells

We have been considering the role of CD4+ T cells as an indirect marker of the critical effector cells that attack the tumor, assuming they themselves have no direct effect on the tumor. They could act directly on tumors if the tumors bore class II MHC expression. We therefore assessed the expression of Class I and II MHC molecules on the surface of the P815 and L5178Y cell lines. **Figure 14** demonstrates that neither P815 nor L5178Y cell lines express MHC class II molecules on their surface. Furthermore, we were unable to induce MHC class II expression on either tumor after treatment with INFγ for 24 hours in culture.
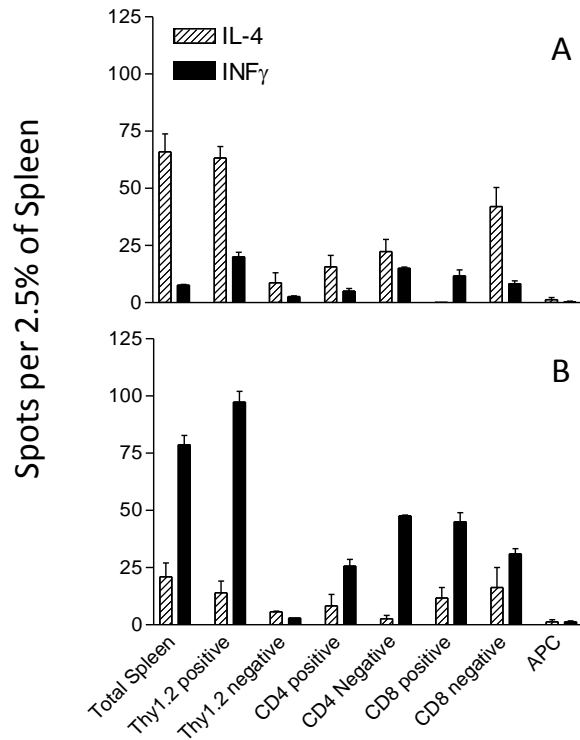
Figure 13: Phenotype of tumor-specific cells producing INFγ and IL-4 cytokines from mice rendered resistant to, or suffering progressive, L5178Y tumor growth.

A) Mice suffering progressive L5178Y tumor growth, after being implanted with 10^6 L5178Y cells intradermally 19 days prior harvest. B) Mice rendered resistant to L5178Y tumor growth after priming intradermally with 10^3 L5178Y cells 6 months, and being challenged 1 month prior to the assay with 10^6 L5178Y cells intradermally. The spleen preparation was separated into different cell populations using midi-Macs. IL-4 and INFγ secretion by the resulting fractions was assessed by the ELISPOT assay. Figure is representative of two independent experiments.
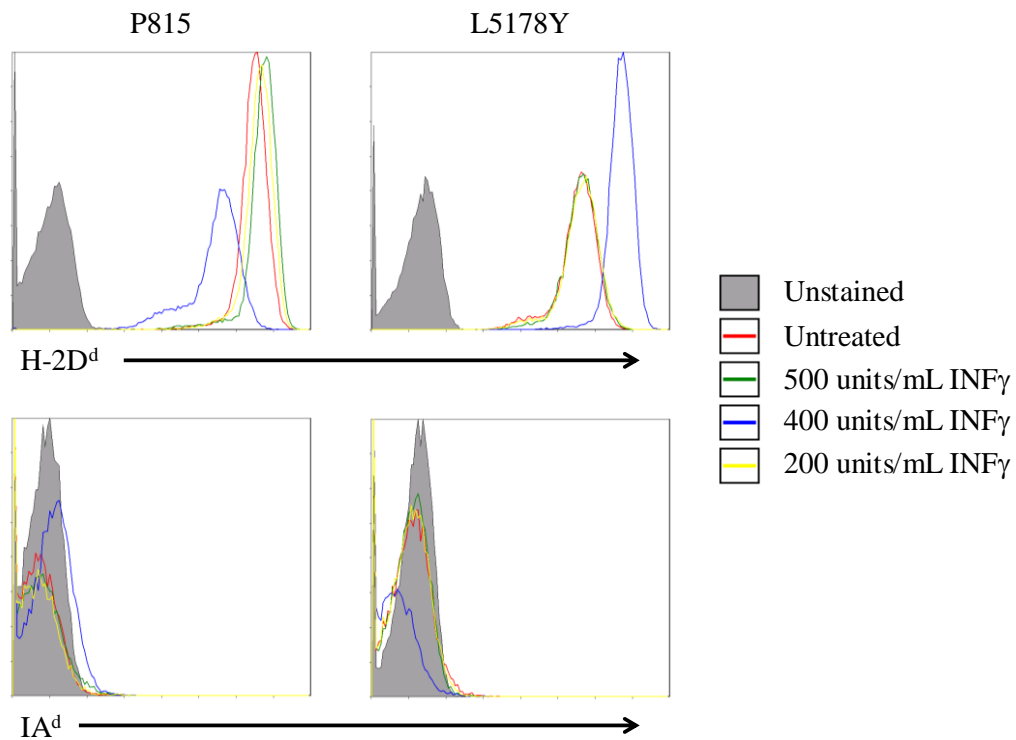
Figure 14: Effect of INFγ treatment on MHC expression by P815 and L5178Y cells

P815 or L5178Y cells were incubated at a cell density of $2x10^6$ cells per well in 24 well plates, and treated for 24 hours with either: 0, 200, 400 or 500 units/ml of recombinant INFγ. Cells were harvested, and were assessed for MHC class I and II expression by flow cytometry. Shaded areas represent the background fluorescence of unstained cells.

4.6   Correlation of tumor-specific antibody subclass with tumor protection

Our hypothesis and results obtained using the ELISPOT assay strongly suggest the Th1/Th2 nature of the anti-tumor immune response correlates with tumor protection or progression. One goal of this line of research is to devise strategies by which one can assess the Th1/Th2 nature of spontaneous anti-tumor immune responses in human cancers, and use such observations to guide cancer treatment. In human cancers the primary source of lymphocytes available are from the peripheral blood. One can question if the Th1/Th2 nature of the peripheral response is characteristic of the true 'systemic' immune response seen in a secondary lymphoid tissue. Using mouse models, it is relatively easy to directly compare the Th1/Th2 nature of antigen-dependent immune responses in the spleen and peripheral blood using the ELISPOT assay. It is however likely our tumor systems are not a useful model in which to test such a hypothesis as it is quite apparent by microscopic analysis that there are many tumor cells circulating in the blood of animals bearing intradermal tumors. Early in my graduate studies, I had done a considerable amount of work using the intracellular parasite, *Leishmania major* (MHOM strain). Using such an experimental model I was able to directly compare the Th1/Th2 nature of the anti-parasite immune response being generated in the spleen as compared to lymphocytes isolated from the peripheral blood. The CBA/J strain of mice is known to be very resistant to infection with

Leishmania major; in fact, mice given a very high dose (3x10^8) of parasites into their hind foot pad show no clinical signs of disease other than some mild footpad swelling which spontaneously resolves. Such resistance correlates with predominant INFγ secretion by spleen cells in response to parasite antigens. However, when we characterize the Th1/Th2 immunity utilizing cells harvested from the peripheral blood from these same animals we observe an exaggeration in the Th2 component of the immune response (**Figure 15**). If such observations are mirrored in humans utilizing cells isolated from the peripheral blood to assess the Th1/Th2 nature of immunity may prove to be misleading. In this regard I began to assess if other methodologies could be utilized to characterize the Th1/Th2 nature of the anti-tumor immune response.

Figure 15: Exaggeration of the Th2 component of the antigen-dependent immune response in the peripheral blood as compared to the spleen.

Groups of CBA/J mice were infected with varying numbers of *Leishmania major* promastigotes on day 0. Four months post infection mice were bled, and their spleens were harvested and immune responses assessed by the ELISPOT assay. A) The number of antigen-dependent INFγ and IL-4 secreting cells in the spleen (upper panel) and peripheral blood (middle panel). B) The ratio of *Leishmania major-*dependent INFγ to IL-4 secreting cells from spleen and peripheral blood leukocytes is depicted.
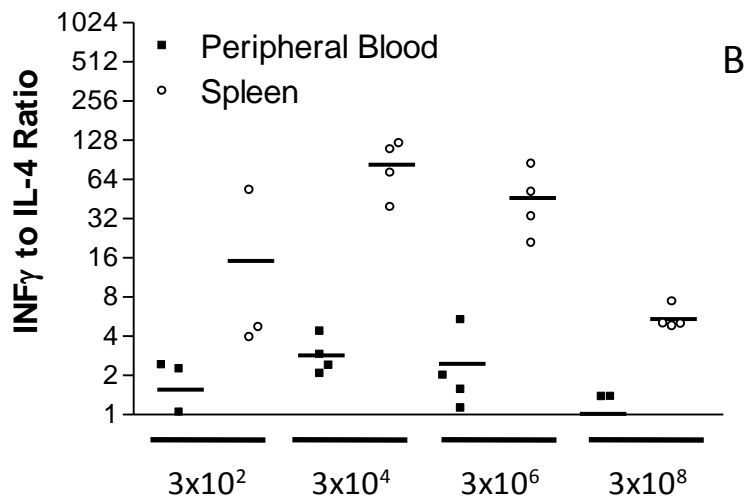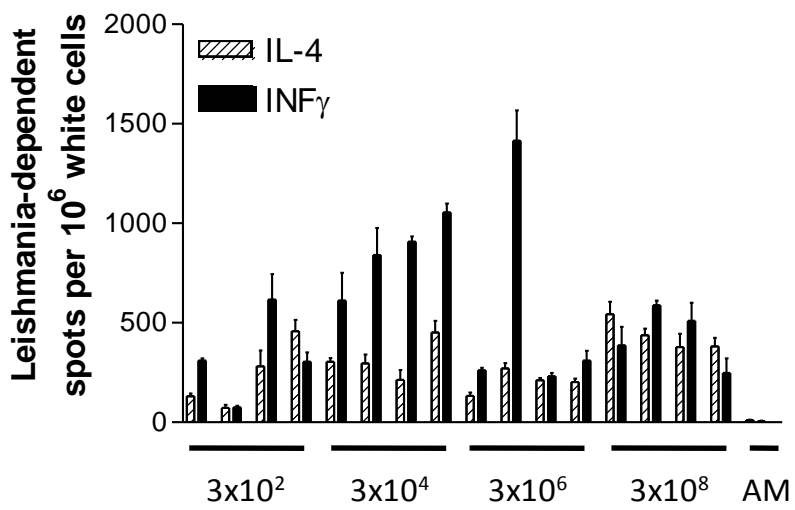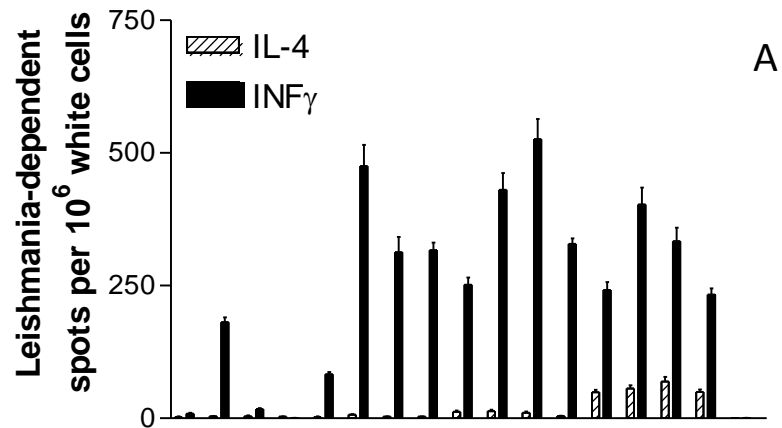
In the mouse models of *Mycobacterium bovis* (BCG strain), and *Leishmania major* our laboratory has previously demonstrated the Th1/Th2 nature of the immune response can be assessed by comparing the relative abundance of pathogen-specific IgG1 and IgG2a antibodies in sera. High amounts of IgG1 antibodies correlate with a response with a large Th2 component, while IgG2a antibodies are associated with a predominant Th1 response (Chen, Gure et al. 1998; Bretscher, Ismail et al. 2001; Uzonna and Bretscher 2001). Based on these observations, I have devised protocols to assess the relative abundance of tumor-specific IgG1 and IgG2a antibodies as a measure of the Th1/Th2 nature of our anti-tumor immune response, and assessed whether there is a correlation of prevalent IgG isotypes with regards to tumor outcomes. I believe such a strategy, if realizable, may prove itself to be very useful in a clinical setting.

4.7 Correlation of tumor-specific antibody subclass with tumor resistance or tumor progression

I have developed a western blotting protocol to determine the predominance of tumor-specific IgG1 and IgG2a antibodies in the sera of animals bearing P815 and L5178Y tumors. Exploiting this western blotting strategy I have been able to identify clear correlates of tumor protection or regression by assessing the relative abundance of IgG1 and IgG2a antibodies to tumor antigens.

It is important to note that the variability with regards to detecting P815-dependent cytokine producing cells in the ELISPOT assay is not mirrored by

variability in our western blotting assay. This simple protocol very consistently detects anti-P815 antibodies beyond those present in P815 non-exposed mice. I compared the relative abundance of tumor-specific IgG1 and IgG2a antibodies in mice rendered resistant as compared to mice suffering from progressive P815 tumor growth. Mice rendered resistant have a predominance of P815-specific IgG2a antibodies, while mice suffering progressive P815 tumor growth have a predominance of tumor-specific IgG1 antibodies (**Figures 16, 17 and 18**). These observations correlate with the cytokine producing cells data obtained utilizing the ELISPOT assay.

Using this western blotting methodology, we were able to demonstrate that the priming strategy, which renders DBA/2J mice resistant to a normally lethal tumor challenge, induces a stable Th1 response to tumor antigens. While rare in occurrence, our priming strategy occasionally results in progressive tumor growth in the footpad. We were able to demonstrate such progressive tumor growth correlates with the development of tumor-specific IgG1 antibodies (**Figure 15B**).

