Molecular Mechanisms of Co-Stimulatory Molecules and Their Application for Tumor Immunotherapy

Molecular Mechanisms of Co-stimulatory Molecules and Their Application for Tumor Immunotherapy

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Abstract

Costimulatory molecules function to stimulate or inhibit T cell activation. A balance between activating and inhibiting receptors determines the functional state of a T cell. PD1 and CTLA4 are the two most well studied inhibitory costimulatory molecules, sometimes termed coinhibitory. Antibodies blocking their inhibitory function have revolutionized the field of tumor immunotherapy. Understanding the pathways governing the coinhibitory molecules and the mechanisms behind antibody action is important for their application in cancer treatment. Though PD1 and CTLA4 have given good clinical benefit in about 20% of patients, there is a high percentage of non responders. Finding better combination strategies for these two molecules is an active field of research. My thesis focuses on the PDL1-B7-1 pathway. I find that a particular structural orientation is needed for the binding of PD-L1 to B7-1. PDL1 and B7-1 have a strong binding interaction when the PDL1 molecule is accessible and flexible but not when constrained. The native conformations of B7-1 and PD-L1 on the cell surface are too constrained to allow binding of B7-1 on one cell to PD-L1 on another cell. My data suggests that, cell surface PD-L1 may interact with cell surface PD-1 *in trans* but not with cell surface B7-1 *in trans.* Instead, interaction of cell surface PD-L1 with cell surface B7-1 *in cis* is possible. In this study I also investigate strategies for PD1 combination tumor immunotherapy. I develop and test a novel mAb and find that treatment with the mAb in combination with PD-1 mAb prolongs the survival of tumor bearing mice better than PD-1 alone. This suggests the combination may be an effective tumor immunotherapy. I further explore the mechanism of action of CTLA4 mAb in cancer immunotherapy. I test a novel CTLA4

mAb that is a non blocker of B7 interaction with CTLA4 but depletes CTLA4 positive cells. I find that this antibody fails to show any therapeutic efficacy. This supports the idea that both blockade and cell depletion are important for therapeutic efficacy by CTLA4 mAb. Together, my findings suggest ways of enhancing cancer immunotherapy.

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This work is for you mom

Chapter 1 Background

1.1 Background

Co-stimulatory molecules bind their respective ligands/receptors on APCs during the process of antigen presentation and promote activation of T cells. The signal 2 provided by costimulatory molecules is required for optimal T cell activation. The most important is of the CD28 family[3]. CD28 binds to B7-1 and B7-2 on activated APCs to fully activate a T cell. This signal goes in parallel with TCR activation. CD28 engagement is required for differentiation and proliferation of antigen specific T cells and regulates a number of downstream cell signaling genes[4].

The co-stimulatory molecules, B7-1 and B7-2 differ in expression. While B7-2 is constitutively expressed on APCs and is rapidly increased in expression on activation of APCs, B7-1 is upregulated several hours later following APC activation. B7-1 and B7-2 share an amino acid identity of twenty five percent. They are expressed on APCs- macrophages, B cells and dendritic cells while the highest expressors are mature dendritic cells[5].

A main mechanism of co stimulation by CD28 is to activate cell survival genes and cytokines. The net result of CD28 co stimulation is- Increase of cell survival genes- Bcl-XL, secretion of IL-2, differentiation of naive T cells to effector and memory cells[6].

Members of the CD28-B7 family are involved in either T cell activation- costimulation or T cell inhibition- coinhibition. A balance is maintained between these activating and inhibitory receptors for T cell activation. The stimulators regulate activation of T cells against pathogens while the inhibitors inhibit T cells for tolerance[7].

Alongside the CD28-B7 family of co-stimulators, the TNF-TNFR family also functions to regulate T cell responses. A number of activating and inhibitory receptors are present on T cells. The major Inhibitors are PD1, CTLA4, LAG3, TIGIT, CD160, 2B4 while the major stimulators are- CD28, OX40, 4-1BB[8, 9].

CTLA4 and CD28 share the same ligands– B7-1 and B7-2. The CTLA4-B7-1 interaction has a fifty fold greater affinity than CD28-B7-1. CD28 is constitutively expressed on T cells but CTLA4 is expressed a few hours after T cell activation. It is mostly a cytoplasmic protein and re cycles to the cell surface via endosomes. This means that CD28-B7 functions in the early stage of T cell response while CTLA4-B7 in the later stage to inhibit the activated T cells.

The inhibitory PD1 receptor binds to its ligands PDL1 and PDL2. The PD1-PDL1 pathway inhibits effector T cells in the periphery while CTLA4-B7 inhibit T cells in the secondary lymphoid organs. The PD1 ligand, PDL1 is present on APCs and tissues while PDL2 is expressed primarily on APCs. PD1 has two inhibitory tyrosine motifs ITIM and ITSM in its cytoplasmic tail[10]. On engagement it translocates to the TCR microcluster[10] and recruits the Src homology domain- SHP2 and SHP1 at the ITIM and ITSM. This blocks CD28 mediated PI3K activation[11].

Both PD1 and CTLA4 are required for maintaining tolerance. Studies in PD1 and CTLA4 KO mice have reported incidences of severe autoimmune phenotypes. CTLA4 KO mice develop severe lymphoproliferative disease soon after birth[9]. Generally, PD-1 deficient mice on wild type backgrounds develop mild autoimmune complications like autoantibodies and kidney damage late in life. PD1 deficient mice on the BALB/c develop autoimmune cardiomyopathy in one animal facility but are fine in other facilities[12]. The CD28-B7 family has been targeted in various disease settings. CTLA4-Ig fusion

protein is used for treatment of rheumatoid arthritis. Antibodies against CTLA4 and PD1 are used in cancer immunotherapy. Structure of co-stimulatory molecules is illustrated in Figure 1[8, 13, 14].

PD1-PDL1, PDL2 pathway

The PD1 binding partners PDL1 and PDL2 each have distinct binding partners other than PD1. PDL1 binds to B7-1,[15] PDL2 does not bind to B7-1 but to RGMb[16]. Soluble forms of PD1, PDL1 and PDL2 have been detected; soluble PDL1 has been shown to be released from DCs and may be immunosuppressive.[17] Important inducers of PDL1 have been identified as IFNy[18], while of PDL2 have been identified as IL-4 and GMCSF[19]. The PD1-PDL1 pathway inhibits a T cell by reducing TCR signal. The tyrosine phosphorylated PD-1 cytoplasmic domain recruits the SHP2 phosphatase. SHP2 then reduces signaling intermediates in the TCR/CD28 pathway, reducing the T cell activation signal, thus downregulating cell survival genes and reducing cytokine production[11]. There are additional mechanisms as well, PDL1 downregulates the Akt/mTOR pathway by upregulating ERK2 and phospho-S6 leading to naive T cell differentiation to iTregs[20]. Recently the PD1 pathway was shown to metabolically reprogram a T cell from aerobic glycolysis to fatty acid oxidation. Further the exhausted phenotype of T cells involves transcriptional reprogramming, upregulation of Blimp1[21] and downregulation of Tbet (transcription factor for Th1 cells)[22] and Eomes (transcription factor for memory T cells).

Programmed death 1 (PD-1, CD279) and its ligand programmed death ligand 1 (PD-L1, B7-H1, CD274) are promising targets in cancer immunotherapy[23, 24]. For tumor immunotherapy as well as chronic infection, antibodies against PD1 and PDL1 have shown remarkable results. PD1 blockade has been FDA approved in different types of cancer- advanced NSCLC, melanoma and kidney cancer and is under FDA review for head and neck cancer, advanced bladder cancer, and Hodgkin's lymphoma[25]. Tumor cells have been shown to upregulate PDL1 expression in response to T cells releasing IFNY as a mechanism for immune escape[25]. Around 30% of solid tumors express PDL1 and PDL2. Some tumors can increase PDL1

and PDL2 expression by gene amplification. In a study on Hodgkin's lymphoma it was shown that the Hodgkin's tumor cell line expressed high PDL1 and PDL2 expression due to amplification of the chromosome 9p24.1 region containing Jak2, PD-L1, and PD-L2, leading to increased JAK-STAT signaling, increased interferon (IFN)-stimulated regulatory element/IFN-regulatory factor 1 [26].

Antibodies against PD1 and PDL1 have both shown clinical benefit in clinical trials. The human antibodies to PD1 are of IgG4 non killer type that function to inhibit PD1 binding with PDL1[27]. Antibodies to PDL1 are mostly of non—killer type though one (Avelumimab) is of human IgG1 killer type. This IgG1 antibody will engage phagocytic receptors and deplete the PD-1 expressing cells. These antigen expressing cells are mainly tumor, myeloid population accounting for the therapeutic efficacy of the antibody. The side effects of using the PD1 antibodies, nivolumab and pembrolizumab, are diverse but autoimmune related including pneumonitis, colitis, hepatitis, nephritis and renal dysfunction[28].

CTLA4 for T cell Inhibition

The structure of CTLA4 has been characterized as containing an extracellular IgV domain, a transmembrane domain and a cytoplasmic tail. The intracellular cytoplasmic tail contains YVKM motif. CTLA4 has a higher binding affinity with B7-1 and B7-2 than CD28. B7-1 has a stronger binding affinity than B7-2[29]. A stable interaction between CTLA4 and B7-1 was observed in crystal lattice structures where a bivalent CTLA4 homodimer interacted with bivalent B7 homodimers resulting in a stable signaling complex[30].

CTLA4 inhibits T cell by an extrinsic mechanism and a cell intrinsic mechanism. In a cell intrinsic mechanism, CTLA4 inhibits T cells by outcompeting CD28 for binding with B7-1. In the cell extrinsic mechanism, CTLA4 removes ligands CD80 and CD86 from antigen presenting cells by transendocytosis [31].

The highest CTLA4 expressing cells are CD4+CD25+ FOXP3+ Tregs. CTLA4 is constitutively expressed on Tregs. CTLA4 mediated mechanism has been identified as dominant for Treg suppression[32]. Mutations in CTLA4 are associated with a loss of its suppressive ability and an inability for transendocytosis of B7-1 leading to immune dysregulation[33]. CTLA4 has a different mechanism of action than PD1, it inhibits T cells Independent of PI3K but rather inhibits AKt activation by PP2A[11].

CTLA4 expression on the surface of activated T cells was identified as transient. *Qureshi et al, 2011* illustrated that CTLA4 was internalized from the plasma membrane in a clathrin dependent endocytosis, on internalization it was recycled and degraded in the endosome and lysosomes respectively. The internalization was mediated by the YVVM motif at the cytoplasmic tail of CTLA4[34].

Monoclonal antibody to CTLA4, Ipilimumab, has been approved for treating patients with metastatic melanoma. These antibodies are of the human IgG1 isotype that can work by depleting CTLA4 expressing immunosuppressive Tregs. There are a high percentage, about 30%, of adverse effects of this immunotherapy including dermatologic toxicity, diarrhea, hepato toxicities, hypophysitis (pituitary inflammation) and hypothyroidism[28].

Tumor Immunology

The immune system's defense against tumors involves three stages- Elimination, Equilibrium and Escape. In the elimination phase the Immune system fights successfully against the tumor cells. Major immune cells involved are cytotoxic T cells, NK cells and M1 macrophages, DCs, and CD4 T cells. The killer function of CD8 and NK cells cause a destruction of the tumor. Release of IFNY creates a pro inflammatory microenvironment recruiting more immune cells. In the equilibrium phase, the immune cells try to keep the cancer cells in check and balance. However cancer cells may acquire immune

mutational escape and eventually grow. The equilibrium phase has mostly cells of the adaptive immune system- NK and cytotoxic T cells. Selective pressure by the immune system leads to immune resistant growth of tumor cells. Lastly tumors outgrow the immune cells in the escape phase. Tumors develop adaptive resistance mechanisms to evade the immune system. 1) They may mutate to eliminate the expression of tumor antigens, increase expression of immune dampening molecules such as FasL and PDL1. 2) Release soluble mediators and growth factors- VEGF, TGFB, IDO to recruit immune suppressive cell types 3) Polarize macrophages to the tumor promoting M2 phenotype. The major two immunosuppressive populations are Tregs and MDSCs. Tregs function to suppress antigen specific CD4 and CD8 cells. Their major mechanisms of action include consuming IL-2 required for CD4 and CD8 survival, expression of CTLA4, release of immunosuppressive cytokines IL-10, and TGFB. MDSCs function by promoting growth of Treg, releasing IL-10, consuming essential amino acids for T cells, cysteine, and arginine[35].

Targeting immune escape mechanisms are potential strategies for cancer immunotherapy. Since tumors utilize an increase in Tregs, MDSCs and increase of exhaustion molecules on CD4 and CD8 T cells, antibodies against immunosuppressive molecules- PD1 and CTLA4, and immunosuppressive cell types serve as treatment strategies. Co-blockade of more than one immune inhibitor can lead to a better therapeutic efficacy than single blockade. The existing PD1 or PDL1 therapy can be used in combination with other co-stimulatory molecule inhibitors such as LAG3[36], Tim3[37], CTLA4[38], and TIGIT[39].

Monoclonal Antibody generation

Antibody production involves a series of steps. The animal for antibody production is immunized with an antigen with an adjuvant. Mice are often used for monoclonal antibody production, immunized every 2-3 weeks. The mice are screened for the required antibody titer by ELISA and flow cytometry. Mice with high levels of antibody titer are euthanized, with removal of the lymphoid organs- spleens for the next steps. The immune cells are fused to an immortalized myeloma cell line in vitro to ensure unlimited

growth and increased antibody production. The non fused cells are sensitive to a selection medium so only successfully fused cells survive. The fusion is done by a chemical method using polyethylene glycol, which causes cell membranes to fuse. The cells are then fed media containing growth factors and selective agent. The antibody producing hybridomas are screened for the desired antibodies and subsequently cloned. Lastly large numbers of hybridoma cells are grown in vitro and mAb purified [40].

Antibody structure and functions

An antibody molecule consists of two identical light chains and two identical heavy chains. The heavy and light chains have variable and constant regions. The variable region is for antigen binding while the constant region is for effector functions. The variable region and the constant region consist of one Ig domain each for the light chains, while in the heavy region there is one Ig variable domain and three to four Ig domains in the constant regions. The Variable regions of heavy and light chain form the antigen binding site. There are two antigen binding sites for each antibody. The constant region is for the effector functions of the antibody. The interaction between an antigen and an antibody are non covalent. The affinity is defined as the strength of binding between an epitope and an antibody. The avidity is the overall strength of multiple affinities of an antibody with its antigens. Antibodies with weak affinity may have a strong avidity as in the case of IgM[5].

The Fc regions of an antibody bind with the FcRs on different cells to mediate antibody dependent cell cytotoxicity (ADCC) or complement mediated cytotoxicity (CDCC). ADCC involves FcR on macrophages and NK cells. CDCC involves complement. Antibody mediated cytotoxicity clears out infections. The current cancer immunotherapy antibodies against CTLA4 work on the principle of ADCC as does one of the PD-L1 antibodies. This property of ADCC is made use of in various disease settings[5].

The B7-PDL1 pathway

Our group discovered B7-1 to be a binding receptor for PDL1[15]. Using protein cross linking and mass spectrometry it was shown that PDL1 bound to B7-1. The binding affinity of this interaction is greater than of B7-1-CD28 interaction but lesser than of the well known binding partners B7-1-CTLA4 and PD1-PDL1. The affinity was around 1.4 μM[15]. This pathway has been shown to have inhibitory effects on T cell activation, and tolerize T cells. In *Butte et al, 2007* the pathway showed decreased T cell proliferation and cytokine production. Specifically the blockade of the PDL1/B7-1 pathway broke T cell anergy and oral tolerance, and in NOD mice it caused a progression to autoimmune diabetes[15, 41, 42, 43]. A dominance of the B7-1 molecule has been observed for transmitting inhibitory signals to T cells[41]. Studies have also shown for the PDL1-B7 pathway to activate T cells and prevent immune suppression by inhibiting PDL1 on tumors from binding with PD1[44, 45, 46]. Conflicting results have also been observed where PDL1 did not bind to B7-1. For instance a study using Biacore showed a weak interaction between PDL1 and B7-1 with a Kd of 18.8 μM, with the conclusion that it is too weak affinity for the pathway to play a functional role[47].

The main questions existing in the field are the exact functions and downstream signaling of the pathways. The crystal structure of the PDL1-B7-1 pathway is not known yet. *Butte et al, 2007* did show that the PDL1-B7-1 binding site was partially overlapping with the binding site of PDL1-PD1[15]. Thus the binding orientation and the functional aspects of the pathway need to be re examined and clarified.

Chemokine receptors for tumor immunotherapy

Chemokines are chemotactic cytokines that activate G protein coupled 7-transmembrane receptor, are divided into C, CC, CXC, and CX3C sets. CX3CL1 (fractalkine) functions by binding to CX3CR1. CX3CL1 exists as either a membrane anchored pro adhesive protein or a clipped soluble form[48]. The structure of CX3CR1 is illustrated in Figure 2. The chemokine- CX3CL1 is secreted via cleavage by

metalloproteinase[49]. CX3CL1 is expressed by endothelial cells, neurons, epithelial cells, smooth muscle cells, DCs, and macrophages. CX3CR1 is expressed by immune cells, NK, T, blood monocytes[50], DC[51]. Expression of CX3CR1 identifies distinct populations of monocyte, macrophages and dendritic cells[52]. The expression of CX3CL1 is increased in inflammation, in particular with tumor necrosis factor stimulation[53]. Downstream signaling of the CX3CR1 pathway proceeds through the PI3K/AKt pathway that results in cell survival and proliferation[54]. On binding with its ligand CX3CL1, CX3CR1 promotes cell adhesion and migration by activating G protein dependent signaling and activation of Beta integrins and FAK[55].

The role of the CX3CR1-CX3CL1 axis had been highlighted in various diseases- Cancer being one of them. High CX3CR1 expression in pancreatic tumors is associated with tumor recurrence and peripheral invasion. It has been proposed for the axis to be a target to prevent tumor dissemination[55]. Breast cancer metastasis to the skeleton has been associated with CX3CR1 on breast cancer cells interacting with CX3CL1 on the stromal cells. In a study published by *Zheng et al*, 2013 mice lacking CX3CR1, showed fewer metastatic tumors and less macrophage infiltration[56]. Since CX3CL1 is widely expressed in various tissues such as lung, bone marrow, brain, these become metastatic sites for tumors expressing high levels of CX3CR1 such as pancreatic[55], breast, renal cell carcinoma[57].

Tumor associated macrophages within the tumor are present at the necrotic and hypoxic regions and switch to an M2 phenotype[58]. M2 macrophages release growth stimulatory factors VEGF, GMCSF, GCSF[59], thus promoting tumor progression, metastasis, migration and angiogenesis[56]. Macrophages enhance tumor growth of cancers- breast, prostate, colorectal and hepatocellular carcinoma. The main mechanism of the macrophage phenotypic switch to M2 s remains elusive. IL-10 has been proposed to cause upregulation of both CX3CR1 in the tumors and macrophages[60]. It is established that macrophages and tumor cells have a bidirectional cross talk through chemokines such as CX3CL1, CCL2 that promote tumor growth[60]. *Hart et al, 2014* showed for CX3CR1 positive MDSCs to accumulate in the tumor micro

environment for tumor progression[52].

Since the CX3CR1-CX3CL1 axis is also involved in the migration of the immune cells to the tumor site, whether it can be blocked from functioning for anti tumor immunotherapy remains in question.

CTLA4 blockade for Tumor immunotherapy

The mechanism of CTLA4 antibodies for anti tumor immunotherapy has remained elusive[61]. *Krummel et al, 1996* proposed for antibodies against CTLA4 to block the binding between CTLA4 and B7-1, B7-2 thus preventing T cell inhibition for efficient anti tumor immunotherapy[62]. Studies also pointed out Tregs to express very high levels of CTLA4 and showed that anti CTLA4 antibodies result in increase of Tregs and Teff cells in the lymph nodes, while decreasing the Tregs in the tumor microenvironment thus altering the Teff/Treg ratio in the tumor[61, 63]. This is mediated by depletion of Tregs mediated by the Fc region of anti mCLA4 antibodies[63]. Mouse IgG2a isotype most efficiently depletes Tregs through mechanisms of ADCC[63] and mouse IgG2b isotype less effectively engages ADCC (previously described). The cells involved in ADCC are NK, monocytes, macrophages within the tumor microenvironment. The IgG2a and the IgG2b isotypes bind to soluble forms of FcyRI, FcyRIIB, FcyRIII, FcyRIV and FcRn[63]. Thus it has been proposed for both the blocking effect of the antibodies and depletion of CTLA4 positive cells to be responsible for tumor immunity[61]. The importance of each individual pathway for anti tumor immunity has not been dissected out and remains in question.

1.2 Schematic Figures

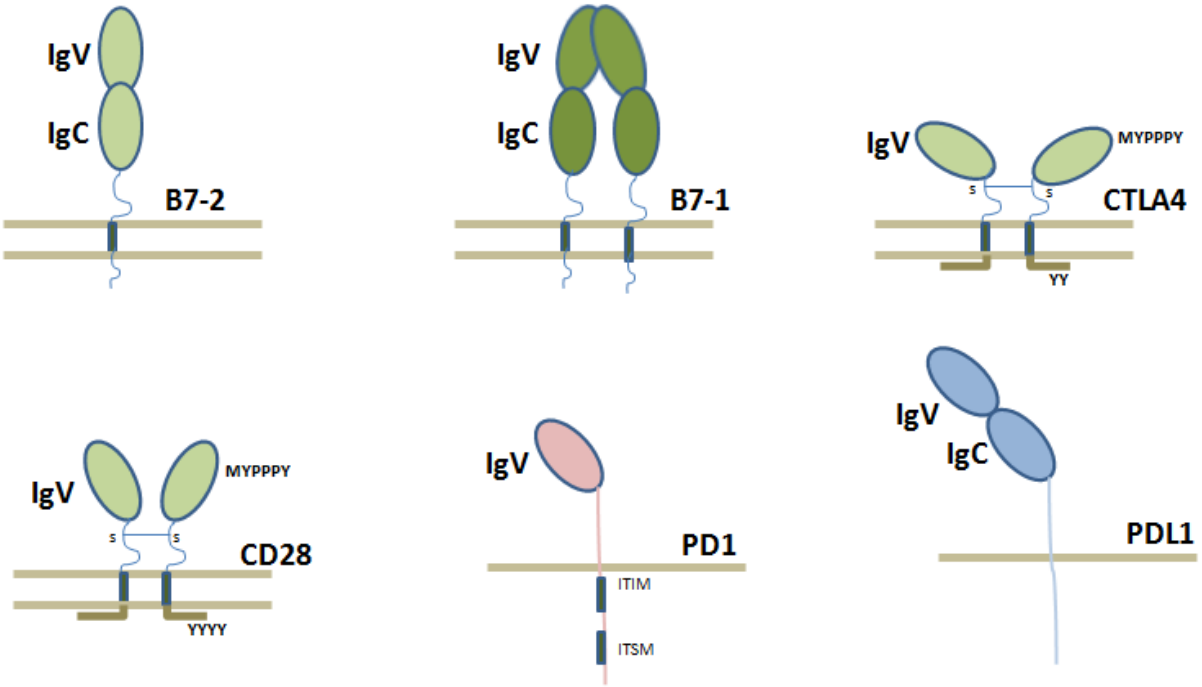


Figure 1. Structure of co-stimulatory molecules[64]

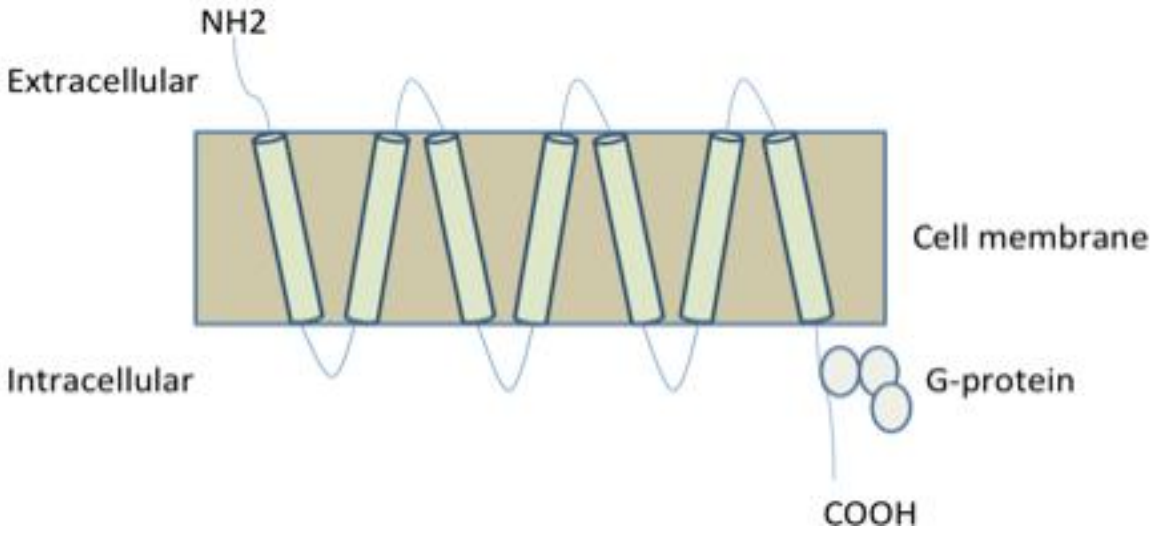


Figure 2. Structure of CX3CR1, a 7 transmembrane GPCR[2]

Chapter 2 Data and Methods

2.1.1 Abstract- Interaction of PDL1 with B7-1

PDL1-PD1 mediated immune suppression is co-opted by tumors to evade immune attack. PDL1 has also been shown to bind to B7-1 and the PDL1:B7-1 pathway functions to help maintain T cell tolerance. Some Biacore studies have shown a moderate affinity for the PDL1:B7-1 interaction but others have reported a very low affinity. To understand this further, we used ELISA and flow cytometry and showed that PDL1 and B7-1 have a strong binding interaction when the PDL1 molecule is accessible and flexible but not when constrained. PDL1 transfected cells did not bind to B7-1 transfected cells. In contrast, PDL1 cells bound to PD-1 cells, and B7-1 cells bound to CD28 cells indicating that the PD-L1:B7-1 interaction can occur in cis when the molecules are on the same cell but not in trans when the molecules are on different cells. Further the PDL1: B7-1 interaction competes for the binding of PDL1 to PD1. Our results confirm the interaction of PDL1 with B7-1 but show it is structurally constrained to occur in cis on the same cell surface rather than in trans between two cells.