Groups of mice implanted with the same lethal dose of P815 cells do not always have the same rate of tumor growth. Using the western blotting protocol we have been able to longitudinally indirectly assess the Th1/Th2 nature of the immune response being generated in animals in which the rate of P815 growth is markedly different. As shown in **Figure 18**, one can observe an increase in the

relative amount of IgG2a antibodies in the animal with a more slowly progressing

P815 tumor as compared to a mouse with a P815 tumor progressing more rapidly.

Figure 16: Effective protection against P815 is associated with predominant IgG2a antibody production against the tumor.

10^5 P815 cells were implanted into the hind foot pad of DBA mice on Day 0. Mice were serially bled over the course of 28 days, and the relative abundance of P815-specific IgG1 and IgG2a antibodies were assessed by western blot. **Panel A** represents a mouse in which there was no evident tumor growth in the foot pad, and which resisted a subsequent normally lethal P815 tumor challenge on Day 28. **Panel B** represents a rare occasion in which the mouse suffers from progressive P815 growth in its footpad.
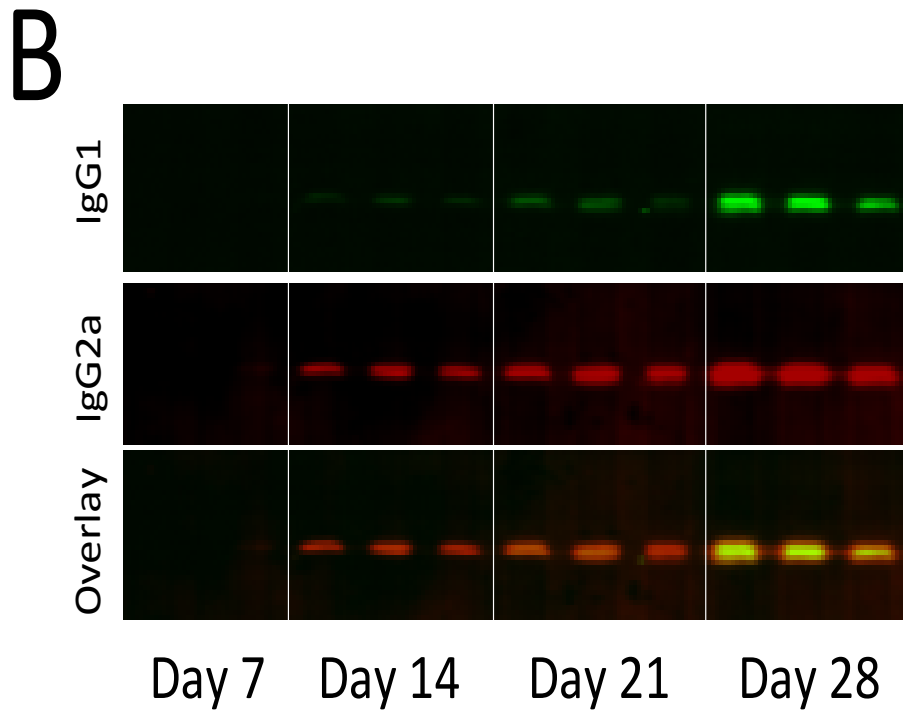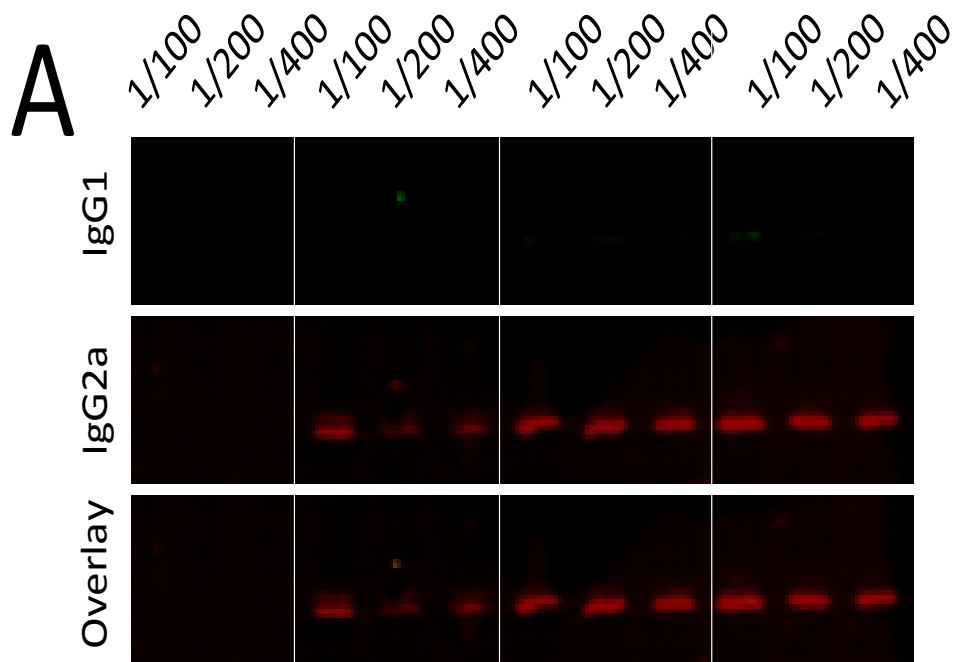
Figure 17: Mice rendered resistant to a normally lethal dose of P815 is associated with predominant Th1-type immunity, as assessed by western blot.

Mice were implanted with 10^5 P815 cells (s.c.) into their hind footpad on Day 0, and challenged with 10^5 P815 (i.d.) into their belly on Day 35. Mice were bled weekly, and the relative abundance of P815-specific antibodies was assessed by western blot with dilution of sera of 1/100, 1/200 and 1/400. Similar experiments have been performed on greater than 40 individual mice with animals falling into one of the three representative groups depicted. The vast majorities of mice are successfully vaccinated and are represented in the top panel showing an initial decrease in the abundance of antibodies, and then a sustained predominance of tumor-specific IgG2a antibodies. The middle panel represents animals with very little or no detectable antibodies. Although more rare in occurrence, it could possibly demonstrate a very strong cell mediated immunity in the absence of detectable tumor-specific antibodies. The bottom panel is the rarest of the three occurrences, where the tumor initially grows out after challenge, and subsequently resolves. This initial outgrowth is associated with tumor-specific IgG1 antibodies, and subsequent resolution correlates with a disappearance of these IgG1, and maintenance of IgG2a antibodies specific for tumor antigens.
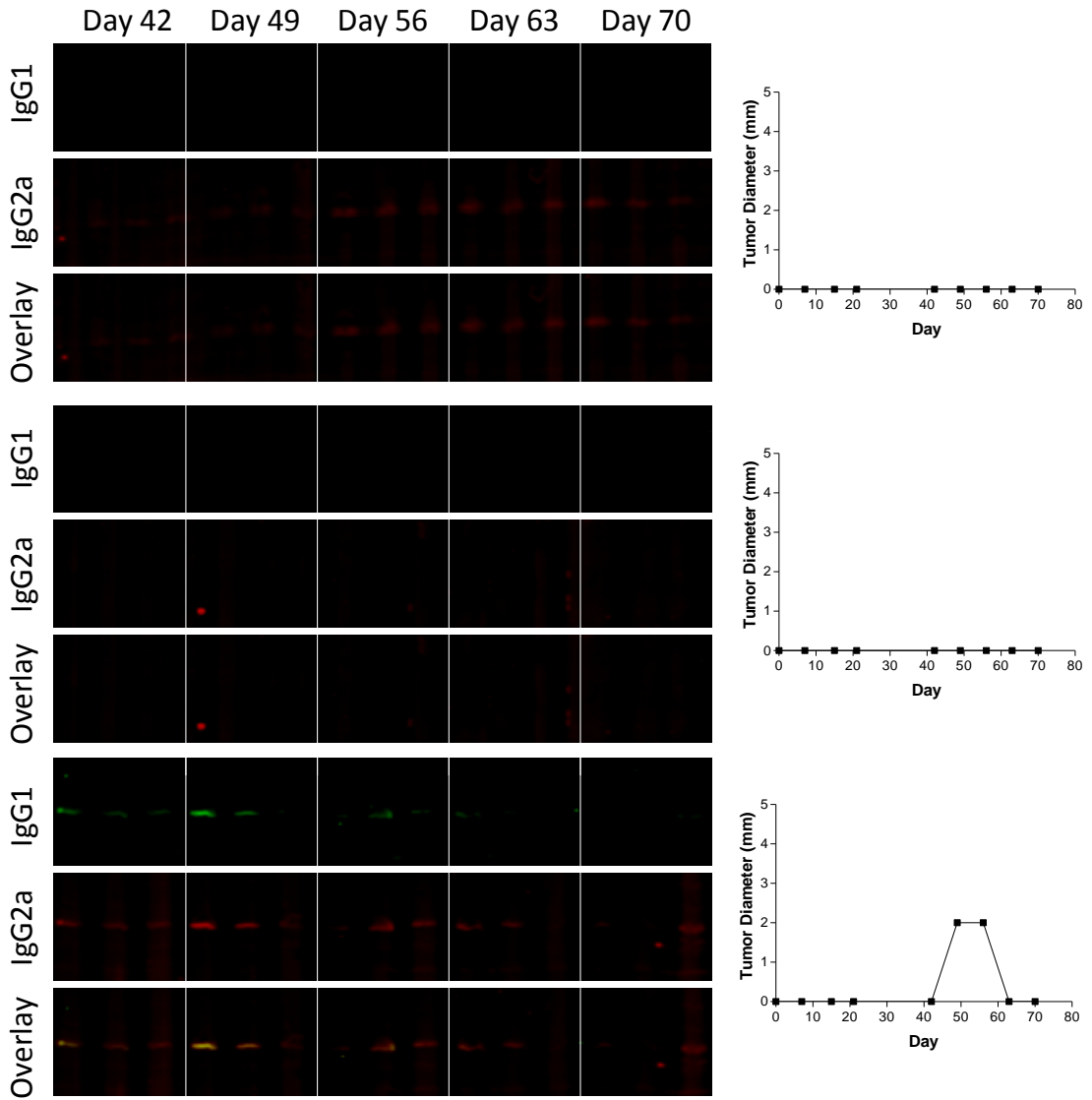
Figure 18: Differing rates of P815 growth is associated with differing relative abundance of tumor-specific IgG1 and IgG2a antibodies.

DBA mice were implanted with 10^5 P815 intradermally into their abdomen on Day 0. The rate of tumor growth was assessed as was the relative abundance of tumor-specific IgG1 and IgG2a antibodies by western blot. As assessed with serum diluted 100 fold. Although individual mice are depicted, the results are indicative of greater than ten mice.

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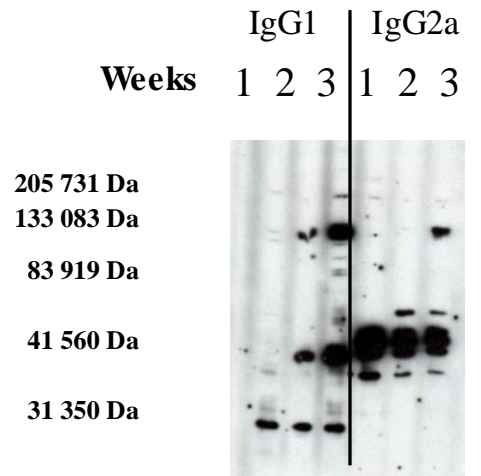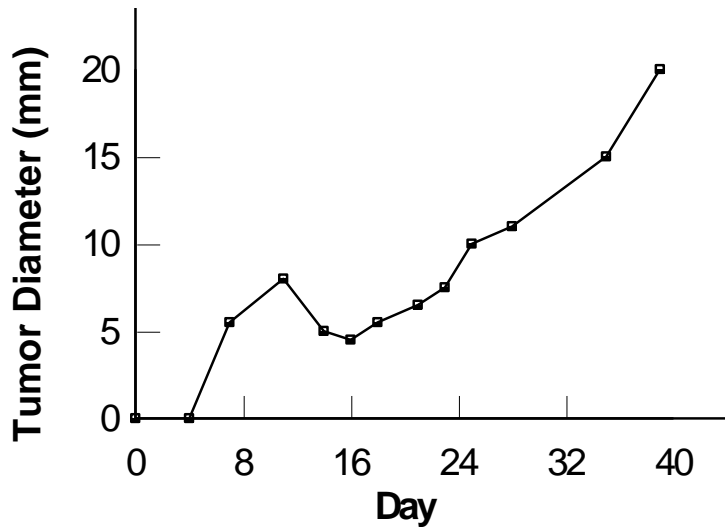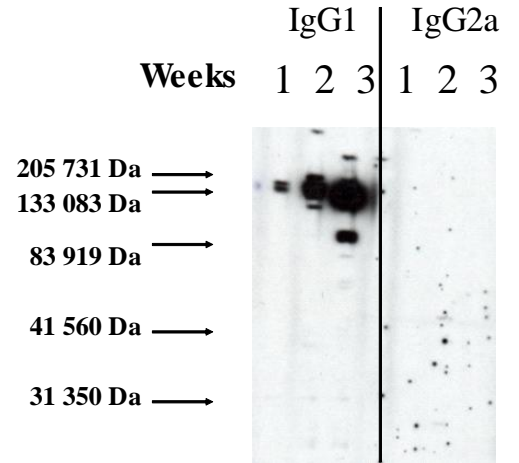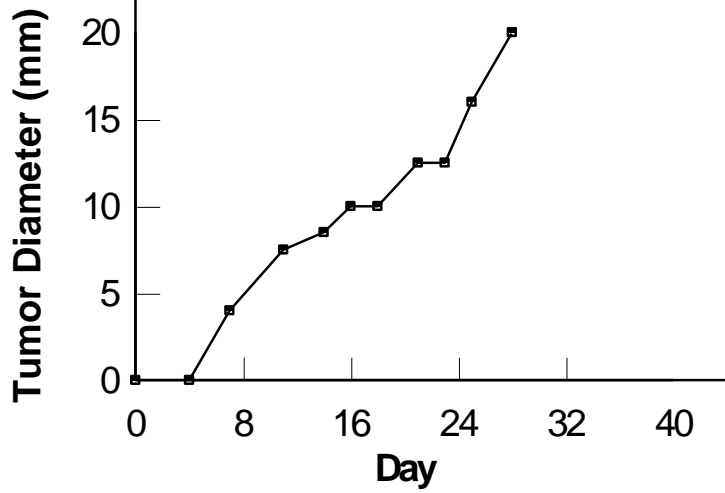
At present, there is no technique by which one can predict the reemergence of a cancer. The observations presented within this thesis suggest tumor reoccurrence might often be associated with the development of Th2 responses against tumor antigens. In this light one could utilize this western blotting strategy to assess this immune failure, and perhaps predict the reemergence of a tumor before this becomes apparent in terms of a mouse's health. In a very rare occurrence, I observed the spontaneous regression of a P815 tumor in a mouse implanted with a normally lethal dose of the tumor. This mouse showed no signs of tumor for 10 days, but the tumor reemerged at the site of implantation and grew progressively. The mouse was bled over the 10 week course of tumor growth, and the Th1/Th2 nature of the anti-tumor immune response was indirectly assessed by our western blotting protocol. I was able to demonstrate that regression of the primary tumor is associated with the predominance of IgG2a antibodies to the tumor antigen. Tumor reemergence and subsequent progressive growth is associated with a very dramatic switch from a predominant IgG2a response to a mixed IgG1/IgG2a, and later to a predominant IgG1 response (**Figure 19**). Although this represents analysis of a single mouse undergoing a very uncommon protracted rate of P815 tumor growth, it very clearly demonstrates the utility of our western blotting method to assess immune failure.