2.1.2 Introduction

The PDL1-B7 pathway was discovered in 2007. Follow up studies threw some light on the functional role of the pathway, and tried to understand its effects on the major immunotherapeutically relevant PDL1-PD1 pathway[43, 44, 45, 46]. Studies have not yet established the exact role as well as the mechanistic basis of the PDL1-B7 pathway. Some of the questions remaining in the field that are yet to be answered are : 1) Is the affinity of B7-1 for PDL1 sufficient to be biologically relevant; 2) the downstream signaling and the functional role of the pathway [47]. Here I sought to examine the orientation of the molecules for a strong, functionally relevant interaction to take place. We use a cell conjugation assay, and two approaches of ELISA and flow cytometry to answer our questions. The cell conjugation assay tests the interaction to see if molecules on two different cells bind to each other. The ELISA and Flow cytometry vary the assay design to mimic the interactions with a more accessible PDL1 molecule versus a naturally occurring PDL1 molecule. I also screen different PDL1 antibodies to distinguish the blockers of PD1 and B7-1 from the non blockers. To determine the functional role of the pathway, I set up a competition assay between PD1 and B7-1 for binding with PDL1. My results suggests a unique orientation under which the pathway may operate. I also delve into the mechanistic aspects of the pathway. I identify different PDL1 antibodies that are blockers and non blockers. My results contribute to the existing knowledge of the PDL1-B7 interactions and change the common view of the interaction.

2.1.3 Materials and Methods

Cells and culture media

The mouse 300.19 pre-B cell line was derived from Swiss Webster mice [65]. The 300.19 cells, 300.19 PDL1 transfected cells, 300.19 PDL1 IgV-Tim3 mucin domain transfected cells, were transfected by electroporation with the relevant mouse or human cDNA constructs in the pEF-Puro or pEF6-Blasticidin expression vectors in our laboratory. Cells were selected in media containing puromycin or blasticidin, sorted with specific monoclonal antibodies (mAbs), and subcloned. Cell-surface expression of the indicated molecules was verified by flow cytometry using specific mAbs [66]. Cells were cultured at 37ºC with 5% CO_2. The medium for transfected 300 cells was RPMI-1640 (Mediatech) supplemented with 10% heat-inactivated FBS (Invitrogen), 1% streptomycin/penicillin, 15µg/ml gentamicin (Invitrogen), 1% glutamax (Invitrogen), 50µM β-mercaptoethanol (Sigma-Aldrich), and 5µg/ml puromycin or blasticidin. For EL4 cells, β-mercaptoethanol was not included.

Fusion Proteins

Recombinant proteins human B7-1-hIgG1 and human PD1-hIgG1 were purchased from R&D systems. Human IgG was purchased from Jackson. hPD1-mIgG2a and hB7-1-mIgG2a were purchased from Chimerigen. hPDL1-mIgG2a was made in our laboratory.

Antibodies

Goat anti-mouse IgG2a, PE-conjugated goat F(ab')2 anti-human IgG (absorbed against mouse Ig) and HRP-conjugated goat anti-human IgG (absorbed against mouse Ig) were purchased from Southern Biotech. Anti-human PDL1 antibodies; anti-mouse PDL1 antibodies were made in our laboratory. Mouse IgG1 isotype control antibody (clone MOPC21) was purchased from BioXcell. Mouse IgG2b and Mouse

IgG2a were purchased from Southern Biotech Secondary antibodies cross absorbed against the other species (mouse or human) were used.

Cell conjugation assay

A cell conjugation assay for cell surface receptor-ligand binding was developed in our previous study [16]. Briefly, cells transfected with cell surface gene 1 were labeled with the red fluorescent dye PKH26 (Sigma), and cells transfected with cell surface gene 2 were labeled with the green fluorescent dye PKH67 (Sigma). Red dye-labeled and green dye-labeled cells were incubated together for 45 min at 37°C in a round bottom 96 well plate in a 5% CO2 incubator[16]. Conjugate formation was analyzed immediately by flow cytometry using the PE channel for the red dye and the FITC channel for the green dye. Data were analyzed using FlowJo 9.5.2 software (TreeStar).

ELISA

The binding of hPDL1 with hPD1 and hB7-1 was assessed using ELISA. Plates were coated with the primary protein at the indicated concentrations in PBS for overnight at 4°, then blocked in 1% BSA. Subsequently washed in ELISA washing buffer(Phosphate buffer saline pH 7.4 with 0.05% Tween 20). Fusion proteins were diluted in PBS plus 1% BSA at the indicated concentrations. The incubation was for an hour. The secondary detection was with Goat anti human IgG HRP. EC-50 values were calculated using 4 parameter variable slope regression curve.

Flow Cytometry

Binding of 300 hPDL1 transfected cells and 300 hPDL1 IgV-Tim-3 mucin domain transfected cells with the fusion proteins was assayed using flow cytometry. The secondary PE Conjugated antibody was used at the indicated concentrations. The binding was analysed using the PE channel of the BD FACS Canto II. EC-50 values were calculated using 4 parameter variable slope regression curve.

2.1.4 Results

PDL1 binds weakly to B7-1 in a constrained orientation but strongly in a flexible orientation

To study the interaction between PDL1 and B7-1 I use an ELISA format. When the fusion protein hPDL1-mIgG2a was adhered to the plate, B7-1-hIgG bound very weakly with an EC-50 of 1.594 µg/ml. EC-50 is defined as the concentration of the antibody where 50 percent of its maximal binding is observed. The PD1-hIgG bound strongly with an EC-50 of 0.022 µg/ml as shown in Figure 3b. The method used is illustrated in Figure 3a. I next developed an assay where the ligand Ig fusion protein was bound to the plate indirectly via an anti-Fc antibody, thereby allowing the ligand protein a greater degree of freedom. I coated the plate with Goat anti mouse IgG2a and then added the fusion protein hPDL1-mIgG2a such that it had more molecular flexibility. The method is illustrated in Figure 4a. In this case the binding affinity of B7-1 was stronger and greater than the previous observed result with directly plate bound PD-L1, with an EC-50 of 0.060 µg/ml (Figure 4b).

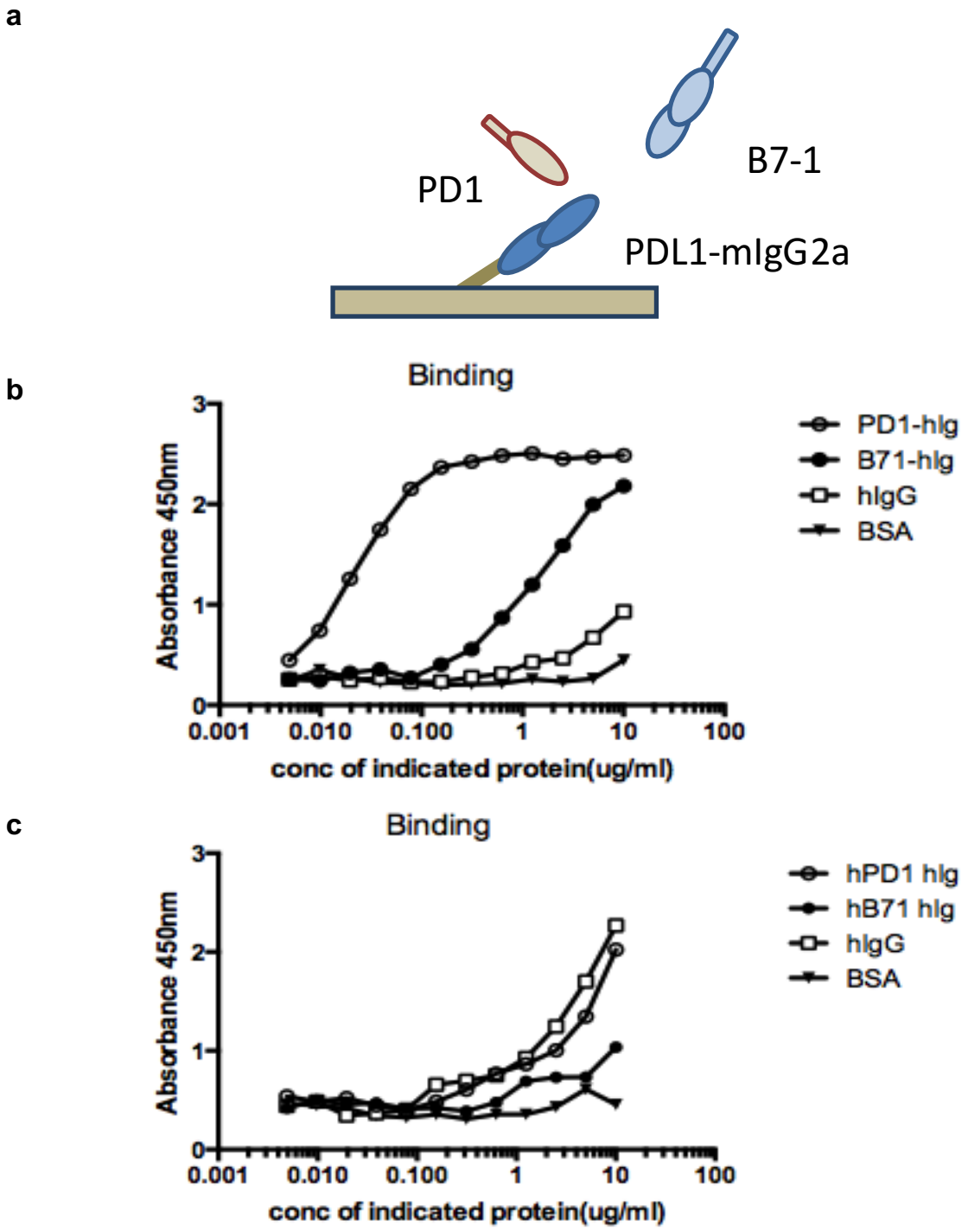


Figure 3. B7-1 binds weakly to PDL1 in a natural trans configuration. (a) Illustration for the ELISA format used; (b) ELISA plate was coated with hPDL1-mIgG2a at 2ug/ml. Fusion proteins hPD1-hIgG1, hB71-hIgG1 and control hIgG were added to the immobilized proteins at 10ug/ml with a series of two fold dilutions. The detection was using Goat anti human IgG HRP; (c) ELISA plate was coated with mIgG2a. Fusion proteins hPD1-hIgG1, hB71-hIgG1 and control hIgG were added to the immobilized proteins at 10ug/ml with a series of two fold dilutions. The detection was using Goat anti human IgG HRP

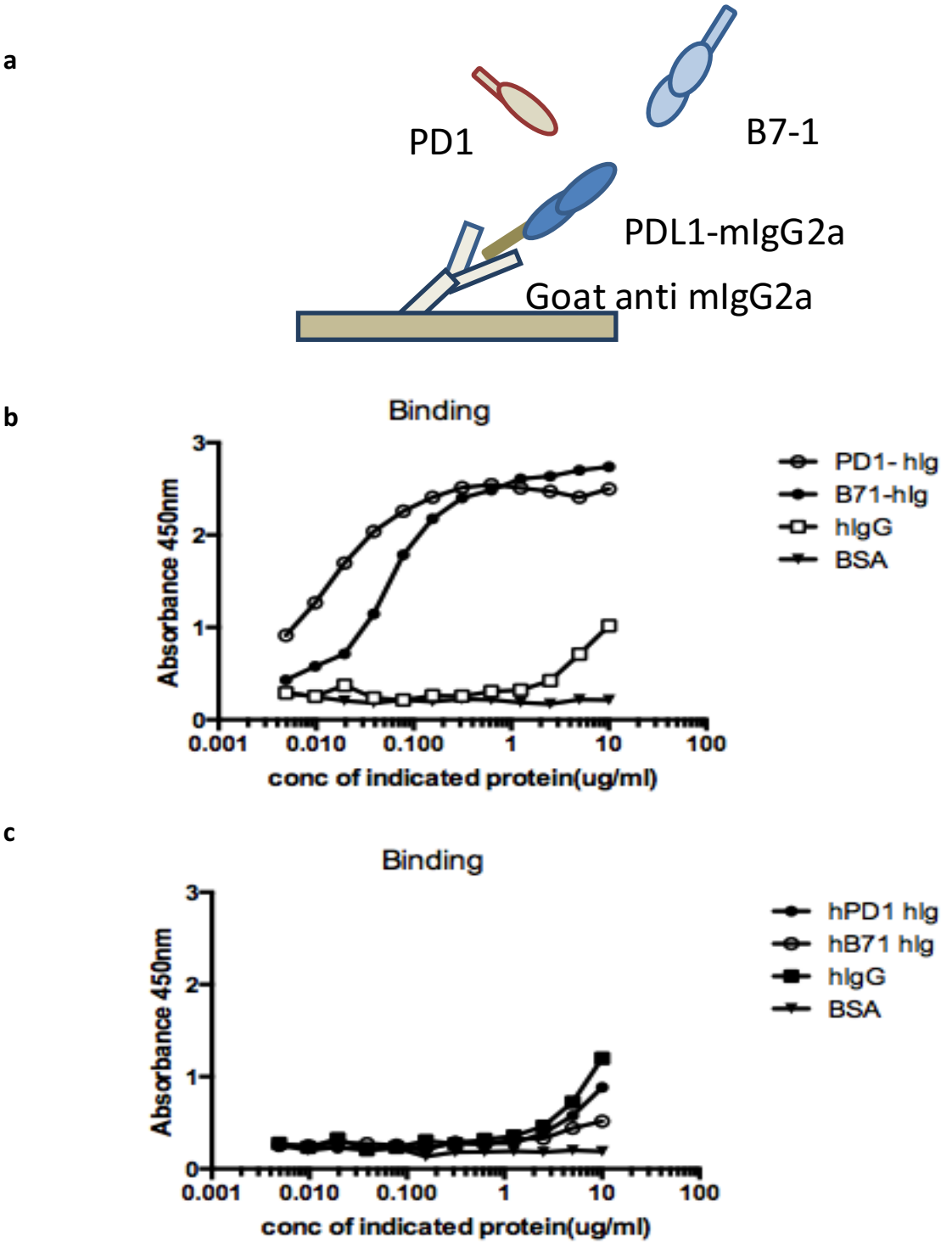


Figure 4. Strong Interaction of B7-1 with PDL1 on increasing PDL1 accessibility. (a) Illustration for the ELISA format used; (b) ELISA plate was coated with 2ug/ml Goat anti mouse IgG2a followed by addition of 10ug/ml hPDL1-mIgG2a. After washing, fusion proteins PD1-hIgG1, B71-hIgG1 and control hIgG were added to the immobilized proteins at 10ug/ml with a series of two fold dilutions. The detection was using Goat anti human IgG HRP; (c) ELISA plate was coated with Goat anti mIgG2a at 2ug/ml followed by addition of mIgG2a. Fusion proteins hPD1-hIgG1, hB71-hIgG1 and control hIgG were added at 10ug/ml with a series of two fold dilutions. The detection was using Goat anti human IgG HRP.

To rule out the possibility of the recognition of mIgG2a by Goat anti hIgG HRP in the above two settings, I performed an experiment and determined that the binding affinities of the fusion proteins, hPD1-hIg, hB7-1-hIg, hIg with immobilized plate coated mIgG2a was minimal (Figure 3c, Figure 4c).

I extended this study to transfected cell lines to mimic the in-vivo molecular orientation of a human PDL1 molecule, and determined PDL1 binding to its ligands PD1 and B7-1. I used 300.19 hPDL1 transfected cells that have a naturally occurring PDL1 on the cell surface (Figure 5a). Here, similarly to the direct ELISA, I observed PDL1 bound very weakly to B7-1 but strongly to PD1. EC-50 of PDL1-B7-1 was non determinable and of PDL1-PD1 was 1.25 µg/ml respectively (Figure 5b). I next used 300.19 cells transfected with hPDL1-Tim 3 mucin domain. Here the PDL1 IgV domain is connected to the flexible mucin domain of Tim 3, allowing PDL1 to be more accessible with greater flexibility (Figure 6a). PDL1 bound strongly to both B7-1 (EC-50, 1.061 µg/ml) and PD1 (EC- 50, 1.423 µg/ml) (Figure 6b). Our data are consistent with the previous finding that PDL1 IgV domain is necessary for binding with PD1 and B7-1 and also show the IgV domain is sufficient for this binding[43]

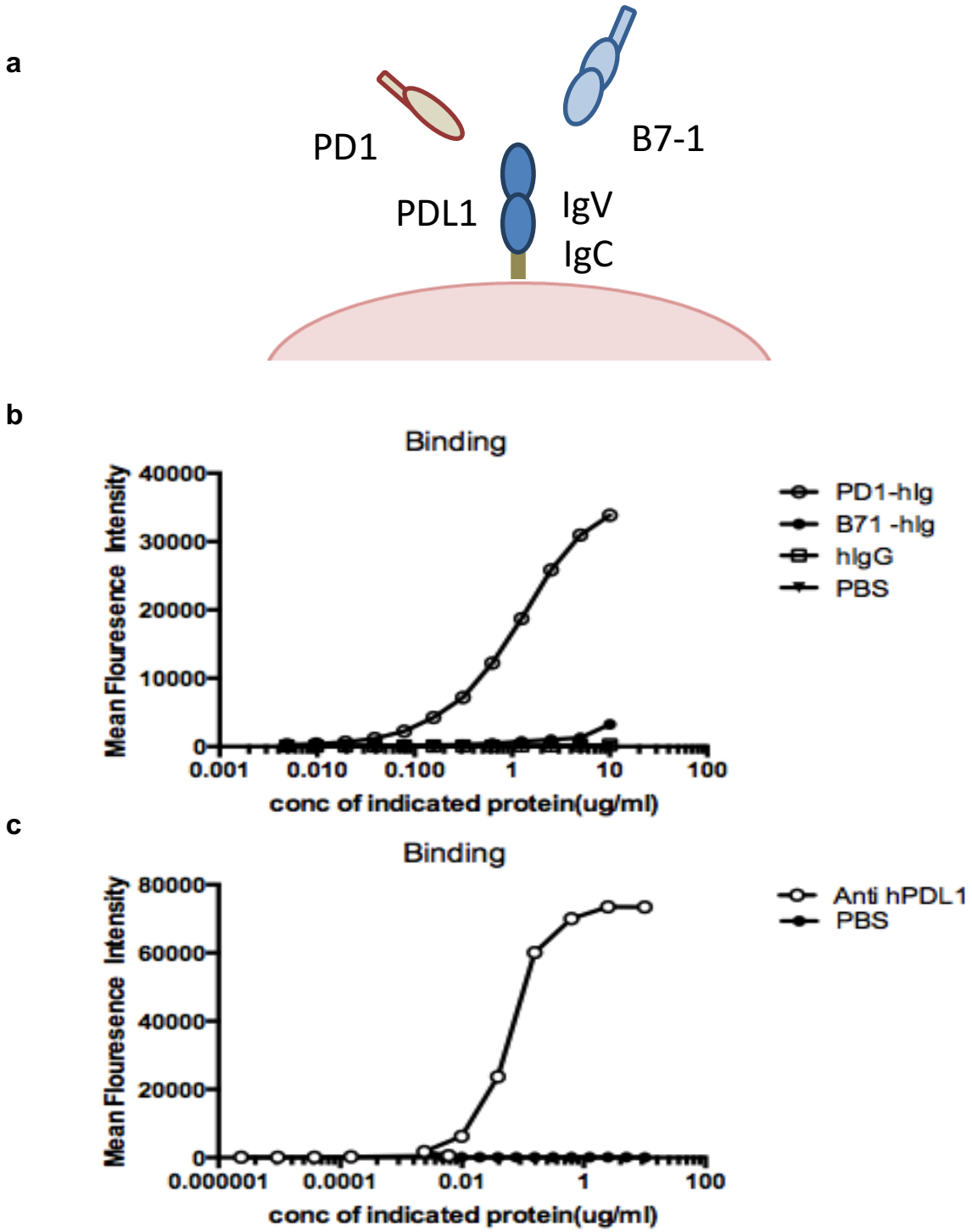


Figure 5. B7-1-Ig binds weakly to cell surface PDL1 in a natural orientation. (a) Diagrammatic representation of 300.19 hPDL1 transfected cells; (b) 300 hPDL1 transfected cells were stained with fusion proteins PD1-hIgG1, B71-hIgG1 and control hIgG at 10ug/ml in a series of two fold dilutions. The secondary antibody used was Goat anti human IgG PE; (c) 300 hPDL1 transfected cells were stained with 1538 antihPDL1-hIgG1 at 10ug/ml in a series of four fold dilutions. The secondary antibody Goat anti human IgG PE was used at 5ug/ml

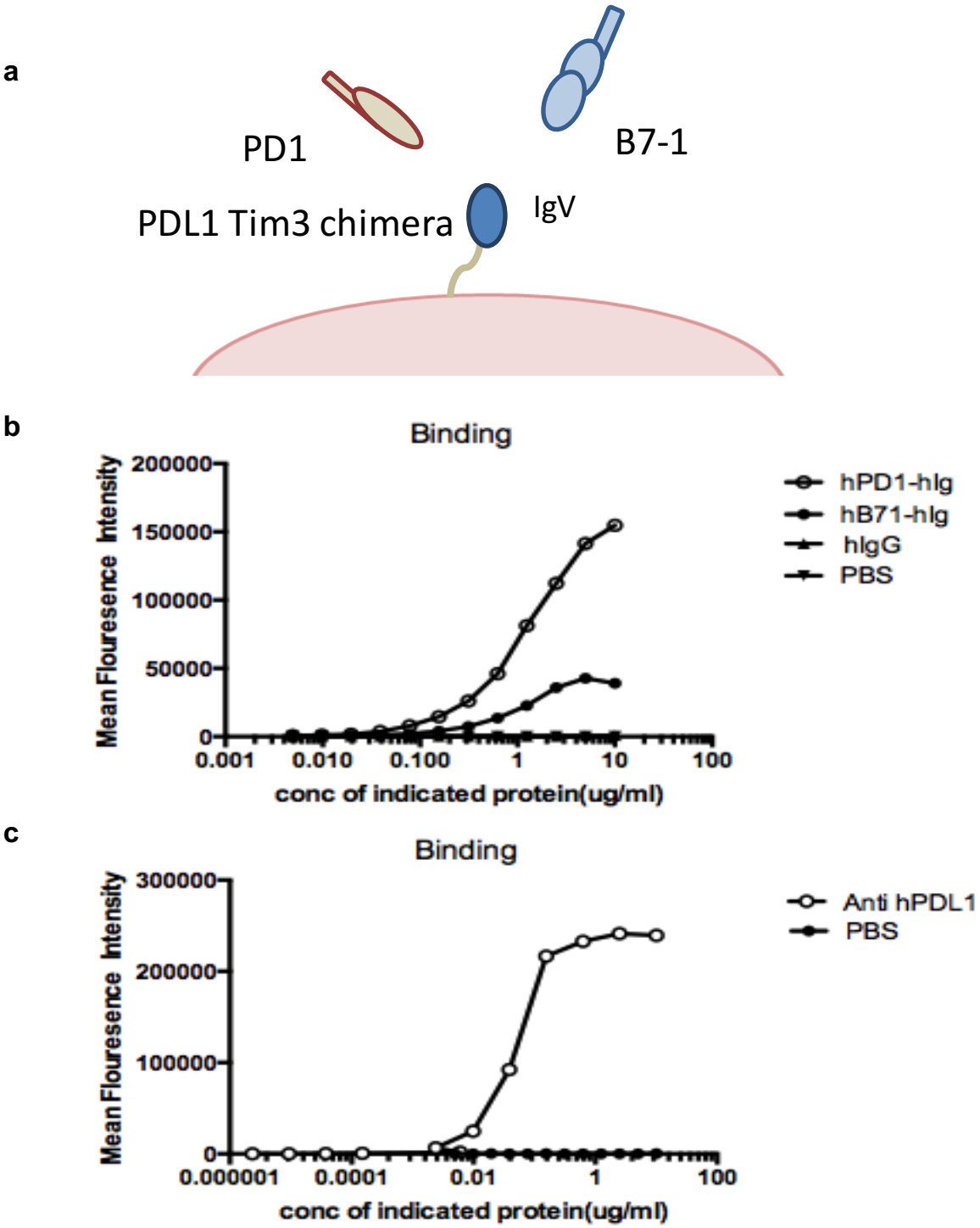


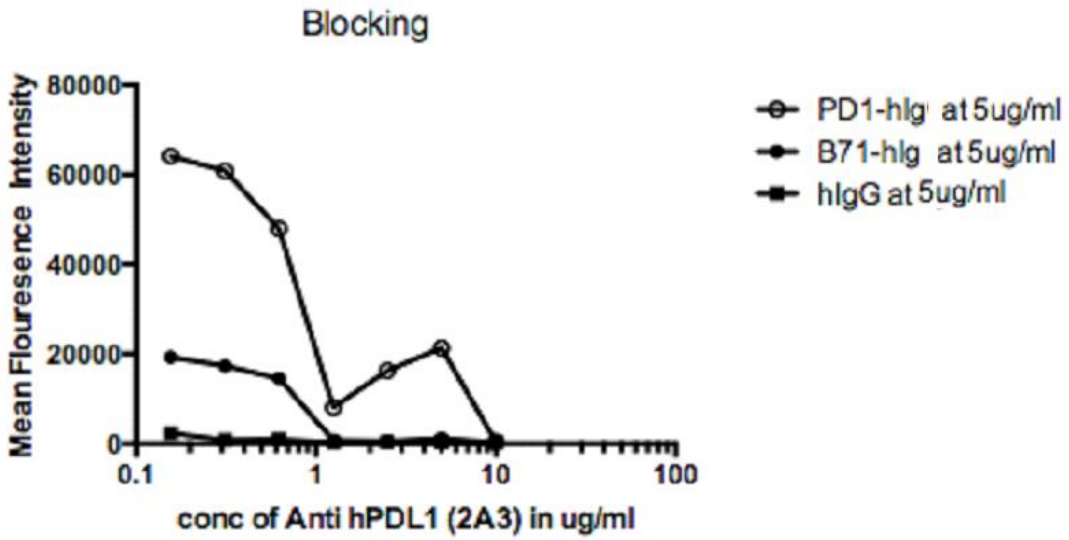
Figure 6. Flexible cell surface PDL1 binds strongly to B7-1. (a) Diagrammatic representation of transfected 300.19 hPDL1-Tim3 chimera cells; (b) 300 hPDL1 Tim3 mucin domain transfected cells were stained with fusion proteins PD1-hIgG1, B71-hIgG1, control hIgG, at 10ug/ml in a series of two fold dilutions. The secondary antibody was F(ab')2 Goat anti human IgG PE; (c) 300 hPDL1 transfected cells were stained with 1538 anti hPDL1-hIgG1 at 10ug/ml in a series of four fold dilutions. The secondary antibody F(ab')2 Goat anti human IgG PE was used at 5ug/ml.

As a positive control I tested the binding of anti human PDL1 mAb (clone 1538) with 300.19 cells transfected with hPDL1 and hPDL1-Tim 3. In both cases as expected I obtained a very high binding affinity of the anti PDL1 antibody with the PDL1 transfected cells (Figure 5c, Figure 6c). The observed difference in the mean fluorescent intensity between the two transfected cells is likely due to the difference in the number of PDL1 molecules expressed on the cell surfaces. This however does not interfere with the conclusions as the binding affinity of hB7-1 as compared to hPD1 is increased in the case of the flexible orientation of hPDL1.