Figure 19: Regression of P815 and subsequent progressive P815 tumor growth is associated with a switch in the relative predominance of tumor-specific IgG1 and IgG2a antibodies.

The mouse was implanted with 10^5 P815 cells on Day 0. The P815 tumor spontaneously resolved, and subsequently reappeared and grew progressively at the site of injection. The mouse was bled over a period of 10 weeks and the relative predominance of P815-specific IgG1 and IgG2a antibodies was assessed by western blot.
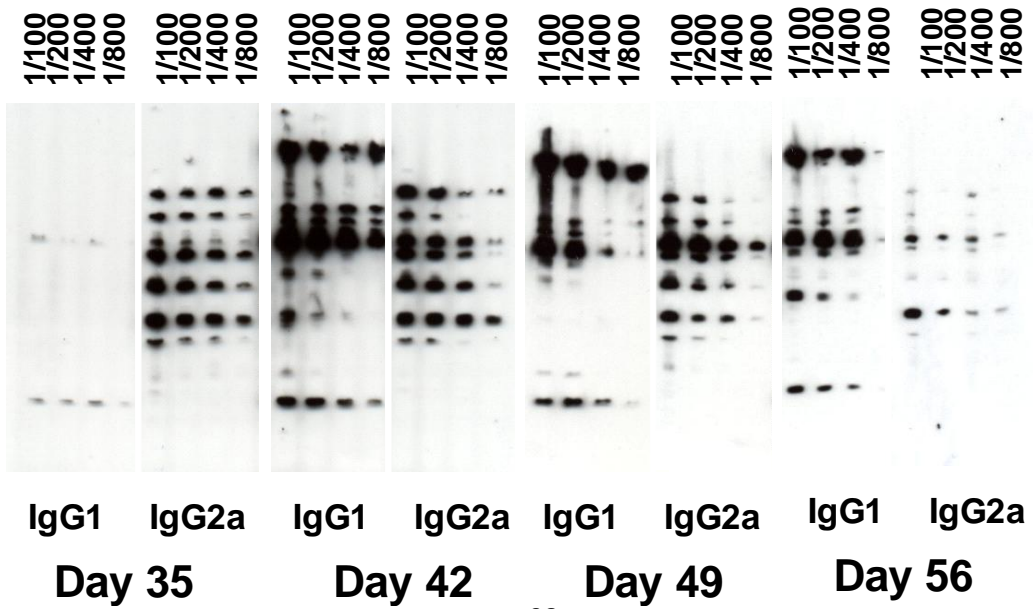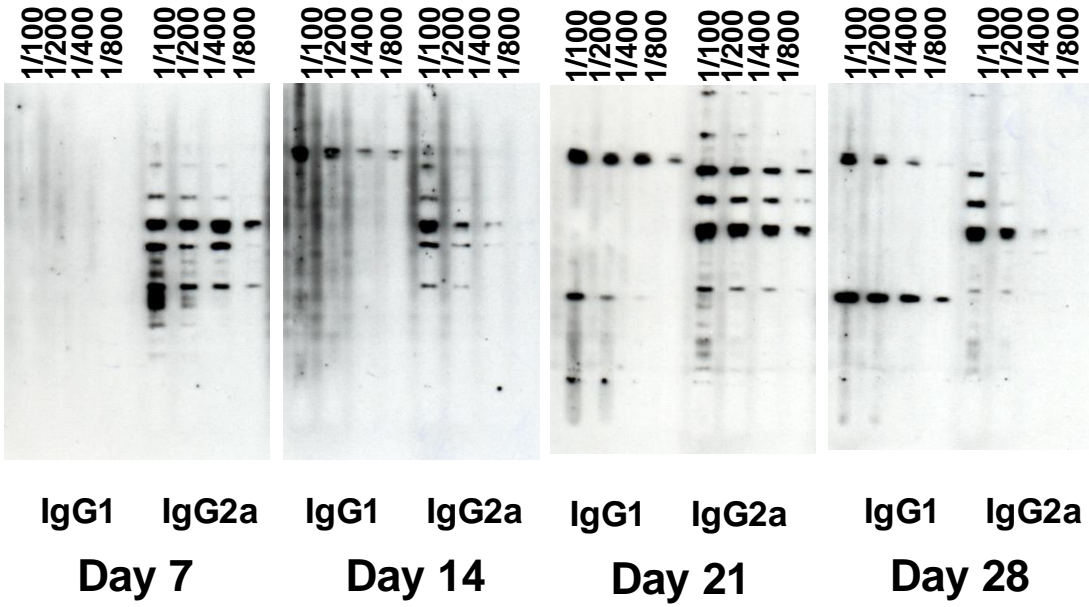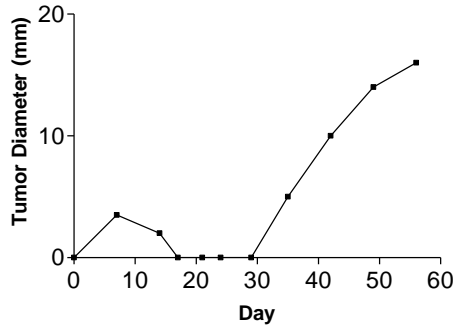
4.8 Correlation of L5178Y-specific antibody subclass and tumor progression and regression

I failed in my attempts to devise an EIA strategy to detect anti-P815 antibodies; however, I have been able to develop an EIA protocol to assess the relative abundance of L5178Y-specific IgG1 and IgG2a antibodies. I believe my ability to devise an EIA protocol for the detection of L5178Y-specific antibodies, and inability for the detection of P815-specific antibodies is due to the fact that antibodies generated in response to the P815 tumor antigens are directed against soluble proteins, whereas mice bearing L5178Y tumors generate antibodies against both soluble and insoluble L5178Y proteins **(Figure 20)**. As such, some of the tumor associated L5178Y antigens may simply bind plastic better than those from P815.
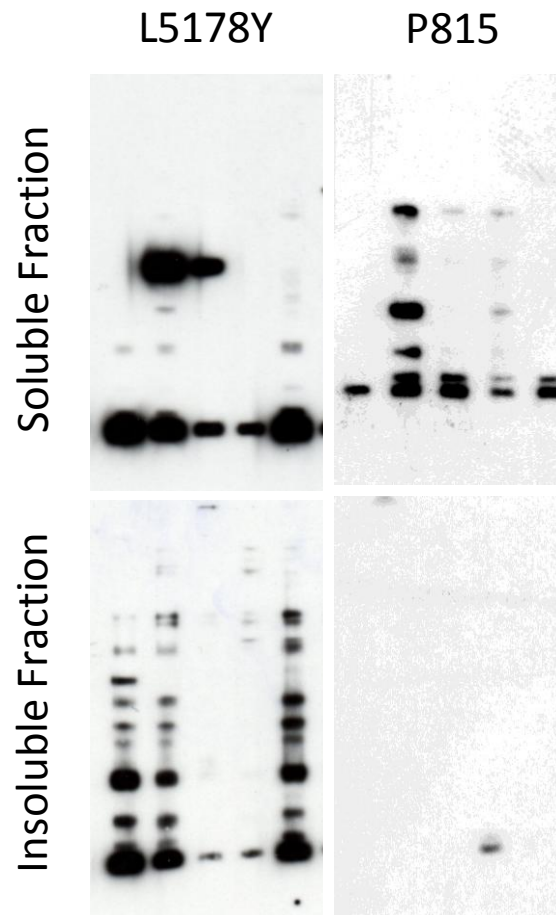
Figure 20: The ability of antibodies from P815 or L5178Y tumor bearing mice to recognize soluble and insoluble components of their respective tumors.

Groups of five mice were primed with either 10^6 L5178Y or 10^5 P815 cells intradermally were bled three weeks later. The ability of tumor-specific antibodies to recognize the soluble or insoluble components of their respective tumors was assessed by western blot. Carried out with sera diluted 100 fold.

Utilizing this EIA technique to assess the relative abundance of L5178Y-specific IgG1 and IgG2a antibodies, I attempted to ascertain if the same correlates we observed with the P815 tumor also apply to mice bearing an L5178Y tumor. As previously discussed, there are instances where mice within the same experimental group either suffer progressive L5178Y tumor growth, or spontaneously reject the tumor. As shown in **Figure 21**, our EIA protocol has allowed us to demonstrate a clear correlation between a predominance of IgG2a antibodies with tumor regression, and between a mixed IgG1/IgG2a or a predominant IgG1 antibody response to L5178Y antigens and tumor progression. These results demonstrate the relatively dynamic nature of the anti-L5178Y immune response. The ratio of L5178Y-specific IgG2a to IgG1 antibodies can change over time. This ratio correlates well with the rate of tumor growth. Mice with spontaneously regressing tumors maintain a relatively high ratio of tumor-specific IgG2a:IgG1 antibodies, while in mice with progressively growing tumors this ratio can initially be high, but subsequently decrease as the tumor progressively grows. These results further suggest that one can utilize this strategy to monitor anti-tumor immune responses, and predict whether a tumor will be contained, or will grow progressively.
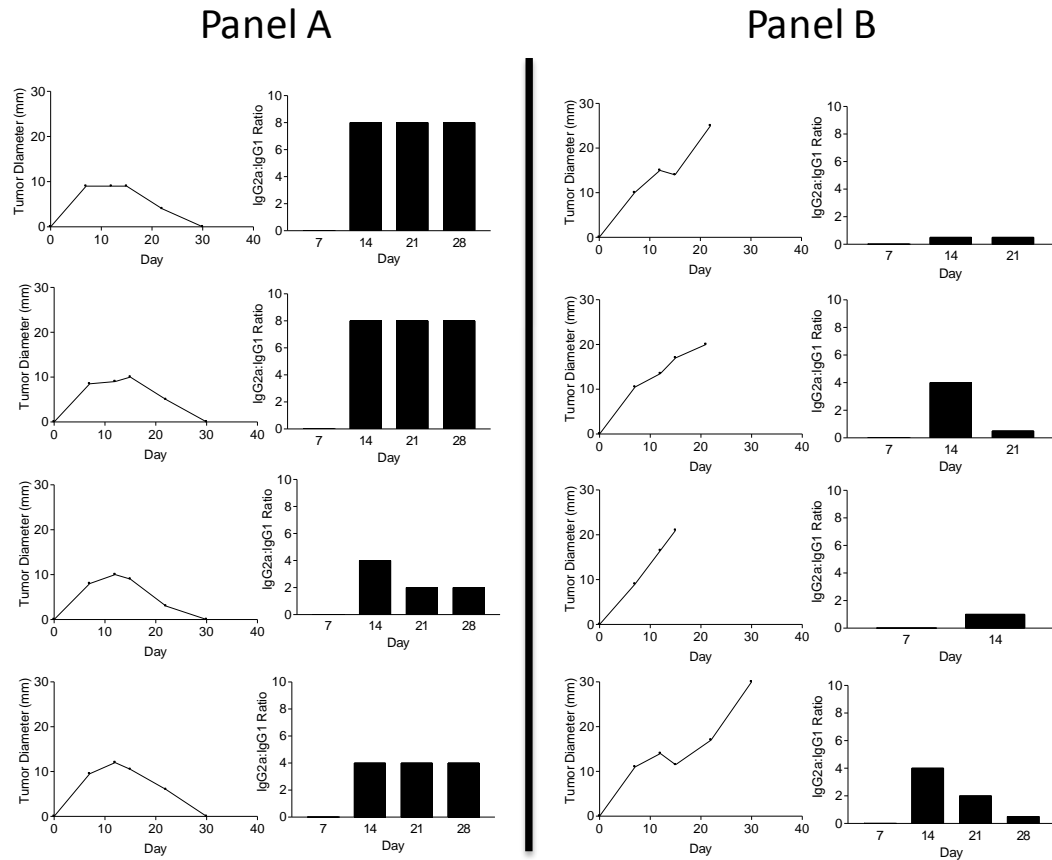
Figure 21: Relative abundance of tumor-specific IgG1 and IgG2a antibodies in mice with either progressive or spontaneously regressing L5178Y tumors.

DBA mice were implanted with $2x10^6$ L5178Y tumor cells on Day 0. Six out of twenty mice suffered from progressive tumor growth. Mice were serially bled over the course of tumor growth, and the ratio of L5178Y-specific IgG2a to IgG1 antibodies was assessed by EIA for four mice rejecting the tumor (Panel A) and four mice showing progressive tumor growth (Panel B). Naïve mice consistently have no detectable anti-L5178Y antibodies in their sera.

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4.9 Low doses of tumor correlate with the generation of protective immunity.

Our observations demonstrate that the generation of Th1 immunity against tumor antigens correlates with protection. In many experimental models the administration of low doses of protein antigens, complex non-replicating antigens, and slowly replicating microorganisms results in the generation of stable antigen-specific Th1 responses (Bretscher, Ismail et al. 2001). In view of this generalization, I gave decreasing doses of tumor antigens and assessed the effect on both tumor outcome, and correlated this outcome with the relative predominance of L5178Y-specific IgG2a and IgG1 antibodies. Mice were implanted with doses of L5178Y tumor ranging from 10^4 to 10^6 tumor cells. Mice given the high, and intermediate doses of tumor cells suffered progressive tumor growth, which correlates with the generation of anti-L5178Y IgG antibody with a low IgG2a:IgG1 ratio. Mice given low doses of L5178Y tumor spontaneously regressed their tumors, and were subsequently resistant to a normally lethal challenge of the L5178Y tumor. This protective anti-tumor immune response correlates with a high ratio of IgG2a:IgG1 antibodies against the L5178Y tumor (**Figure 22**).
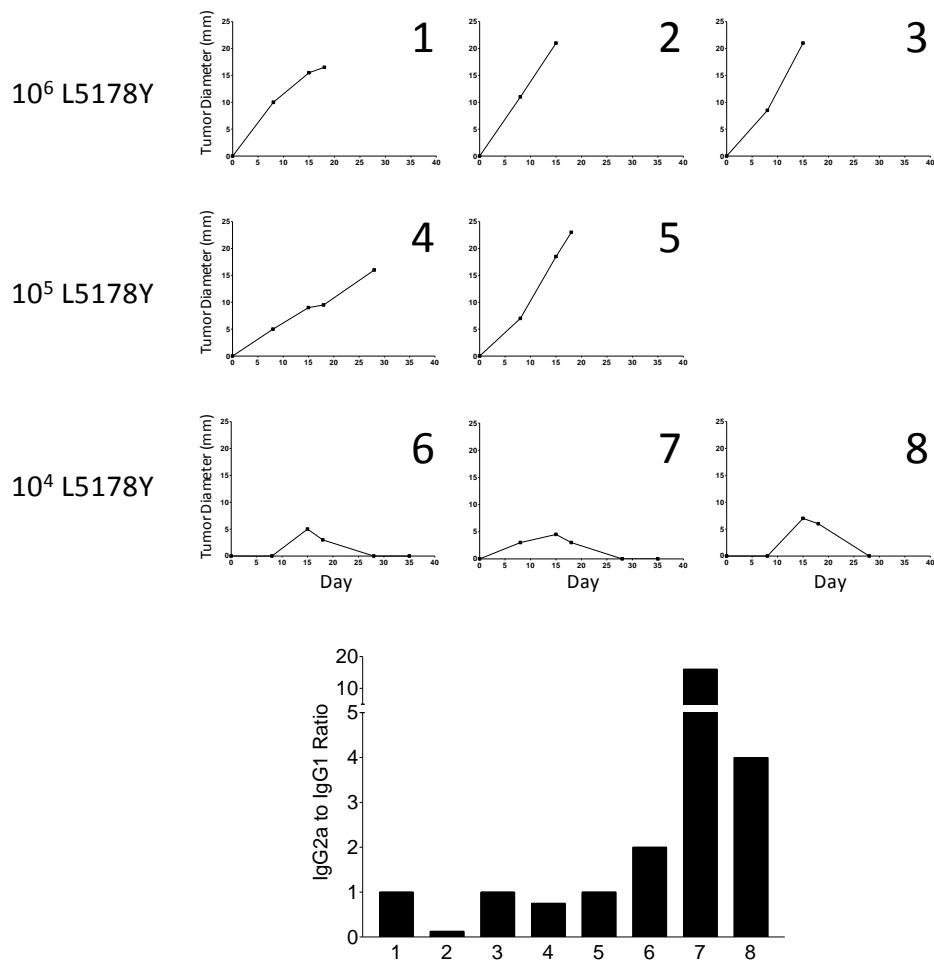
Figure 22: Administration of low doses of L5178Y tumor cells is associated with tumor regression, and an increase in the relative predominance of tumor-specific IgG2a antibodies.

Mice were implanted with varying doses of L5178Y cells intradermally into their belly on Day 0. The rate of tumor outgrowth was assessed, and the IgG2a:IgG1 ratio of L5178Y-specific antibodies was assessed by EIA at 14 days after tumor challenge.

I have also utilized the western blotting strategy to assess the relative predominance of L5178Y-specific IgG1 and IgG2a antibodies. These results obtained by western blot correlate very well to those obtained by EIA. Progressive growth of the L5178Y tumor is associated with a predominance of tumor-specific IgG1 antibodies. Our results also demonstrate an increase in the relative abundance of L5178Y-specific IgG2a antibodies in mice suffering from a more protracted rate of tumor growth (**Figures 23 and 24**). Mice undergoing spontaneous tumor regression have an initial mixed response to tumor antigens, which subsequently is replaced with a predominant IgG2a antibody response against L5178Y tumor (**Figure 25**).
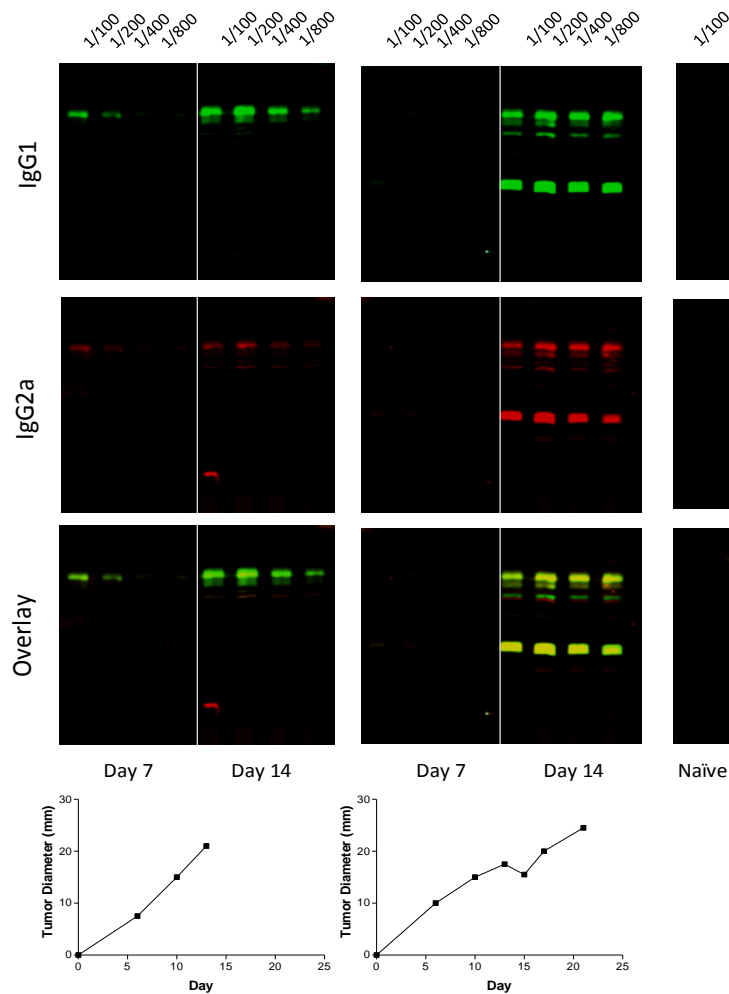
Figure 23: Rapid progression of the L5178Y tumor correlates with an increase in tumor-specific IgG1 antibodies as compared to the isotypes of antibodies present in the serum of animals with prolonged survival.

Mice were implanted with 1.2x10^6 L5178Y tumor cells intradermally on Day 0. The rate of tumor growth was assessed along with the development of tumor-specific IgG1 and IgG2a antibodies by western blot over the course of tumor growth.
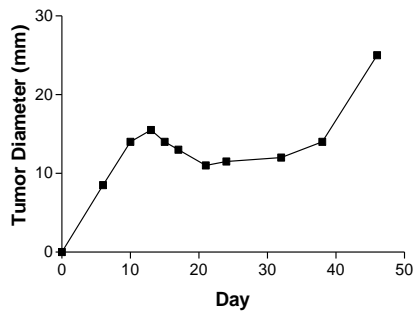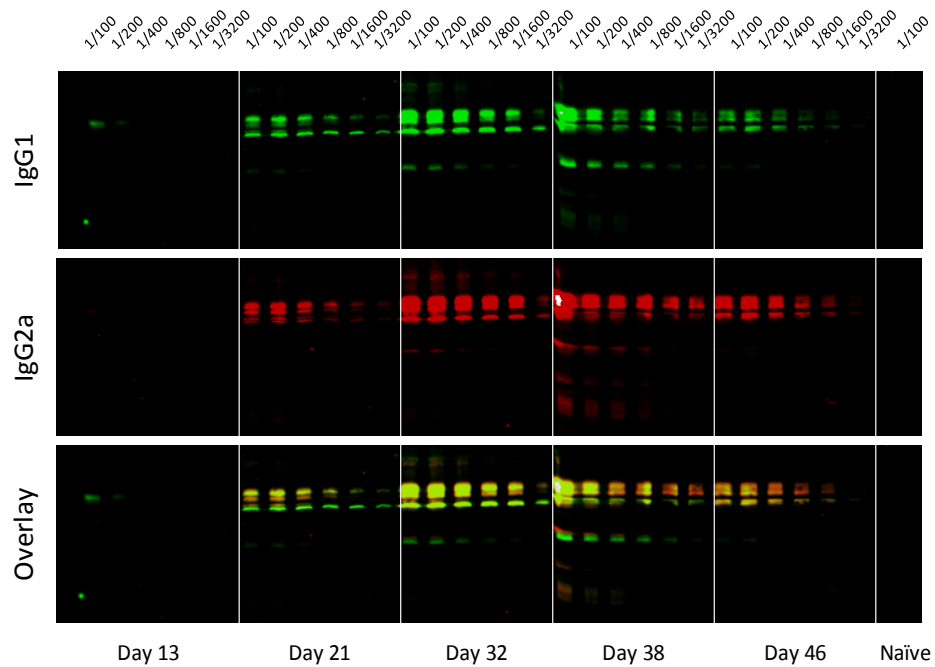
Figure 24: Prolonged L5178Y tumor growth is associated with an increase in tumor-specific IgG2a antibodies.

The mouse was implanted with 10^6 L5178Y tumor cells intradermally on Day 0. The rate of tumor growth was assessed along with the development of tumor-specific IgG1 and IgG2a antibodies by western blot over the course of tumor growth.

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Figure 25: Tumor regression is associated with prolonged predominance of L5178Y-specific IgG2a antibodies.

Mice were implanted with 10^3 L5178Y cells on Day 0. Mice were bled over the course of tumor growth, and the relative abundance of tumor-specific IgG1 and IgG2a antibodies were assessed by western blot. **A)** The delay of tumor outgrowth is associated with a predominance of tumor-specific IgG2a antibodies. Subsequent progressive tumor growth correlates with the development of tumor-specific IgG1 antibodies. **B)** Tumor regression is associated with a strong predominance of tumor-specific IgG2a antibodies is stable over time, while IgG1 antibodies to tumor antigens decrease over time.

A

IgG1

IgG2a

Overlay

Day 13   Day 19   Day 27

B

IgG1

IgG2a

Overlay

Day 13   Day 19   Day 27   Day 35   Day 42   Day 64

109
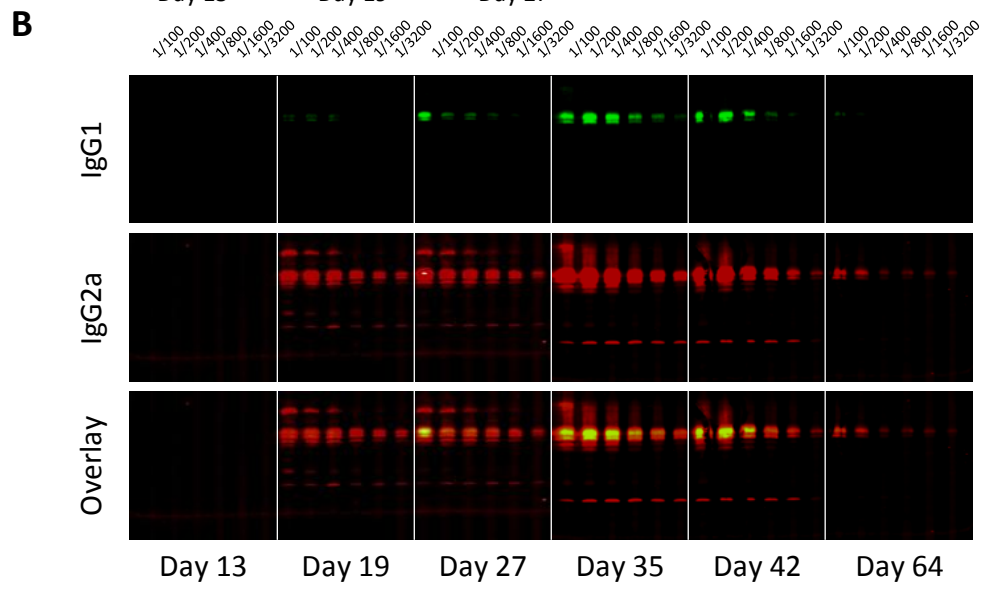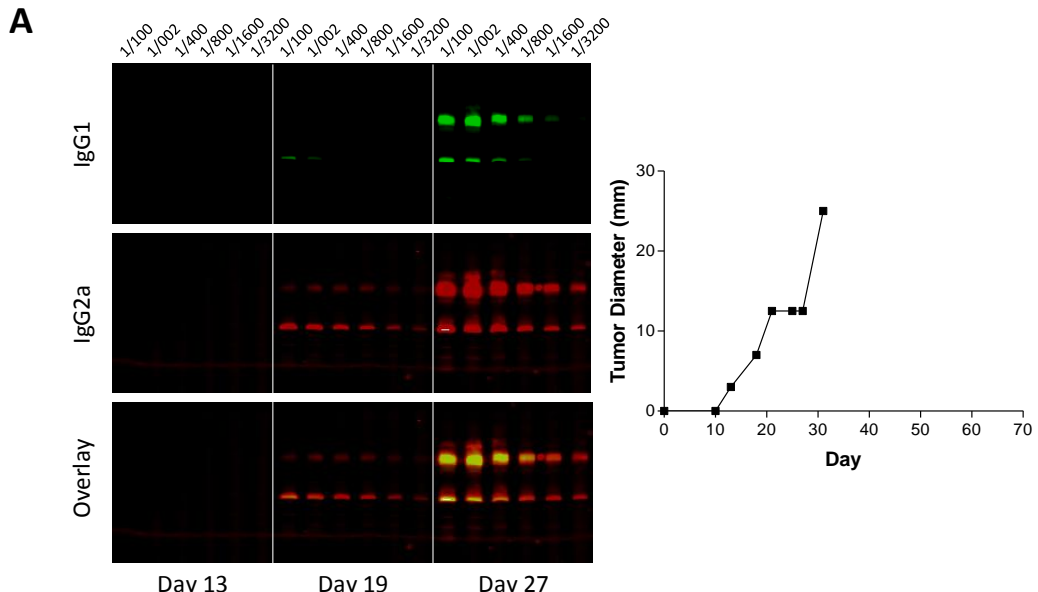
4.9.1 Exploiting tumor-specific antibody subclasses to define immune correlates of protection, and implications for human disease