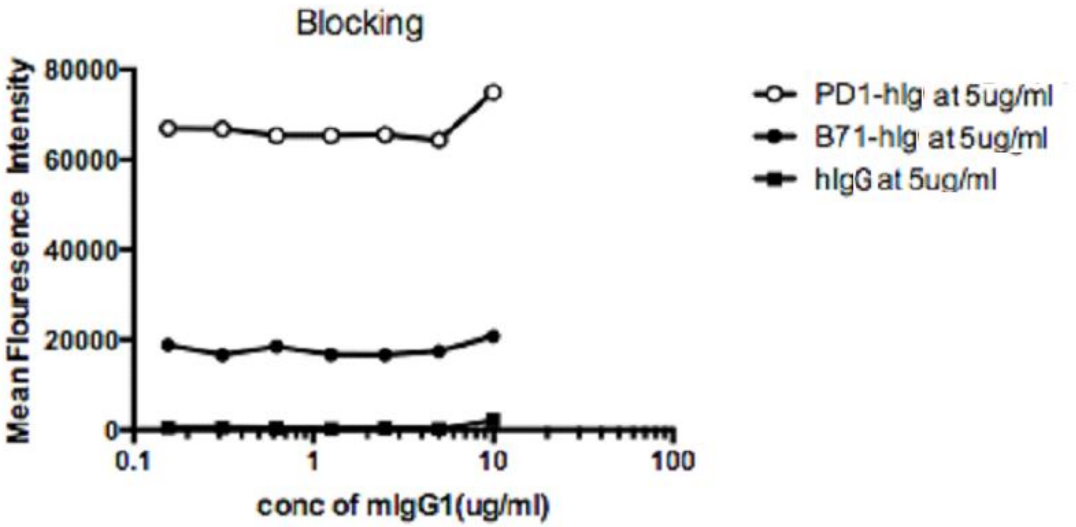
PDL1 antibodies block the binding of B7-1 to PDL1 in a concentration dependent manner

To assess the nature of the binding of B7-1 to PDL1 I used a series of anti hPDL1 antibodies. I observed many anti PDL1 antibodies block the binding of both PD1 and B7-1 to PDL1 and this blockade decreased with decreasing mAb concentrations (Figure 7a). One such example of anti hPDL1 mAB 29E.2A3, is illustrated in Figure 7a. I checked the possibility of the anti PDL1 antibodies, and of the isotype controls, mIgG2a and MOPC21 mIgG1, to be detected by PE conjugated antibody. As expected no significant binding interaction with 300.19 hPDL1-Tim3 transfected cells was detected, showing the secondary is specific for human IgG and does not detect mouse IgG (Figure 7b, 7c, 7d). My data is consistent with the previously obtained results of the binding site on PDL1 to be in the same vicinity on the IgV domain for both PDL1 and B7-1[43]. I further observed some PDL1 antibodies to be non blockers of B7-1 and PD1 to PDL1 (Figure 8a, 8b). One specific non blocker 4C10 bound to the IgV domain (Figure 9a, 9b). The summary of the PDL1 antibodies as well as their specific domain binding regions on PDL1 are illustrated in Table 1, 2. The non blockers in Table 1 did not bind to the IgV domain, it is likely that they bind to the IgC domain on the hPDL1 molecule.

a


Blocking
Mean Flouresence Intensity
80000
60000
40000
20000
0
0.1
1
10
100
conc of Anti hPDL1 (2A3) in ug/ml
PD1-hIg at 5ug/ml
B71-hIg at 5ug/ml
hIgG at 5ug/ml

b


Blocking
Mean Flouresence Intensity
80000
60000
40000
20000
0
0.1
1
10
100
conc of mIgG1(ug/ml)
PD1-hIg at 5ug/ml
B71-hIg at 5ug/ml
hIgG at 5ug/ml

c

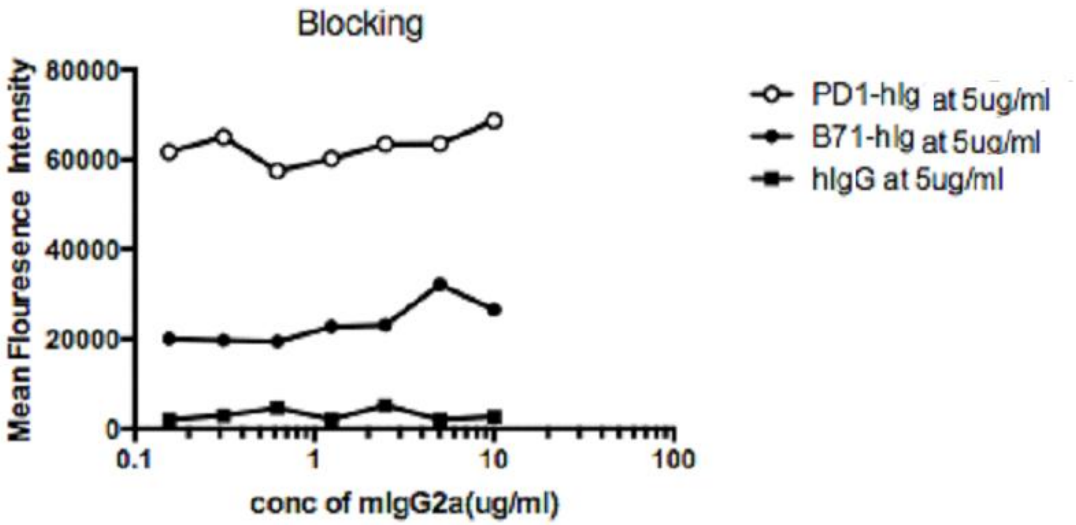

Blocking
Mean Flouresence Intensity
80000
60000
40000
20000
0
0.1
1
10
100
conc of mIgG2a(ug/ml)
PD1-hIg at 5ug/ml
B71-hIg at 5ug/ml
hIgG at 5ug/ml

Figure 7 Continued

c

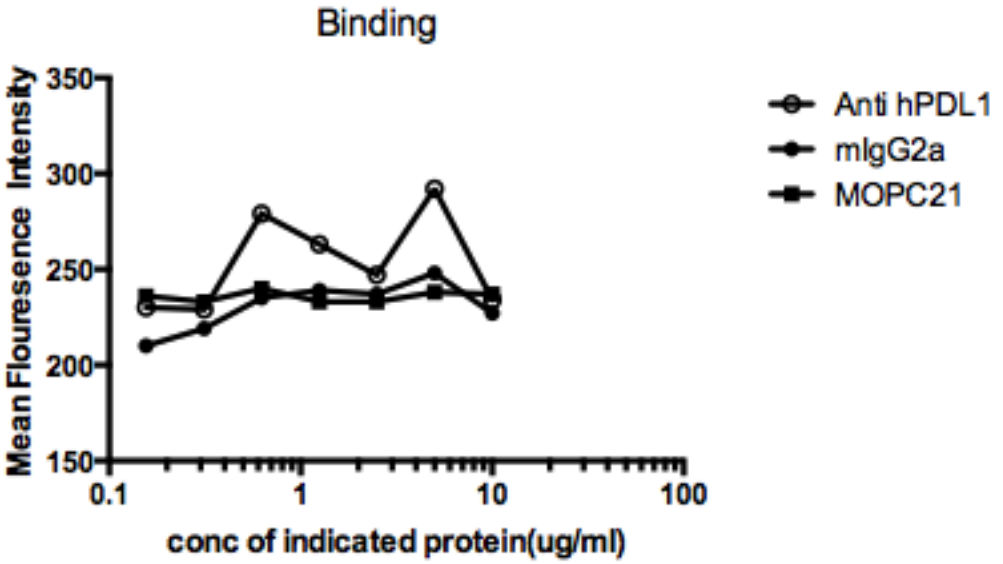
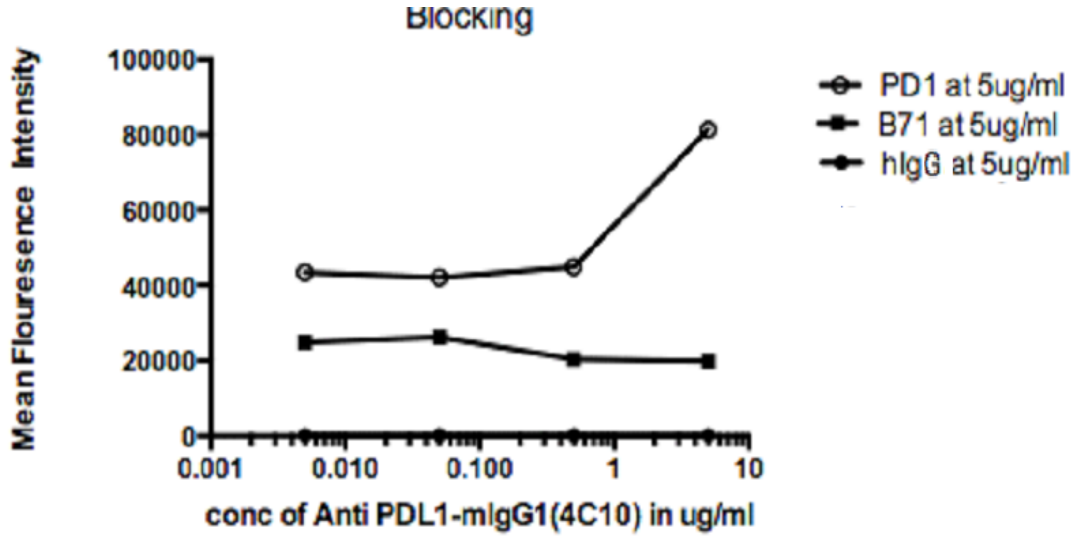


Figure 7. PDL1 antibodies block the binding of PD1 and B7-1 with PDL1 (a) 300 hPDL1 Tim3 domain transfectants were incubated with 10ug/ml of PDL1 antibody, 29E.2A3 in two-fold dilutions. The fusion proteins PD1-hIgG1, B7.1-hIgG1, control hIgG were added at 5ug/ml. The secondary antibody used was goat anti human IgG PE; (b) 300 hPDL1 Tim3 domain transfectants were incubated with 10ug/ml of mIgG2a in two-fold dilutions. The fusion proteins PD1-hIgG1, B7.1-hIgG1, control hIgG were added at 5ug/ml. The secondary antibody used was goat anti human IgG PE; (c)300 hPDL1 Tim3 domain transfectants were incubated with 10ug/ml of MOPC21; mIgG1 in two-fold dilutions. The fusion proteins PD1-hIgG1, B7.1-hIgG1, control hIgG were added at 5ug/ml. The secondary antibody used was goat anti human IgG PE; (d) 300 hPDL1 Tim3 domain transfectants were incubated with 10ug/ml of mIgG2a, 29E.2A3, MOPC21; mIgG1 in two-fold dilutions. The secondary antibody used was goat anti human IgG PE.

B7-1 blocks the binding of PD1 with hPDL1

I set up a competition assay between PD1 and B7-1 to further understand the in vivo biology of the two molecules for binding to PDL1. Both PD1 and B7-1 blocked each other's binding to PDL1 at higher concentrations. PD1 was able to compete for the binding of B7-1 with PDL1. As expected the EC-50 for competition of B7-1 was greater than PD.1 (6.978 μg/ml and 1.460 μg/ml respectively)(Figure 10a, Figure 11a). As positive controls I used anti hPDL1 antibodies and observed that they blocked the binding of both B7-1-mIgG2a and PD1-hIg with PDL1 (Figure 10b, 11b).

a



b

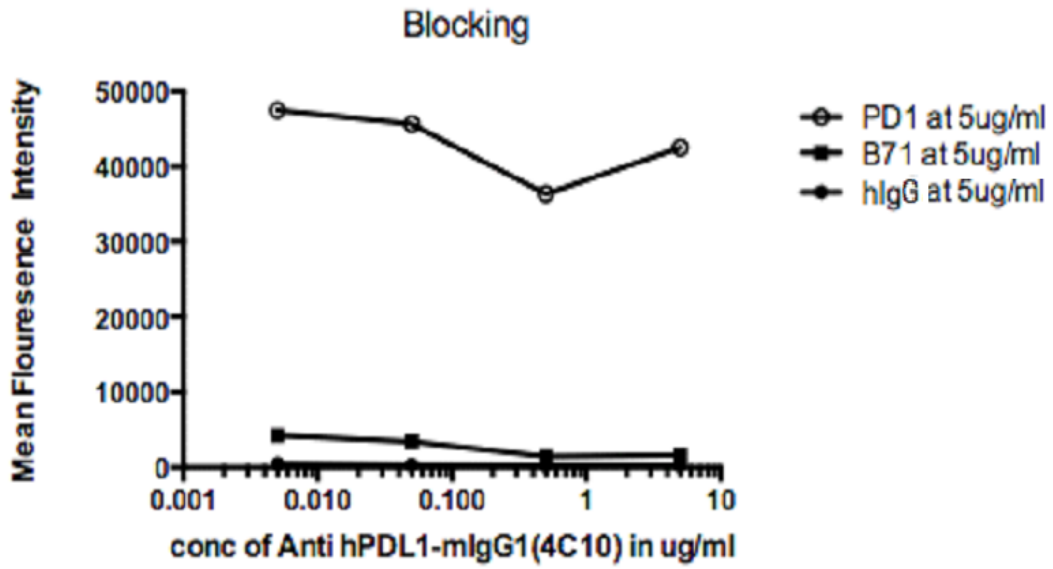
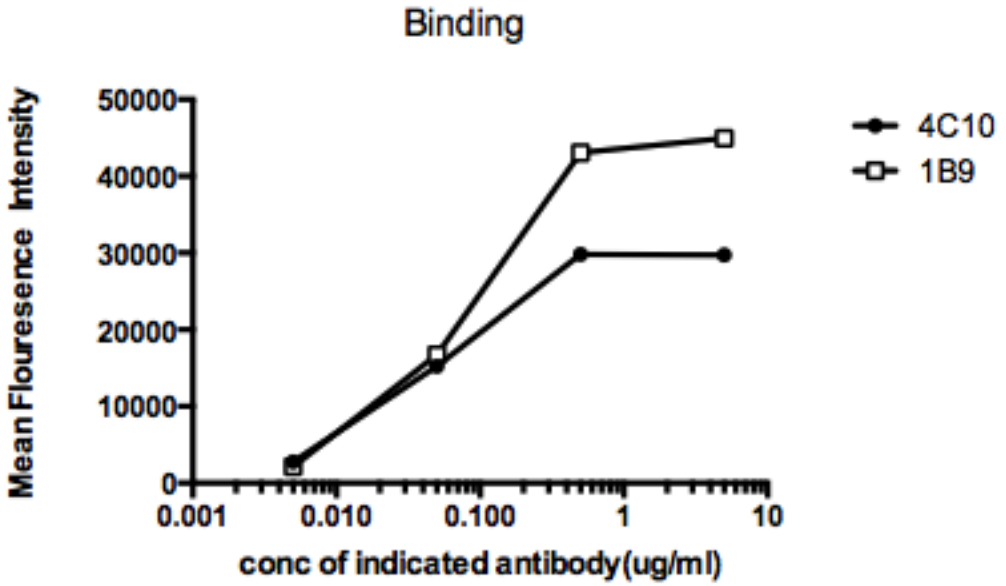