There are obvious potential concerns when using tumor-specific antibodies rather than cytokine-secreting cells in defining immune correlates of protection. Tumor-specific antibody isotypes allow one to indirectly assess the Th1/Th2 nature of the systemic immune response. It is important to keep in mind that most subclasses of antibodies have a fairly long half life in the serum of mice. IgG, IgA, IgM, IgD and IgE subclasses have half lives of approximately 23, 5.5, 5, 2.8 and 2 days respectively in the serum of mice (Benjamini and Sunshine 1996). As such one must be aware that antibodies effectively give us an average view of the type of immunity present previously over the last few weeks. In contrast an analysis of cytokine producing cells at one time gives a snapshot picture. As most human cancers progress relatively slowly, such a hindrance may prove itself to be not important; however, it is more of an issue in the mouse models of cancer, which are selected to exhibit more rapid tumor regression/progression than would probably occur in a natural murine model of cancer.

Most mouse models of cancer utilize isogenic tumors which induce rapid tumor growth. We observe, utilizing rapidly progressing tumors, a clear correlation between progression and the development of tumor-specific IgG1 antibodies. In mice suffering from a more protracted course of tumor growth, we observe the presence of a large amount of tumor-specific IgG2a antibodies. Such correlates are useful, but often with such mixed responses we are unable to confidently predict

whether the tumor will spontaneously resolve, or subsequently progress. **Figure 25 A** depicts a situation in which the mouse suffers a rather protracted course of tumor growth. There is a period of roughly 10 days in which the animal is able to control the growth of the tumor. This transient control of tumor growth, as expected, correlates with a substantial amount of tumor-specific IgG2a antibodies; however, the tumor subsequently undergoes rapid tumor progression. Our western blotting protocol detected the development of tumor-specific IgG1 antibodies, but we also detected the development of IgG1 antibodies in **Figure 25 A**, where the tumor subsequently resolved. The critical difference appears to be that the IgG1 antibodies decrease over time, while tumor-specific IgG2a antibodies are sustained. Although the observations support our hypothesis we were unable to predict using western blots, at earlier time points, which of the tumors would ultimately resolve or progress. As most human cancers do not progress as rapidly, I believe very strongly that serial analysis of the Th1/Th2 nature of the anti-tumor immune response using this western blotting protocol may prove to be very useful in predicting the course of disease.

4.10 Common tumor antigens

As discussed in the introduction, it is widely accepted that many tumor cells of different etiologies share common antigens against which the immune system mounts a response. In this regard, we assessed if we could detect antigens shared by both L5178Y and P815 using our western blotting strategy. As shown in **Figure 26 A**, mice bearing a P815 tumor produce antibodies capable of recognizing L5178Y antigens which are not present in naïve sera. Conversely, similar cross-reactivity with P815 is seen with antibodies obtained from mice bearing a L5178Y tumor. To assess if such cross-reactivity is also present at the T cell level I assessed the ability of T cells from mice primed to either P815 or L5178Y tumors to produce cytokine when exposed to the other tumor in our *ex-vivo* ELISPOT assay. As shown in **Figure 26 B**, the cross-reactivity between the L5178Y and P815 tumors observed at the level of antibodies is not readily observed at the level of cytokine secretion. Such an observation is somewhat perplexing, and could perhaps be perceived as arguing against the possibility of a universal vaccine for the prevention of many common cancers, and may be responsible for the lack of cross-protection commonly observed between different tumor cell lines sharing common tumor-associated antigens (Prehn and Main 1957; Ramarathinam, Sarma et al. 1995). It could also be argued that the shared antigens apparent by western blot may be mirrored at the level of cytokine secretion but that the relative abundance, the

efficiency of presentation, and immune dominance of these common tumor antigens may differ significantly among the two cell lines.

There are grounds for optimism that a general strategy of vaccination against cancer may be realizable. Thierry Boon and his colleagues at the Ludwig Institute have discovered cross-reactive CTLs among patients sharing common HLA restriction elements (Skipper, Hendrickson et al. 1996). In light of these observations the prospect of a cancer vaccine remains a definite possibility.

Figure 26: Cross-reactivity of P815 and L5178Y tumor antigens as assessed by western blot is not complemented by cross-reactivity at the T cell level as assessed by the ELISPOT assay.

A) Four mice primed to either P815 or L5178Y tumors generate antibodies that cross-react with tumor-associated antigens expressed by the other tumor, and two naïve mice were used as controls. Sera was used at a dilution of 1:75 **B)** P815-primed mice were implanted with 10^5 P815 cells intradermally into their belly 14 days prior to assay. L5178Y-primed mice were implanted with 10^3 L5178Y cells intradermally into their belly, and were challenged 4 months later with 10^6 L5178Y cells. An assessment of crossreactivity in the ELISPOT assay was assessed one month post-challenge.

A

Priming *in-vivo*:

P815   L5178Y   Naïve   P815   L5178Y   Naïve

205 731 Da →
133 083 Da →
83 919 Da →

41 560 Da →

31 350 Da →

P815 Antigen   L5178Y Antigen

B

115
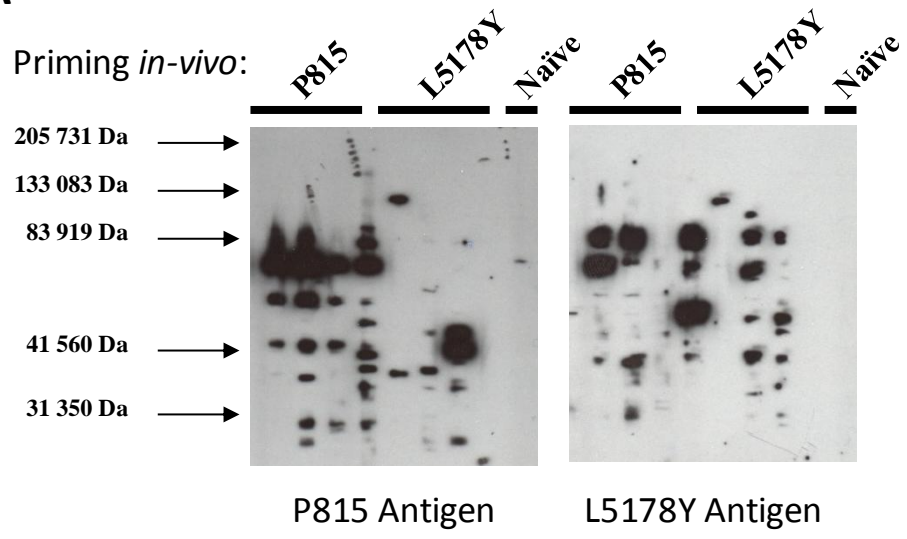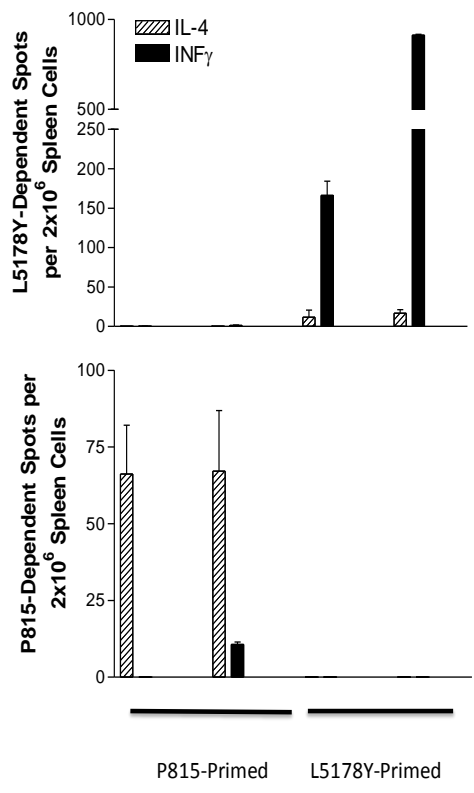
4.11 Transfer of P815 resistance to naïve animals.

We have examined the cell types required to transfer the protection we generated in P815 resistant mice to irradiated recipients. We can readily transfer protection using whole spleen cell preparations from mice rendered resistant. By depleting cell populations using midi MACS prior to transfer I have been able to demonstrate that protection is mediated by T cells, and that both CD4+ and CD8+ cells from primed mice are required to transfer this resistance (**Figure 27**). Although tumor-specific CTLs are most likely responsible for the direct killing of P815 cells such an observation is not entirely unexpected as it has been demonstrated in other systems that specific CD4+ T cells are required to maintain effective CD8+ T cell memory (Janssen, Lemmens et al. 2003; Shedlock and Shen 2003; Sun and Bevan 2003).

Figure 27: Characterization of the cell populations mediating protection against P815 tumor upon transfer into naïve, irradiated recipients.

Donor mice were rendered resistant to P815 by priming with 10^5 P815 cells into their hind footpad 2 weeks prior to challenge with $2x10^6$ P815 cells intradermally. Transfer was performed 2 months after challenge. Cells were nylon wool purified, and fractionated using midi-Macs (histogram inserts are a flow analysis of the cell populations transferred in each group). Recipient mice were given 850 rads of gamma-irradiation 24 hours prior to transfer. Recipient mice received an equivalent of 10^8 spleen cells from donor mice (iv), and were challenged 1 hour post-transfer with 10^6 P815 cells given intradermally into their belly. Tumor development was assessed.

Thy1.2 Depleted
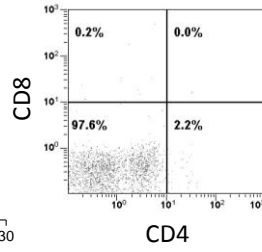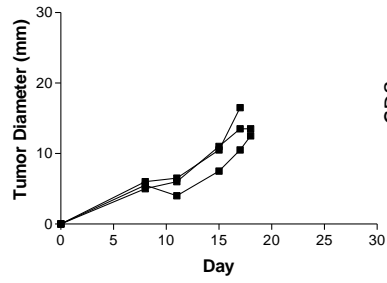
CD8 Depleted
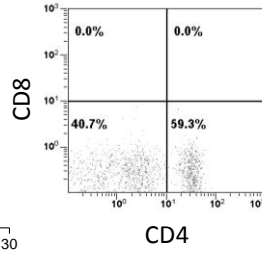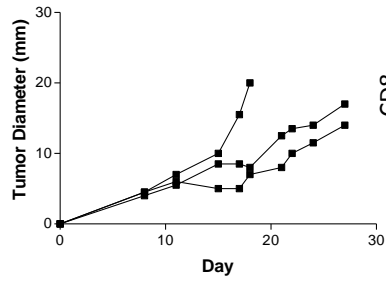
CD4 Depleted
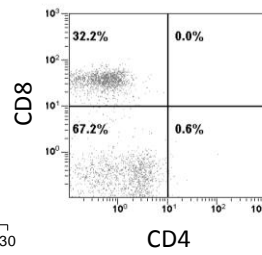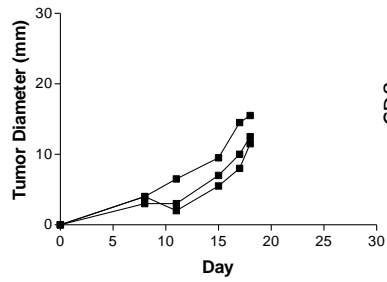
Non-Depleted

Naive Cells

118
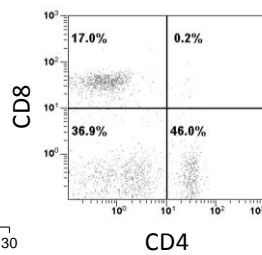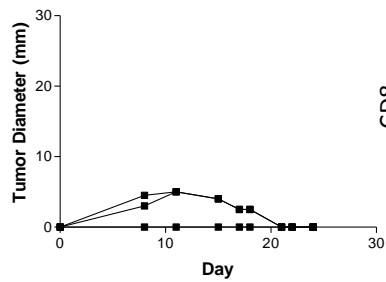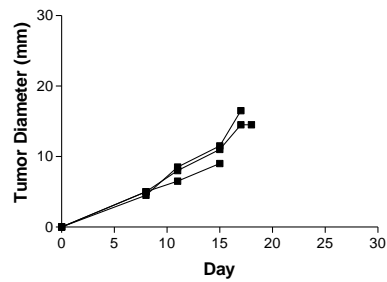
4.12 Modulation of the Th2-type response associated with progressive P815 tumor growth utilizing neutralizing anti-IL-4 antibodies as a therapeutic treatment

If tumor-dependent IL-4 production by lymphocytes is indeed responsible for progression, one can examine whether immunotherapeutic strategies effective in other model systems where a mixed Th1/Th2-type immune response is associated with disease and a predominant Th1 response with resistance are effective in our tumor systems. *Leishmaniasis* is a parasitic disease caused by infection with a species of *Leishmania* ssp. The murine model of leishmaniasis has been exhaustively studied, and the correlates of protection are very well known. Mouse strains that mount a strong, stable Th1-type immune response to parasite antigens are able to contain the outgrowth of the parasite, while other strains that have a substantial Th2-type component to their immune response suffer from progressive/fatal disease. In this model, it has been recently shown that one can cure established stable lesions using neutralizing anti-IL-4 antibodies. This curing was shown to be due to a 'switch' of the immune response to the parasite away from the Th2 pole, towards a protective Th1-type immune response (Bretscher, Ismail et al. 2001; Uzonna and Bretscher 2001). In our experimental system, we strongly believe that the immune failure is very similar to that seen in *Leishmaniasis*. Consequently, we propose that anti-IL-4 therapy will effectively

switch the phenotypic anti-tumor immune response towards what we consider to be the protective Th1 pole, and cause the regression of an established tumor.