Figure 8.PDL1 antibodies are also non blockers and do not inhibit PD1 and B7-1 from binding with PDL1. (a) 300 hPDL1 Tim3 domain transfectants were incubated with 10ug/ml of PDL1 antibody, 4C10 in two-fold dilutions. The fusion proteins PD1-hIgG1, B7.1-hIgG1, control hIgG were added at 5ug/ml. The secondary antibody used was goat anti human IgG PE; (b) 300 hPDL1 Tim3 domain transfectants were incubated with 10ug/ml of PDL1 antibody, 4C10 in two-fold dilutions. The fusion proteins PD1-hIgG1, B7.1-hIgG1, control hIgG were added at 5ug/ml. The secondary antibody used was goat anti human IgG PE

a



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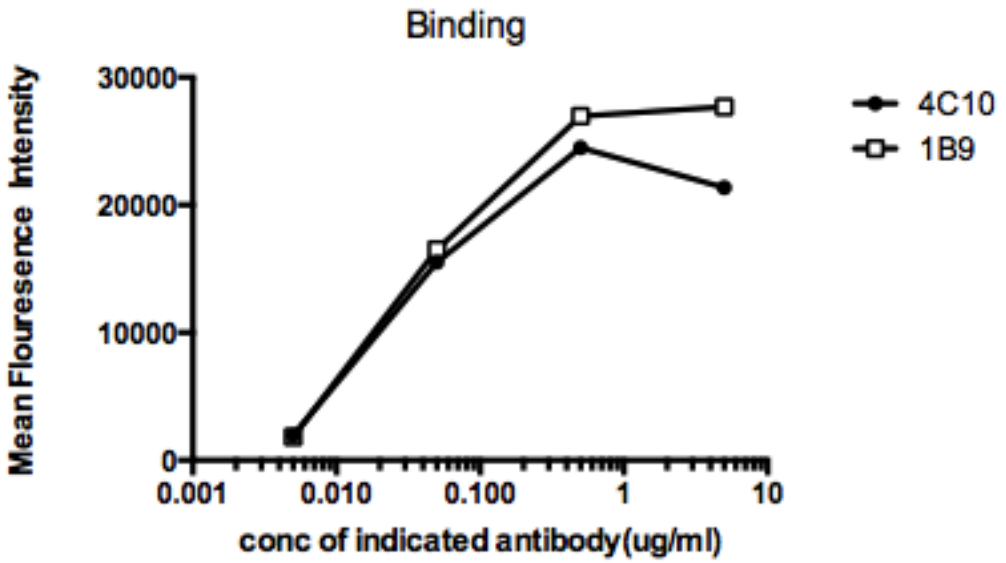
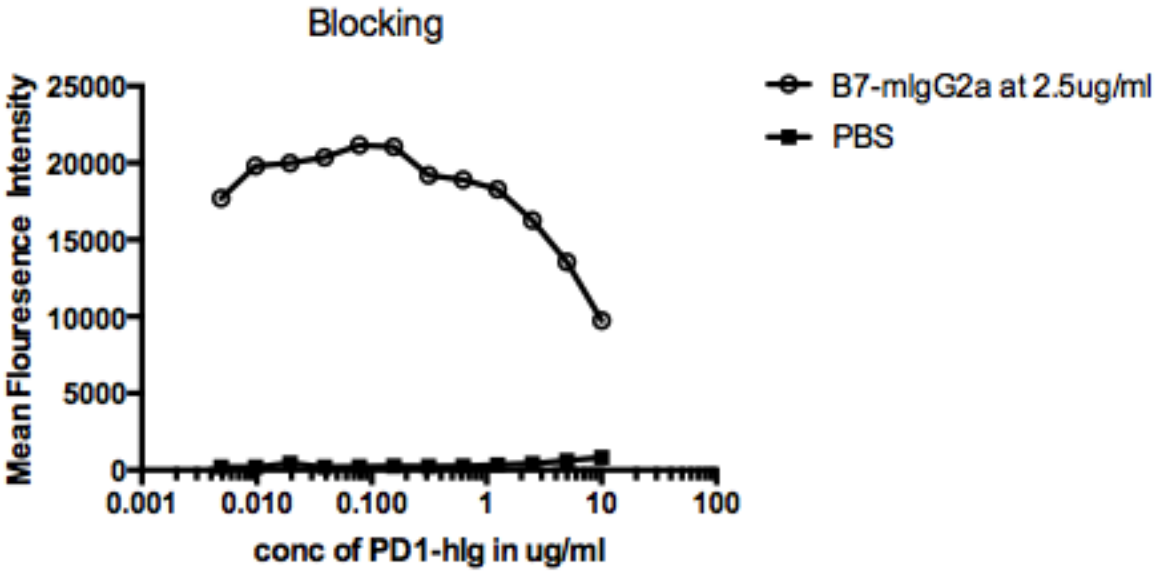


Figure 9. PDL1 mAb 4C10 binds to the IgV domain of hPDL1. (a)300 hPDL1 Tim3 domain transfectants were incubated with 10ug/ml of PDL1 antibody, 4C10 and 1B9 in two-fold dilutions. The secondary antibody used was goat anti mouse IgG PE; (b)300 hPDL1 were incubated with 10ug/ml of PDL1 antibody, 4C10 and IB9 in two-fold dilutions. The secondary antibody used was goat anti mouse IgG PE

a



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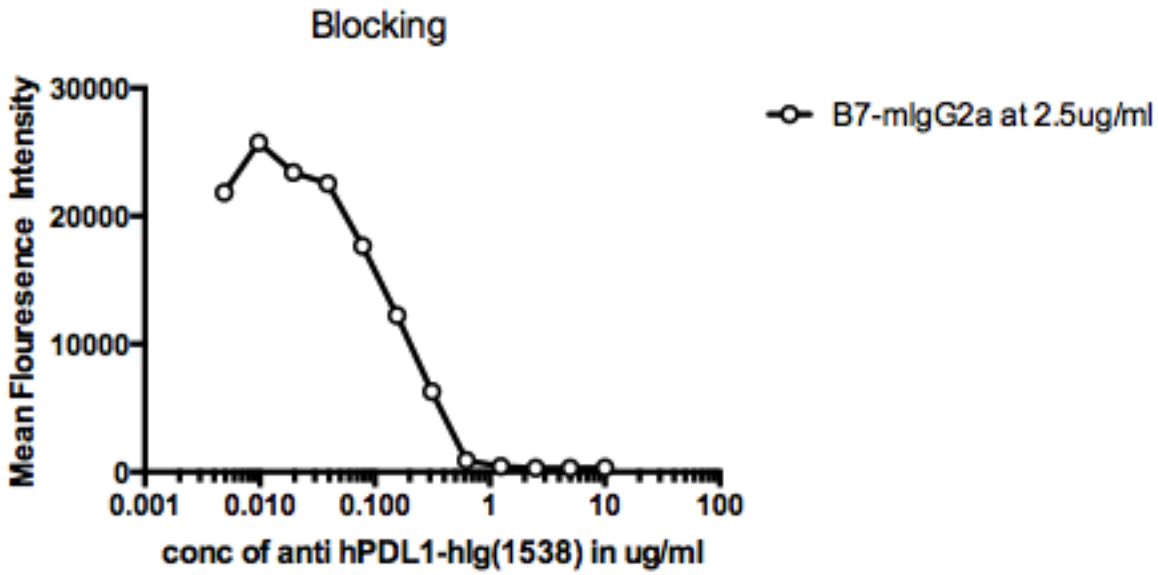


Figure 10. PDL1 competes for the binding of B7-1 with PD1 (a) 300hPDL1 Tim3 transfectants were incubated with 20ug/ml of PD1-hIg in a series of two fold dilutions. The fusion protein B7-mIgG2a were added at 2.5ug/ml. The detection reagant was $F(ab)_2$ anti-mIgG2a PE; (b) 300hPDL1 Tim3 transfectants were incubated with 20ug/ml of 1538 with a series of two fold dilutions. The fusion protein B7-mIgG2a were added at 2.5ug/ml. The detection reagant was $F(ab)_2$ anti-mIgG2a PE.

Table 1. Characterization of hPDL1 antibodies

Antibody	Binding of hPD1 to hPDL1	Binding of hB71 to hPDL1	PDL1 Domain Specificity
29E.1D5	blocker	blocker	IgV
29E.2A3	blocker	blocker	IgV
29E.5A9	blocker	blocker	IgV
29E.10D9	blocker	blocker	IgV
29E.11D12	blocker	blocker	IgV
29E.12B1	blocker	blocker	IgV
368A.1.1B9	blocker	blocker	IgV
368A.1.4H1	blocker	blocker	IgV
368A.1.5A4	blocker	blocker	IgV
368B.3.1D11	non blocker	non blocker	not IgV
368B.3.2C10	non blocker	non blocker	not IgV
298.1D5	blocker	blocker	IgV
298.3D12	blocker	blocker	IgV
298.3H2	blocker	blocker	IgV
298B.3C6	non blocker	non blocker	not IgV
298B.3E10	blocker	blocker	IgV
298B.3E12	blocker	blocker	IgV
298B.3G6	non blocker	non blocker	not IgV
298B.4C1	anti m PDL1; very weak for h PDL1	anti m PDL1; very weak for h PDL1	not determined
298B.8C3	blocker	blocker	IgV
298B.8E2	blocker	blocker	IgV
339.4C10	non blocker	non blocker	not IgV
339.4D6	blocker	blocker	IgV
339.6A2	blocker	blocker	IgV
339.7C3	blocker	blocker	IgV

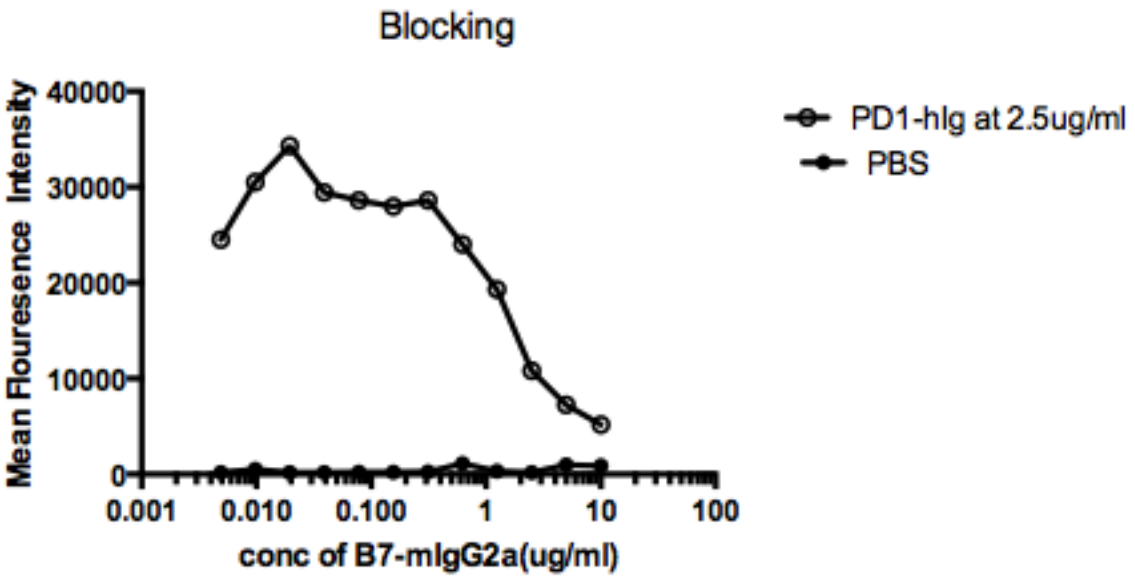
Table 2. Non blocking hPDL1 antibodies

Antibody	Binding of hPD1 to hPDL1	Binding of hB71 to hPDL1	PDL1 Domain Specificity
368B.3.1D11	non blocker	non blocker	not IgV
368B.3.2C10	non blocker	non blocker	not IgV
298B.3C6	non blocker	non blocker	not IgV
298B.3G6	non blocker	non blocker	not IgV
339.4C10	non blocker	non blocker	not IgV

PD-L1 transfected 300 cells do not bind to B7-1 transfected 300 cells

To study the cell surface receptor-ligand binding of PD-L1 to B7-1, I used a cell conjugation assay [16]. The parental 300.19 cells and transfectants grow as non-adherent single cells. One transfected cell was labeled with a red fluorescent dye and the other transfected cell with a green fluorescent dye. The binding of the two cells was assessed by flow cytometry and indicated by double positive events (yellow dots) with higher forward scatter (FSC), indicating conjugate formation. We found that hPD-L1 transfected 300 cells did not bind to hB7-1 transfected 300 cells (Figure 12a). In contrast, hPD-L1 transfected 300 cells bound to hPD-1 transfected 300 cells (Figure 12b). hCTLA4 transfected 300 cells bound to hB7-1 transfected 300 cells(Figure 12c). As a control hCTLA4 transfected cells did not bind to hPD-1 300 transfected cells (Figure 12d). We also observed for mB7-1 transfected cells to bind to mCD28 transfected cells (data not shown). Taken together, our results with transfected B cell and T cell lines show that the structural orientation of PD-L1 and B7-1 is not compatible with binding *in trans* between two cells (cell surface to cell surface binding).

a



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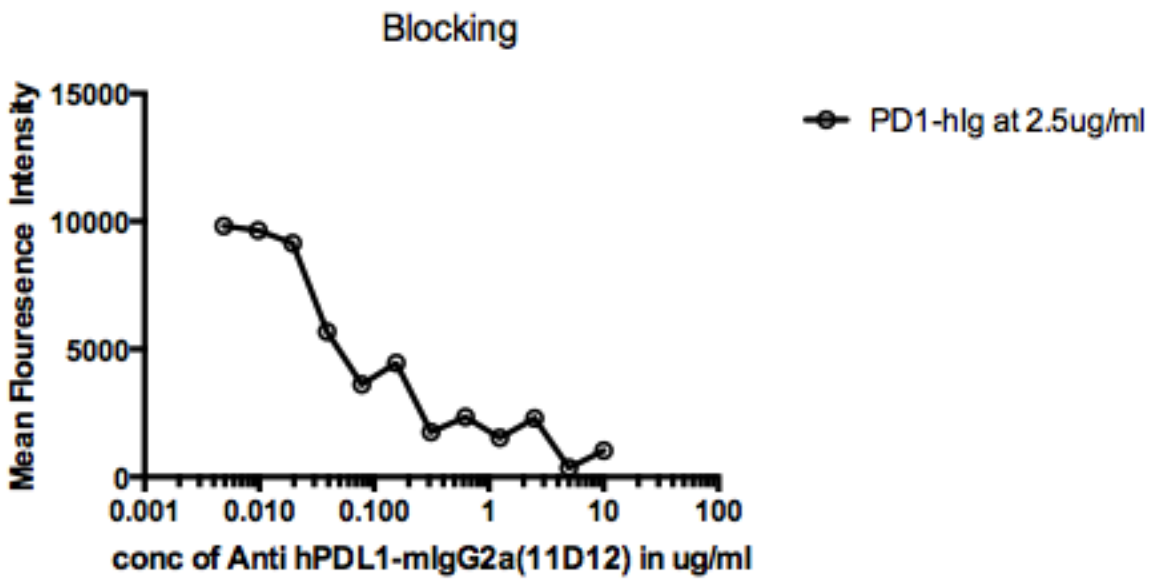


Figure 11 B7-1 competes for the binding of PD1 with PDL1. (a) 300hPDL1 Tim3 transfectants were incubated with 20ug/ml of B7-mIgG2awith a series of two fold dilutions. The fusion protein PD1-hIg were added at 2.5ug/ml. The detection reagant was $F(ab)_2$ anti-hIg PE; (b) 300hPDL1 Tim3 transfectants were incubated with 20ug/ml of 11D12 with a series of two fold dilutions. The fusion protein PD1-hIg were added at 2.5ug/ml. The detection reagant was $F(ab)_2$ anti-hIg PE.

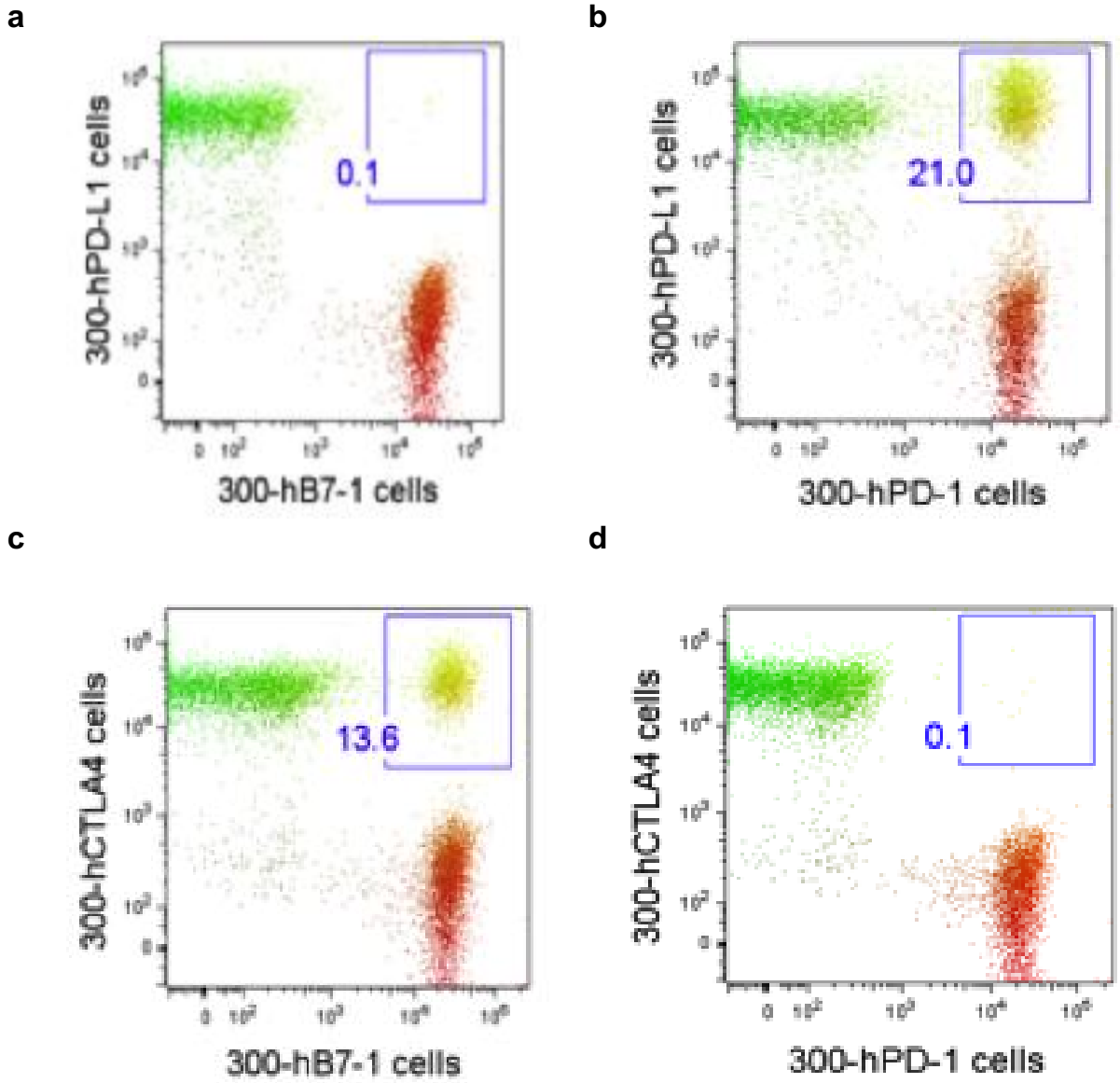
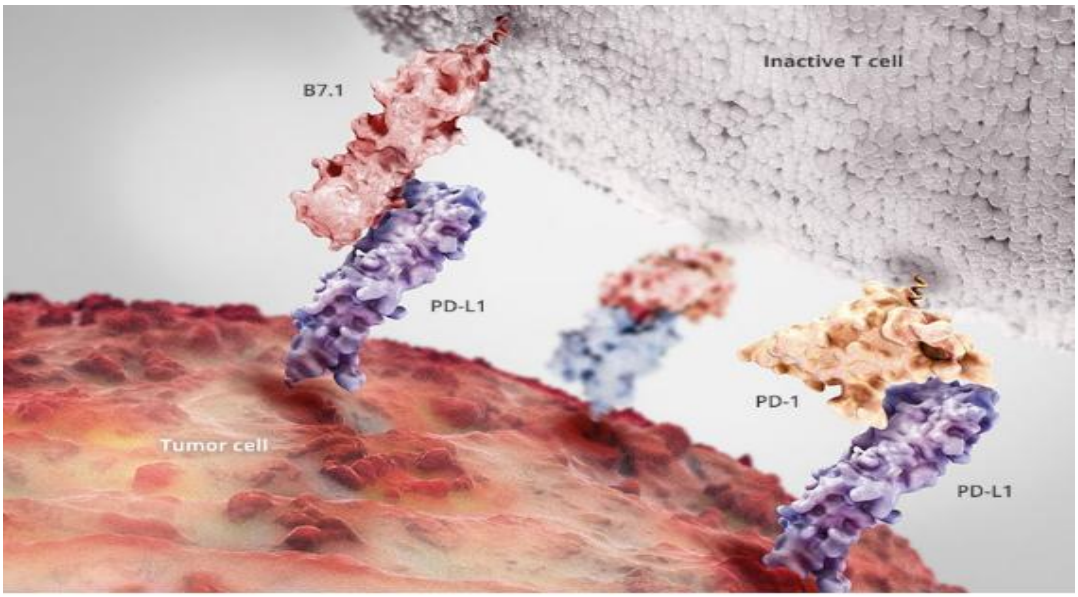


Figure 12. hPDL1 and hB7-1 do not bind in a cell to cell interaction (a) Cell to cell binding of the hPDL1 to hB7-1 was analyzed by cell conjugation assay. The binding of the red dye-labeled cells and the green dye-labeled cells was assessed by flow cytometry and indicated by double positive events (yellow dots) with higher forward scatter (FSC); (b) Binding of hPDL1 to hPD1 as assessed by cell conjugation assay; (c)Binding of hCTLA4 to hB7-1 as assessed by cell conjugation assay; (d) hCTLA4 does not bind to hPD1 as assessed by cell conjugation assay

Model for PD-L1 binding to B7-1 *in cis*

I propose that PD-L1 turns via its flexible stalk to bind to B7-1 *in cis* (Figure 13b), in a way that can competitively block the binding of PD-L1 to PD-1 or of B7-1 to CD28 or CTLA4 [15]. According to our data, cell surface PD-L1 may interact with cell surface PD-1 *in trans* but not with cell surface B7-1 *in trans*. Instead, interaction of cell surface PD-L1 with cell surface B7-1 *in cis* is possible. My study revises our previous understanding of the PDL1-B7-1 pathway as illustrated in the figure taken from the web (13a).

a



* https://www.biooncology.com/pathways/pdl1.html[1]

b

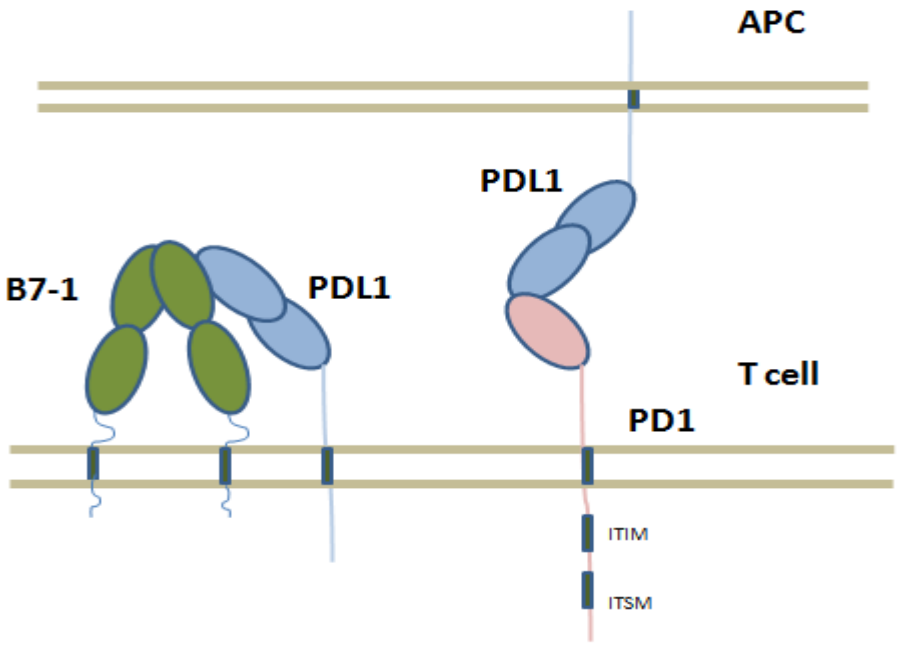


Figure 13. Old and new Models of PDL1 binding with B7-1. (a) The previous understanding of the B7-PDL1 binding interaction in trans taken from web; (b) PDL1 binding with B7-1 in cis but not trans based on my results

2.1.5 Discussion

My work suggests that PDL1 binds to B7-1 in cis on the same cell surface rather than in trans as a cell to cell interaction. This binding feature structurally supports previous findings that B7-1 binding to PD-L1 on the same tumor cell or soluble B7-1 binding to PD-L1 on tumor cells can competitively block PD-L1 and PD-1 interaction thus overcoming PD-L1 mediated immunosuppression and enhancing anti-tumor immunity [44, 45, 46].

Previous studies have also reported two cell surface molecules interacting in cis to execute their biologic function. For instance *Cheung et al, 2009* showed that the two molecules, HVEM and BTLA interact in cis. This complex prevents HVEM from binding with its trans acting ligands such as LIGHT preventing downstream activation of cell signaling pathways thereby playing a role in T cell tolerance.