Mice implanted with 10^5 P815 cells intradermally into their belly were treated with neutralizing anti-IL-4 antibodies (11B11 clonotype) at various times and concentrations. The effect of such treatments on the survival of P815 bearing animals was assessed. As shown in **Figure 28**, our treated mice, on average, show prolonged survival as compared to control animals. As shown in **Figures 29 and 30**, this prolonged survival correlates with reduced rate of tumor growth, and an increase in the relative predominance of P815-specific IgG2a antibodies in their sera when compared with control animals. These observations suggest our treatment is having an effect on the phenotypic anti-tumor immune response which results in a prolongation of life.
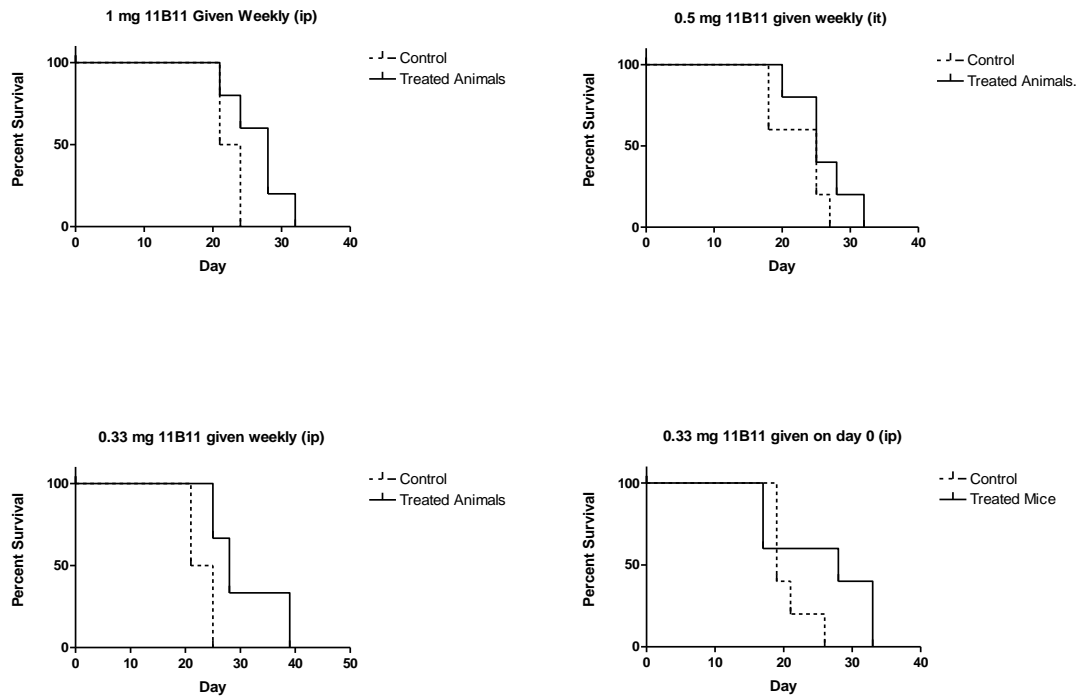
Figure 28: Effect of anti-IL-4 treatment on the survival of P815 tumor bearing animals.

Mice were implanted with 10^5 P815 cells intradermally on day 0. As indicated, treated mice were given different doses of neutralizing anti-IL-4 antibodies (11B11 clonotype) at varying intervals. The effect of our treatment strategies were assessed as percent survival as compared to non-treated control animals. Survival curves are representative of five animals per group.
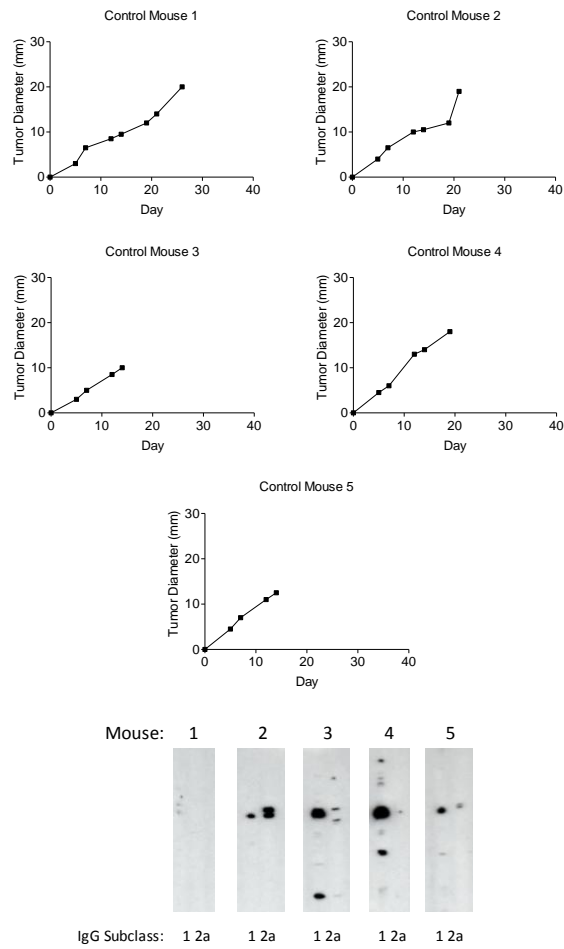
Figure 29: Relative abundance of P815-specific IgG1 and IgG2a antibodies in control animals.

Mice were implanted with 10^5 P815 cells on day 0. Tumor development was assessed, and a Western blot to assess the relative predominance of P815-specific IgG1 and IgG2a antibodies was performed on day 14 of tumor growth at a serum dilution of 1:100.
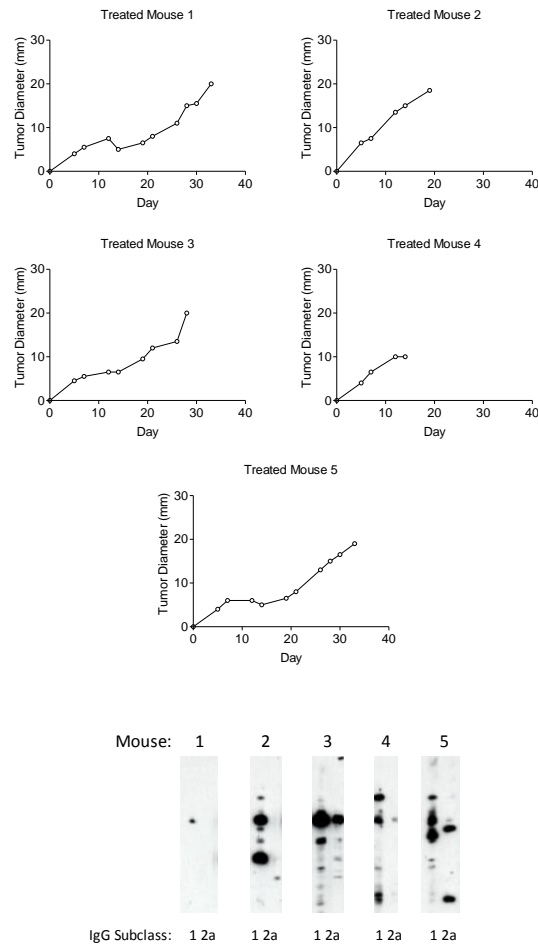
Figure 30: Relative abundance of P815-specific IgG1 and IgG2a antibodies in P815 bearing animals treated with anti-IL-4 antibodies.

Mice were implanted with 10^5 P815 cells on day 0 and were given 0.3 mg of anti-IL-4 antibodies (11B11 clonotype) on day 0 of tumor growth. Tumor development was assessed, and a Western blot to assess the relative predominance of P815-specific IgG1 and IgG2a antibodies was performed on day 14 of tumor growth at a serum dilution of 1:100.

4.12.1 Potential concerns regarding anti-IL-4 treatment of the P815 tumor

In our model system, P815 itself secretes IL-4 and for this reason we must appreciate the possibility that our anti-IL-4 treatments may be more complex than simply modulating the immune response towards the protective Th1 pole. This endogenous IL-4 production by a minority of P815 cells may potentially have many roles *in-vivo* with regards to promoting tumor progression. It is possible P815 utilizes this IL-4 as a growth factor. By neutralizing its effects we may simply decrease the rate of P815 replication, creating an environment more favorable for the generation of tumor-specific Th1-type cells. In an attempt to partially address this possibility, we incubated P815 cells in-vitro with various concentrations of neutralizing anti-IL-4 antibodies and assessed if such treatment had an effect on tumor growth. As shown in **Figure 31**, it appears anti-IL-4 antibodies have very little effect on the rate of P815 growth, as compared to exposure to a control antibody (anti-CD4). One could also argue this IL-4 secretion may promote the development of a 'pro-Th2-type' tumor environment which may increase the tumorogenicity of P815. A third possibility is that the IL-4 produced has other biological roles not associated with immune modulation. There are reports in the literature that suggest IL-4 may be involved in the development of angiogenesis, thereby actively promoting the development and subsequent metastasis of the primary tumor (Fukushi, Morisaki et al. 1998).
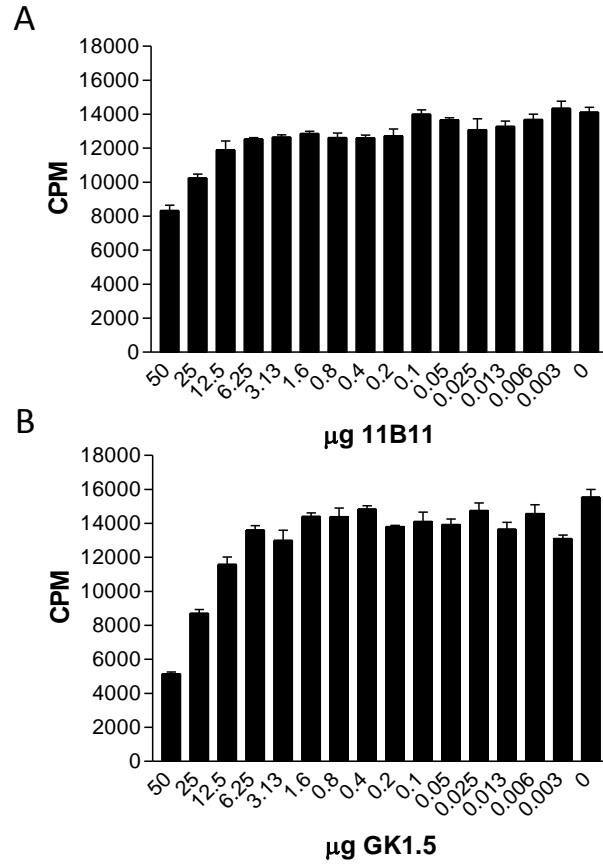
Figure 31: Effect of anti-IL-4 and anti-CD4 antibodies on the rate of growth of P815 cells *in-vitro*:

10^4 P815 cells were incubated with varying concentrations of neutralizing anti-IL-4 antibodies (11B11) or anti-CD4 antibodies (GK1.5) for 24 hours in a 96 flat-bottomed tissue culture plate. Cells were labeled with 0.5 µCi ^{3}H Thymidine for the last 4 hours of culture before harvesting.

4.12.2 Modulation of the immune response against established, normally lethal, L5178Y tumor utilizing a low dose of gamma Irradiation to induce tumor regression

Robert J. North has previously shown that a mouse, given 500 Rads of gamma-irradiation six days after a normally lethal dose of the L5178Y, rejects the tumor (North 1984; North and Bursuker 1984; North 1986). As is shown in **Figure 32**, we have been able to reproduce this observation using 250 rads of whole-body gamma irradiation. Utilizing our EIA and western blotting protocol, we have demonstrated that the regression mediated by this treatment is associated with a switch in the type of immune response from a mixed Th1/Th2 type immune response to a predominant Th1 type response against tumor antigens as compared to the predominant Th2 in non-treated mice suffering from progressive L5178Y tumor growth.
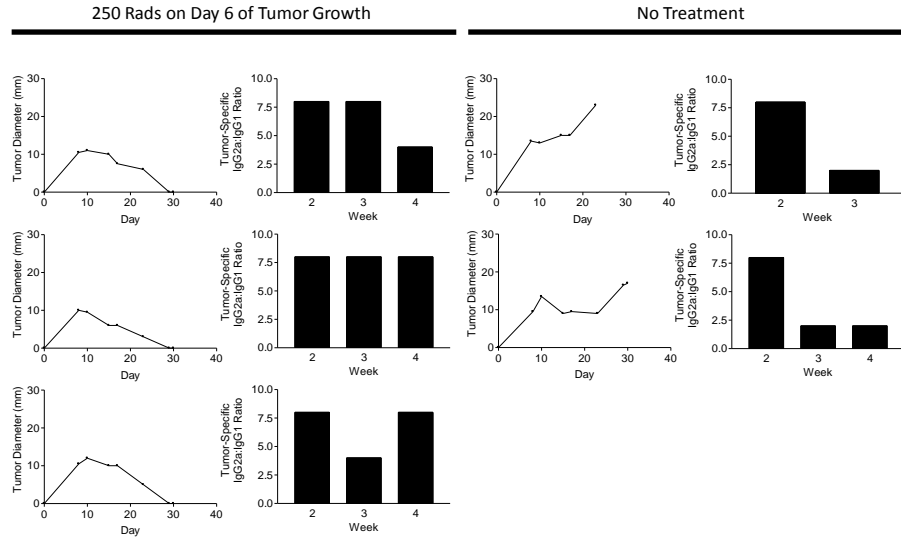
Figure 32: Low-dose irradiation of L5178Y tumor-bearing animals induces immune-mediated tumor regression. This regression is associated with a switch in the phenotype of the anti-tumor immune response towards the Th1 pole, as inferred by IgG isotypes of anti-tumor antibody.

Mice were implanted on Day 0 with 10^6 L5178Y tumor cells. Treated mice were given 250 Rads of whole-body gamma-irradiation on day 6 of tumor growth. The effect of the treatment was assessed by comparing the tumor growth in treated animals as compared to untreated controls. Mice were bled and the relative predominance of L5178Y-specific IgG2a and IgG1 antibodies were assessed by EIA. The observed tumor regression after low-doses of gamma-irradiated has been repeated in three independent experiments. The relative abundance of tumor-specific IgG1 and IgG2a antibodies in induced tumor regression as compared to control animals has been repeated twice.

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5 Discussion

Despite decades of intensive research, the mechanism(s) responsible for immune failure against cancer remains poorly understood, and the literature reflects a view that this is a highly complex issue. The literature is composed of a spectrum of theories attempting to explain the inability of the immune system to control tumor growth. There are reports suggesting T cells are tolerized against tumor antigens (Bogen 1996; Staveley-O'Carroll, Sotomayor et al. 1998). However, such specific 'tolerance' for tumor antigens may often be considered misleading, as it has been appreciated for more than a decade that spontaneous anti-tumor immune responses are often detectable in patients with progressively growing tumors (Ferradini, Mackensen et al. 1993; Mackensen, Ferradini et al. 1993; Zorn and Hercend 1999; Valmori, Dutoit et al. 2000; Griffioen, Borghi et al. 2001). Immune failure in the presence of a vigorous antigen-specific immune response can be a perplexing observation, but it is one not unique to tumors. In many microbial infections, clinical disease results not from a lack of specific immunity, but rather from the generation of a class of immunity ineffective at controlling microbial growth (Bretscher, Ismail et al. 2001). Observations made since the early 1960s have led immunologists, such as George Klein (Klein 1968), to propose that strong

cell mediated immunity against tumor antigens is protective, while ineffective humoral responses are associated with tumor progression.

Utilizing experimental systems whereby we could render mice resistant to normally lethal challenges of either the P815 or L5178Y tumor cell lines, both of which are syngeneic in DBA/2J mice, we developed techniques to assess the Th1/Th2 phenotype of the anti-tumor immune response in mice resisting or succumbing to the tumor. Using such systems, we attempted to ascertain if one could correlate resistance/progression with Th1 and Th2 immunity specific for tumor antigens. Such knowledge would allow for a better understanding of the mechanisms of immune failure, and allow one to develop strategies of prevention through vaccination and also immunotherapeutic strategies aimed at exploiting this endogenous anti-tumor immune response to improve cancer treatments.

At present, there are no clinically useful and simple techniques to effectively and longitudinally assess the Th1/Th2 phenotype of a patient's anti-tumor immune response. In this respect, it has been difficult to define any clear correlates of protection. We have developed ELISPOT, EIA and western blotting protocols which allow us to directly assess, or indirectly infer, the Th1/Th2 phenotype of an ongoing anti-tumor immune response. Exploiting these experimental tools, we have been able to define very clear immune correlates in our two tumor models. Of perhaps most interest, because of their practical simplicity, is our western blotting and EIA protocols which reveal our unique ability to longitudinally assess the Th1/Th2

phenotype of the anti-tumor immune response in the same animal as the tumor develops over time. Utilizing such techniques, we have been able to clearly demonstrate that cellular Th1 responses are associated with effective immunity, while the generation of mixed Th1/Th2 immunity against tumor antigens is associated with progressive tumor growth. The proposal that the immune correlates of resistance/tumor progression correlate with cell mediated (Th1) and humoral (mixed Th1/Th2) responses is not novel (Klein 1968); however, the majority of modern tumor immunologists support the idea that other causes of immune failure are more important.

The *in-vivo* role of CD25+ T regulatory cells in dampening ongoing immune responses, in a purported effort to maintain self tolerance to peripheral self antigens, has become a very topical subject (Sakaguchi, Sakaguchi et al. 2001). Most current reports in the literature suggest that the generation of CD25+ T regulatory cells specific for tumor antigens act to inhibit the generation of effective anti-tumor immunity. Many of these conclusions are based on the increased frequency of CD4+CD25+ T cells in patients with progressively growing tumors (Woo, Chu et al. 2001; Liyanage, Moore et al. 2002; Javia and Rosenberg 2003; Sasada, Kimura et al. 2003; Wolf, Wolf et al. 2003; Curiel, Coukos et al. 2004; Marshall, Christie et al. 2004). How or if such a correlation reflects the primary cause of immune failure in these patients remains to be determined. The present study does not directly assess a possible role of T regulatory cells; however, our

results suggest that a specific loss of immunity is not associated with tumor progression as would be expected if tumor-specific T regulatory cells were the primary factor in immune failure. We observe a phenotypic difference in the class of immunity generated against tumor antigens in mice rendered resistant to, as compared to normal mice suffering progressive tumor growth after being given a normally lethal dose of the syngeneic tumor. Based on our observations, one could surmise immune failure is not due to a specific loss of immunity, but rather the generation of ineffective immunity in response to tumor antigens.

A decrease in the level of expression of tumor associated antigens is another mechanism leading to immune failure much reported and discussed in the literature (Biddison and Palmer 1977; Uyttenhove, Maryanski et al. 1983). It is very likely that such processes occur late during the course of tumor progression. Both the P815 and L5178Y tumor cell lines have been passaged *in-vitro* and *in-vivo* numerous times during the past roughly 60 years. One might hypothesize that both these cell lines are very adept at spontaneously changing their antigenic profile to evade the immune system. We have been able to consistently render mice resistant to tumors challenges, with both these cell lines, with challenges many fold above what is a semi-lethal dosage. These challenges with a high number of tumor cells ($2x10^6$), would favor the outgrowth of rare variants that have lost the expression of tumor associated antigens (Boon, Coulie et al. 2006). Furthermore, as shown in **Figure 19**, we observed one occurrence when mice, implanted with a

normally lethal dose of 10^5 P815 tumor cells, resulted in regression of the primary tumor. Subsequently, the tumor reemerged, and grew progressively at the site of implantation. It would be natural to suppose that such an occurrence followed a randomly generated tumor cell variant whose antigenic profile is no longer recognized by the immune system, so that it simply grew out from the quasispecies of the tumor cells still under immune control. We made two observations pertinent to evaluating this possibility utilizing our western-blotting protocol to assess the relative abundance of tumor-specific IgG1 and IgG2a antibodies. Firstly, the initial tumor regression correlated with a predominance of production of IgG2a antibodies, and as the tumor re-emerged, and progressively grew, we detected a switch to a predominant production of IgG1 antibodies against tumor antigens. Secondly, it appears upon a detailed examination of the western blots with sera taken at different times post tumor implantation, as though only the subclass of antibodies against the tumor antigens is changing, and not the antigens against which immunity is being generated. Such observations suggest there is no role for tumor variants affecting the class of immunity induced as a cause for tumor progression in this particular and unusual case.

5.1 Implications for the development of cancer vaccines

The ever-expanding cancer immunome has identified a number of common tumor antigens against which patients commonly mount immune responses. Such observations have helped to fuel the enthusiasm for the potential development of a possible vaccine to prevent the development of many common cancers. One caveat in the development of such vaccines are reports that rendering mice resistant to one tumor does not cross protect against a different syngeneic tumor with which it shares common tumor antigens (Prehn and Main 1957; Ramarathinam, Sarma et al. 1995; Srivastava 1996). I observed the presence of tumor antigens shared between the P815 and L5178Y cell lines as assessed by western blot, and in a small preliminary experiment I wanted to assess if resistance to the L5178Y tumor could effectively vaccinate against a normally lethal challenge with the P815 tumor. As is shown in **Figures 33**, both control mice suffered rapid tumor growth. Such tumor growth was associated with predominant IgG1 responses generated against the P815 tumor antigens. Interestingly, we observe no cross-reactive antibodies recognizing L5178Y antigens. I observed a much slower rate of P815 tumor growth in both mice vaccinated three months prior with 10^3 L5178Y tumor cells (**Figure 34**) as compared to the control mice. One vaccinated mouse was able to resolve, while the other had an uncharacteristic unprogressive tumor growth for three weeks, but ultimately suffered from progressive growth. One is able to clearly observe a much higher IgG2a:IgG1 ratio

against tumor antigens in the mouse which successfully contained the P815 challenge, and a much lower ratio in the mouse that succumbed from tumor growth.
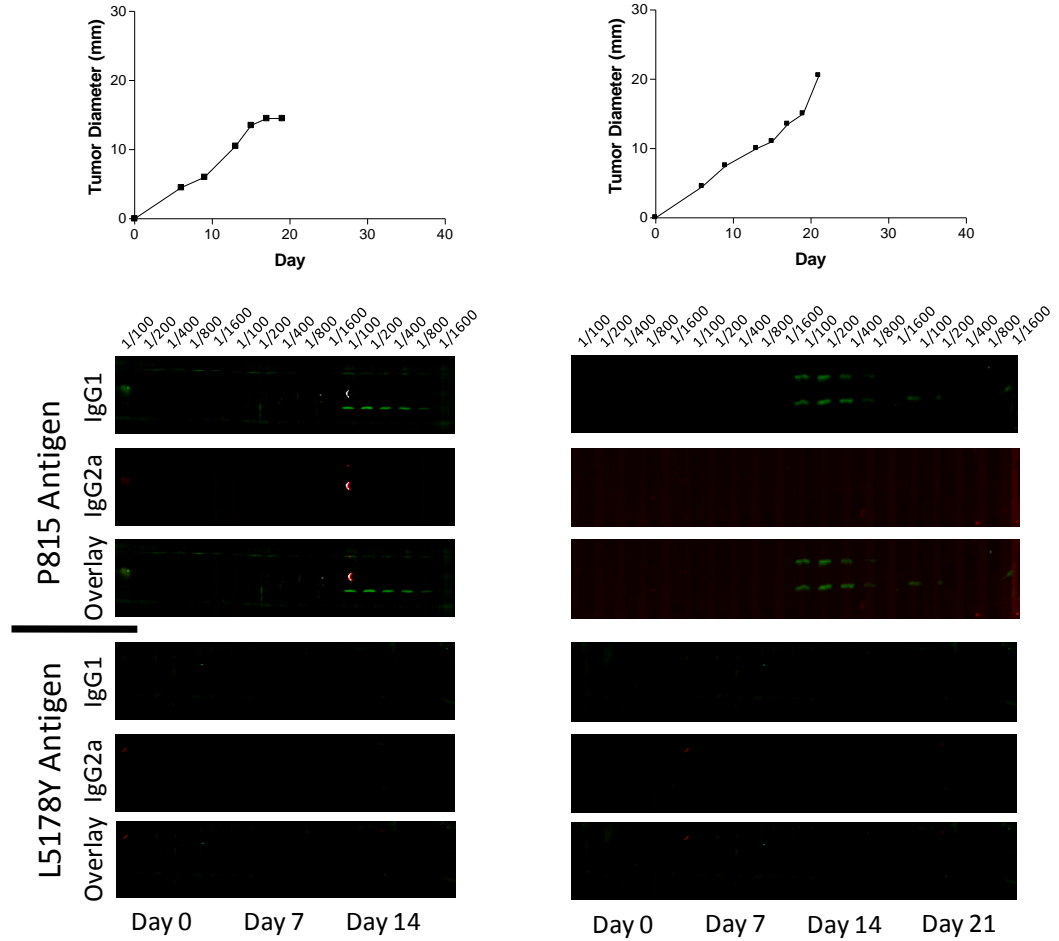
Figure 33: Progressive P815 tumor growth is associated with the predominance of IgG1 antibodies specific for P815 antigens.

Mice were implanted with 10^5 P815 cells intradermally into their belly on Day 0. Rate of tumor growth was monitored, and Sera was collected weekly to assess the relative abundance of tumor-specific IgG1 and IgG2a antibodies by western blot using either P815 or L5178Y as antigen.

Figure 34: Effect of L5178Y vaccination on the growth of a normally lethal challenge with the P815 tumor

Mice were vaccinated with 10^3 L5178Y cells intradermally into their belly 3 months prior to challenge with 10^5 P815 cells intradermally into their belly on Day 0. Rate of P815 tumor growth was monitored, and sera was collected weekly to assess the relative abundance of tumor-specific IgG1 and IgG2a antibodies by western blot using either P815 or L5178Y as antigen.

Day 0   Day 7   Day 14   Day 21   Day 28   Day 35   Day 42   Day 49

Day 0   Day 7   Day 14   Day 21   Day 28   Day 35   Day 42
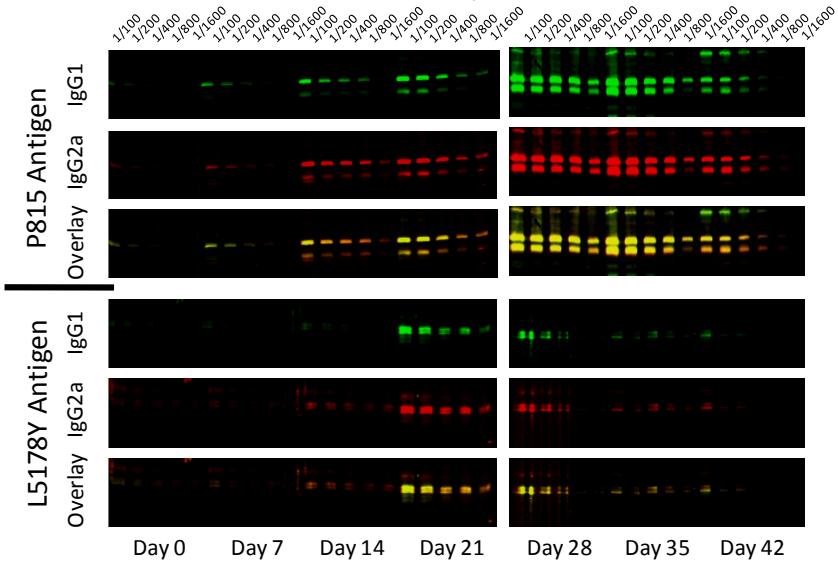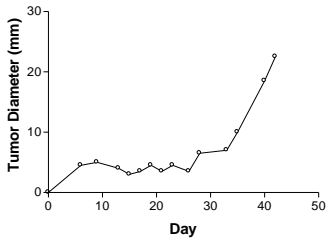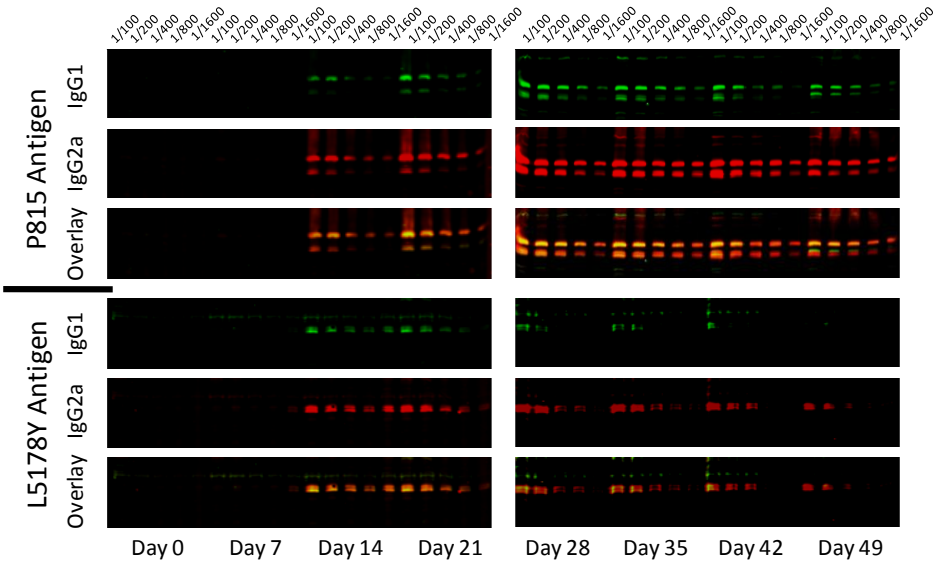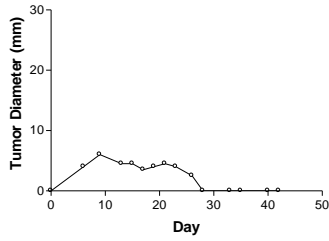
Perhaps and most interestingly, by assessing the ratios of tumor-specific IgG1 and IgG2a antibodies over the course of the experiment, one could have tentatively predicted which of the two mice was going to contain, or suffer progressive tumor growth, at times where both had roughly the same size tumor. I recognize that this is a preliminary experiment involving very few animals, and only present it here as an interesting observation showing cross-protection between the P815 and L5178Y tumors in contrast to a report by RJ North and colleagues who observed no such cross reaction (Dye and North 1984). Such cross-protection between tumor cell lines, which share common antigens, provides hope that vaccines against several common antigens may be capable of preventing much cancer. As discussed in the previous section, it should be kept in mind that for such vaccines to be effective, they must induce strong, Th1 responses against tumor antigens (see **Figure 35** bottom panel, (Bretscher, Hamilton et al. 2002).

5.2 Implications for cancer therapy

R.J. North's classical studies on the kinetic development of concomitant immunity, and its subsequent decline associated with the generation of tumor-specific suppressor CD4+ T cells, demonstrates a highly dynamic aspect of the anti-tumor immune response (North and Bursuker 1984). One must appreciate that the types of immunotherapeutic strategies that are most effective at treating cancers may be different depending on the phenotype of the endogenous anti-tumor immune response at the time of treatment. As such, the ability to assess the

nature of the anti-tumor immune response is critical for the development of effective, clinically relevant, immunotherapeutic strategies. Utilizing western blots, we have developed a uniquely simple methodology to assess the Th1/Th2 nature of ongoing anti-tumor immune responses in mice, and place them within the conceptual framework of concomitant immunity described by R.J. North.

The relative prevalence of specific antibody belonging to different IgG subclasses, has proven itself useful as a measure of the Th1/Th2 nature of the immunity in human disease. In their studies on visceral leishmaniasis, Bretscher and colleagues demonstrated one could easily distinguish patients from healthy contacts based upon the relative abundance of parasite-specific antibody subclasses. Interestingly, one healthy contact had an antibody profile similar to patients. It was noted that this individual subsequently developed disease (Hailu, Menon et al. 2001). Observations such as these demonstrate that such techniques are not only useful in simply defining immune correlates, but can also be diagnostic.

In the case of our tumor models, one could explore the utilization of a similar strategy that examines how the ratio of tumor-specific IgG2:IgG1 antibodies develop over time. I hypothesize that disease progression could be predicted if this ratio decreases rapidly over time. In situations where the ratio remains high, or increases over time, I would predict a favorable outcome. In contrast, ratios when low or where they decrease rapidly, one might be able to predict exacerbation of

tumor growth prior to the development of overt symptoms. If such a technique is proved useful, it would represent a very powerful diagnostic tool in the fight against cancer.

Assuming our observed correlates of regression/progression are mirrored in humans, one can envisage the potential for developing immunotherapeutic strategies to treat cancer by utilizing interventions that switch established, mixed Th1/Th2 responses towards a protective, Th1 response against the tumor. It is a somewhat underappreciated that established Th1/Th2 responses in chronic infections are not cast in stone. Through simple procedures, one can effectively modulate the immune response towards a Th1 mode (Hailu, Menon et al. 2001; Uzonna and Bretscher 2001). Such treatments, when optimized, should be capable of switching the response towards a Th1 mode, leading to increased effectiveness of current anti-tumor treatment strategies.

Exploiting an experimental system developed by Robert North (North 1986), we have been able to induce regression of an established, progressively growing L5178Y tumor, using low doses of gamma-irradiation. We have been able to demonstrate that such regression is associated with the development of a high ratio of tumor-specific IgG2a to IgG1 antibodies, while in untreated control mice we observe a decreasing ratio as the tumor progresses. Some may interpret the low-dose irradiation, and subsequent tumor regression, as preferentially depleting tumor-specific CD4+ CD25+ regulatory T cells (Ghiringhelli, Larmonier et al. 2004;

Turk, Guevara-Patino et al. 2004). I did not assess the direct effects of these low doses of irradiation on the development of T regulatory cells. It is possible such low dose irradiation depletes T regulatory cells to allow for the generation of protective immunity; however, as discussed in the introduction, I personally do not favor such an interpretation. It has been reported that T regulatory cells are capable of directly suppressing B cell activation (Lim, Hillsamer et al. 2005). In mice suffering from a progressively growing L5178Y tumor, we never observe a lack, or decrease, in the anti-tumor immune response as assessed by production of antibody. I have consistently observed an increase in the Th2-component of the anti-tumor immune response; furthermore, there is no evidence in the literature suggesting regulatory T cells have any such effect on the switching of antibody subclasses.

Contemporary chemotherapeutic agents are, in general, not appreciated for their potential effects on the immune system. It is often assumed that the powerful anti-proliferative properties of traditional chemotherapeutic agents act primarily upon the rapidly proliferating tumor cells. Often it is not appreciated that such drugs also have a profound impact upon the concurrent anti-tumor immune response. It has been shown that such treatments, for example the administration of cyclophosphamide, when utilized appropriately, are capable of enhancing delayed type hypersensitivity at the expense of humoral immunity (Turk, Parker et al. 1972; Lagrange, Mackaness et al. 1974; Mitsuoka, Baba et al. 1976; Goto,

Mitsuoka et al. 1981). These studies for the most part involve non-replicating antigens such as xenogenic red blood cells. The effect of such agents cannot in this case be indirect, acting on the replicating antigen and thus indirectly reducing its effective 'dose'. Rather, the anti-mitotic agents in these cases are more likely to act on replicating cells of the immune system. The effects of these agents are comprehensible in the context of a cooperative model of T cell activation. In such a model, T cells specific for a given antigen cooperate not only to induce the generation of effector cells, but also the 'strength' of antigen-mediated CD4 T cell cooperation determines the Th1/Th2 phenotype of the effector T cells generated. Limiting cooperation either by limiting antigen concentration or numbers of specific CD4+ T cells has been shown to preferentially generate Th1 cells. Conversely, by increasing either antigen concentration or numbers of specific CD4+ T cells one can preferentially generate Th2 cells (Bretscher, Ismail et al. 2001; Ismail and Bretscher 2001). Traditional anti-cancer treatments that inhibit division of lymphocytes, such as administration of cyclophosphamide, or irradiation, have the potential, if given at the correct level, to modulate mixed Th1/Th2 responses to a Th1 mode. In this respect, being able to assess the effects of chemotherapeutic agents on the endogenous anti-tumor immune response may allow clinicians to tailor their treatment strategies to facilitate the promotion of protective Th1 responses at the expense of ineffective, Th2 immune responses specific for tumor antigens.

This dissertation forms a foundation for many potential future studies. An obvious route of investigation is to see if one can detect tumor-specific antibodies in cancer patients, and determine if one can correlate the relative predominance of tumor-specific Ig subclasses with disease progression and/or response to treatment. Our simple western blotting protocol would allow us to longitudinally assess the effects that traditional cancer therapies have on the Th1/Th2 nature of the anti-tumor immune response. Such information may allow one to tailor the cancer treatment regimen to fully exploit the endogenous anti-tumor immune response. Such experimental data may prove very significant in helping to improve the effectiveness of current strategies of cancer treatment.

The immune correlates presented within this thesis may also allow the development of effective cancer immunotherapies. The direct modulation of ongoing anti-P815 immune responses, as presented in this thesis, is somewhat inconclusive. In some anti-IL-4 treated mice we have observed a decreased rate of tumor growth, as compared to control animals. This decrease in the rate of growth often correlates with an increase in the predominance of P815-specific IgG2a antibodies. Such observations suggests one can modulate ongoing anti-tumor immune responses; however, it is likely P815's inherent aggressiveness does not lend itself well to being a model for immunotherapeutic strategies, and most likely is not representative of most human cancers. The immune response generated against L5178Y has shown itself to be easily modulated by gamma-irradiation to

induce immune-mediated clearance of the tumor. This suggests it is an ideal system in which to assess the utility of immunotherapeutic strategies to induce regression of an established tumor. Such treatments could include the above-mentioned neutralizing anti-IL-4 treatment, and partial depletion of CD4+ T cells, both maneuvers having been shown effective at modulating established mixed Th1/Th2 immune responses towards the Th1 pole (Uzonna and Bretscher 2001).

Many current cancer immunotherapeutic strategies, such as those developed by Rosenberg and colleagues, involve the ex-vivo expansion of tumor-reactive lymphocytes (Dudley and Rosenberg 2003). Such strategies have had some success at inducing tumor regression (Rosenberg, Yannelli et al. 1994); however, as was reported by RJ North (North, Dye et al. 1982), often treatment also requires reduction in the number of T cells in the patient to improve efficacy (Dudley, Wunderlich et al. 2005). Based upon the observations discussed within this thesis, I would argue that such procedures are not effective by causing the simple inflation of effector cells, but rather the intervention to be effective must act to switch the phenotype of the anti-tumor immune response from an ineffective Th2 or mixed Th1/Th2, towards an effective, Th1 mode. One may be able to greatly improve the efficacy of such immunotherapeutic strategies.

Observations made by Bretscher and colleagues have demonstrated that it is possible to switch a humoral to a strong cellular immune response *in-vitro*. It was observed that such a switch was associated with the generation of antigen-specific

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CD8+ T cells able to inhibit antibody response in an antigen-specific manner (Tuttosi and Bretscher 1992). The ability to effectively assess the Th1/Th2 nature of the anti-tumor immune response could allow one to develop *in-vitro* strategies to promote the expansion of cells capable of successfully inducing immune clearance of established tumors.

The development of the cancer immunome has really allowed one to envisage how vaccines might be developed that prevent the outgrowth of many common cancers. Our results suggest that immunity to such antigens is not enough to induce protection against cancers. To induce protective anti-tumor immunity, one must ensure the development of the correct type of immune response. Previous work in this laboratory has demonstrated mechanisms by which one can consistently induce Th1 immune responses in genetically diverse mice (Menon and Bretscher 1996; Menon and Bretscher 1998). By exploiting these systems, one could potentially devise vaccination strategies by which one could induce protective Th1 immunity against tumor associated antigens in a diverse population (Menon and Bretscher 1998; Power, Wei et al. 1998; Bretscher, Ismail et al. 2001).

5.3 Concluding Remarks

It seems to me that there has been a loss of optimism over the past few decades in the field of tumor immunology. By approaching the field of tumor immunology, from a particular perspective that we thought might be too simplistic, we have been able to utilize very simple techniques to define correlates of protection. We do not know how generalized our observations might be. Nonetheless, we feel such simple techniques have the potential to reshape current thinking, leading to improvements in traditional cancer treatments, and allow for the development of new cancer immunotherapeutic and vaccination strategies to treat and perhaps prevent the frank occurrence of many common cancers.

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