The binding between PDL1 and B7-1 might also have additional functions. The pathway has been reported to be inhibitory on T cells[15, 41, 42]. A role as a self inhibitory mechanism is possible[43]. The pathway may thus function before the PDL1-PD1 pathway, as a tuning mechanism of T cell exhaustion. In previous studies it is not clear if the pathway operates between an antigen presenting cells and a T cell or if it is operating within a cell. Our study shows that the pathway cannot operate via an APC-T cell interaction but is likely to be within a cell.

My data (Figure 7) supports the previous finding that both B7-1 and PD1 bind to PDL1 in the same vicinity[43]. The concentration dependent blocking of PDL1 interaction with B7-1 by anti PDL1 antibodies also suggests the binding to be functionally relevant rather than it being a chance homology binding. The reason for B7-1 to block PD1 interaction with PDL1 at higher concentrations is likely due to a steric hindrance effect due to partial overlap of the binding sites. In line with the previous finding by *Paterson et al, 2011* I see the requirement of only the IgV domain of PDL1 for binding with PD1 and B7-1 (Figure

6).

I identify a series of anti-PD-L1 antibodies that are either dual blockers or non blockers of the PD1 and B7-1 binding to PDL1 (Table 1). I also report a specific non blocker of PD1 and B7-1 binding with PDL1 that does bind to the IgV domain of PDL1 (Table 2).

In summary, the binding features of PD-L1 and B7-1 shown here structurally support the importance of co expression of B7-1 and PD-L1 on the same cell. Interaction in cis of PDL1 and B7-1 and not in trans changes our understanding of the two pathways and the action of the immunotherapeutic monoclonal antibodies that bind to the respective receptors of PD1 and CTLA4. Our findings may help better utilize PD-L1 and B7-1 pathways to overcome PD-L1–mediated immunosuppression in cancer immunotherapy.

2.2.1 Abstract - Combination blockade of CX3CR1 with PD1

While 20-30% of patients benefit significantly from PD1-PDL1 immunotherapy, a large percentage do not respond creating a need for more effective combination therapies. It is well known that chemotactic molecules mediate a cross talk between cancer cells and immune cells; a major mechanism for tumor progression. One such example is CX3CR1 and its ligand CX3CL1 (fractalkine). This pathway has been shown to be involved in migration and metastasis of tumor cells. We made CX3CR1 mAbs and showed they blocked the binding of CX3CL1 to CX3CR1. I tested whether CX3CR1 antibody blockade increased the effectiveness of tumor immunotherapy in combination with anti PD1 mAb in a mouse colon cancer model. My results suggest the combination of anti CX3CR1 and anti PD1 is an effective combination therapy. Further work focuses on the mechanism of this combination.

2.2.2 Introduction

Chemokines are generally small secreted proteins that mediate leukocyte migration. They are divided by structure into three sub families of CXC, CC, C[67] with multiple members while CX3CL1 (fractalkine) occupies a subfamily with only itself as a member. CX3CL1 binds to CX3CR1, a 7 transmembrane G protein coupled receptor. The chemokine receptor CX3CR1 and its ligand CX3CL1 are involved in the migration of lymphocytes. CX3CR1/CX3L1 are widely expressed but the expression varies across tissues. CX3CL1 promotes function as a chemokine as well as an adhesion molecule[68], in particular CX3CL1 promotes integrin independent adhesion[69]. CX3CL1 is synthesized as a transmembrane protein but also released as a soluble protein through proteolytic cleavage unlike other chemokines that are secreted[69]. As a soluble form it functions as a chemokine. G-protein receptor signaling is required by CX3CR1 to mediate migration but not adhesion[50]. Recently the role of this axis has been seen in tumor progression as tumor cells use the axis for migration and metastasis. A bidirectional interaction between cancer cells and macrophages was observed, the main receptor mediators were CCR2 and CX3CR1[60]. Previous studies have also highlighted the expression of CX3CR1 on the immunosuppressive cell types- M2 macrophages[58] and MMDSCs as well on the tumor cell itself[55]. It has been shown that CX3CR1 mediates monocyte homeostasis and macrophage survival[56]. Thus CX3CR1 is a potential candidate for tumor immunotherapy. My group developed a novel CX3CR1 monoclonal antibody by immunizing CX3CR1 knockout mice (mIgG2c isotype) and showed it binds an accessible extracellular motif of CX3CR1. Here I confirm previously obtained results on CX3CR1 expression and use a combination blockade approach of antibodies against CX3CR1 with PD1 to see if the combination has a better therapeutic effect than single agent anti murine PD1 in the CT26 mouse tumor model. I also investigate the tumor immune infiltrates for the expression of CX3CR1 to understand the underlying mechanism behind our antibody blockade approach. Together my results suggest the potential use of blockade against CX3CR1 in tumor immunotherapy.

2.2.3 Materials and Methods

Cells and cell culture

CT26 mouse tumor cell line was from ATCC. It was maintained in RPM1-1640 at 5% CO_2. Jurkat mCX3CR1 and 300-mCX3CR1, 300 mPDL1 cells were made in our laboratory and maintained in RPMI-1640 plus puromycin in 5% CO_2.

Antibody

The antibodies for mCX3CR1, clones 455.2E8 and 455.1C11 were made in our laboratory by immunizing CX3CR1 knockout mice (kindly provided by Uli von Andrian) with 300-mCX3CR1 cells and screening for reactivity with Jurkat-mCX3CR1 cells but not untransfected Jurkat cells. Since the knockout mouse is of C57BL/6 origin, the monoclonal antibodies have the IgG2c isotype. Goat anti mouse IgG PE was purchased from Southern Biotech. MOPC21, an IgG1 control antibody was purchased from BioXcell. For flow cytometry, flourochrome conjugated antibodies were made by Biolegend and their isotype controls were purchased from Biolegend.

Antibody Titration experiments

The cell lines, Jurkat mCX3CR1 and CT26 were incubated with 455.1C11 antibody. The detection reagent was Goat anti mouse IgG PE. The tumor cell line CT26 were also screened for PDL1 expression. The antibody binding affinity was tested at 10 µg/ml. A flow cytometric analysis using BD Facs Canto was done.

Antibody Blocking experiments

The cell line jurkat mCX3CR1 were incubated with 1C11 and 2E8 in a two fold dilution series. This was followed by the subsequent addition of CX3CL1-hIgG at 2 ug/ml. The binding of the CX3CL1 fusion protein was detected by Goat anti hIgG PE.

Western Blot

Cell lysates were resolved on Nu PAGE Novex bis tris 4-12% gel followed by transfer to nitrocellulose membrane by dry transfer method (Bio-Rad). The membrane was blocked in 5% milk, 1% goat serum in TBST. Next, the primary antibody at 1 µg/ml was incubated at 4 C overnight. The secondary antibody was Goat anti Mouse IgG HRP at 1 to 40,000. The blot was developed with super signal west pico stable peroxide solution/ super signal west pico luminal enhancer.

Tumor Infiltrating lymphocyte isolation

Tumors were disaggregated using collagenase and red blood cells were lysed using RBC lysis buffer. Cells were Fc blocked and stained with antibody for 30 min and permeabilised for intracellular staining. Acquisition was performed on an LSR Fortessa SORP HTS flow cytometer. Data analysis was performed using FlowJo X.

Antibody Treatment

CT26 tumor cells were injected at 0.25 million/mouse in Balb/c mice. At day 7 the antibody treatments were started for a series of 5 treatments once every 3 days. 200ug of antibodies were injected. Tumor growth was monitored. For study of tumor immune infiltrate, mice were sacrificed when the tumors were about 1 cm.

2.2.4 Results

Characterization of anti m CX3CR1 - 455.1C11 binds with high affinity to mCX3CR1

To determine the binding affinity of our anti mCX3CR1 antibodies. I tested two clones 1C11 and 2E8 for their respective binding affinities with cell surface CX3CR1. As expected the two clones had a very high binding affinity with CX3CR1, while no binding was observed with the IgG control. The antibody binding followed a concentration dependent dose curve. 1C11 had the highest affinity with an EC-50 of 0.1020 µg/ml (Figure 14). I therefore selected 1C11 for further studies. We performed a blocking assay to see if 1C11 blocked CX3CL1 from binding with CX3CR1. We observed both 1C11 and 2E8 to prevent the binding of a CX3CL1 fusion protein to 300 mCX3CR1 cells (Figure 15).

Expression of CX3CR1 and PDL1 on Tumor cell lines

I wanted to test if the tumor cell lines expressed CX3CR1 and PDL1. I chose CT26 and 4T1 for testing by flow cytometry with the 1C11 anti m CX3CR1 at 10 µg/ml. Both 4T1 and CT26 expressed CX3CR1 while CT26 had a much greater expression of CX3CR1. The IgG control did not show any binding. The tumor cell lines also showed expression of PDL1 (Figure 16).

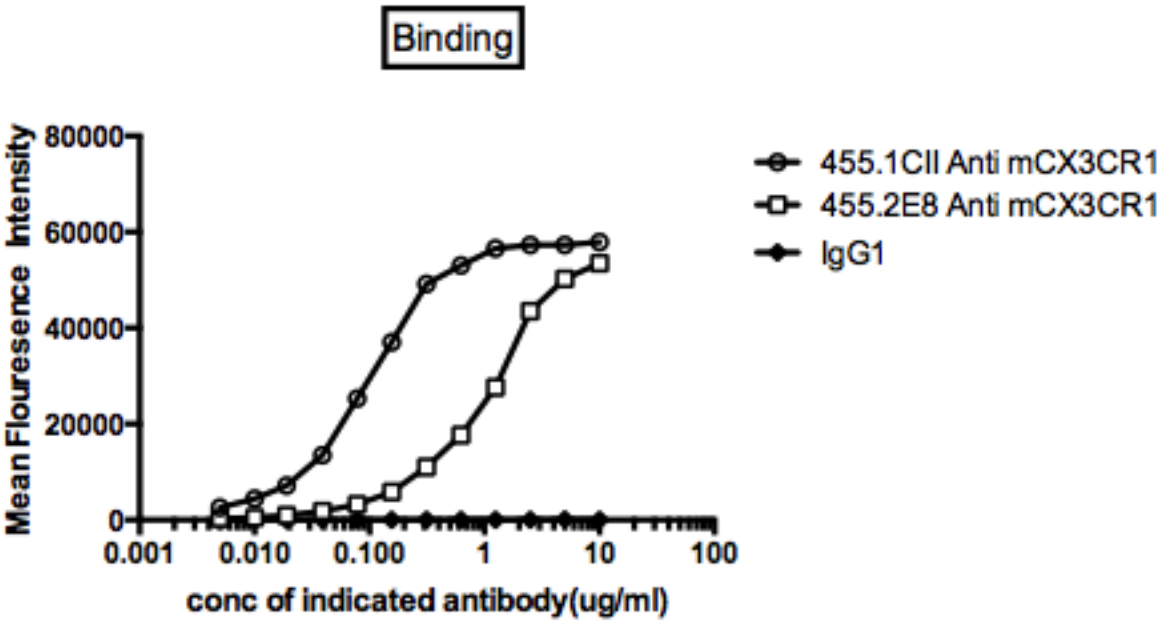


Figure 14. Anti mCX3CR1 clone 1C11 binds to cell surface expressed mCX3CR1. Jurkat mCX3CR1 transfected cells were treated with 1C11 anti mCX3CR1 and 2E8 anti mCX3CR1 at two fold dilutions.Detection reagant was Goat anti mIgG PE

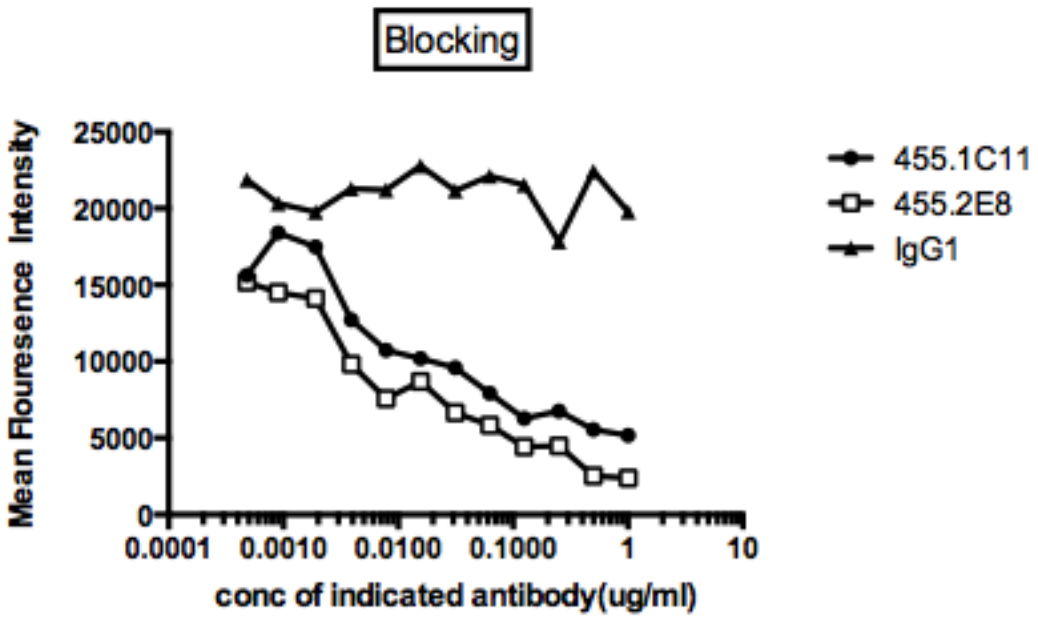


Figure 15. Anti mCX3CR1 blocks the interaction of CX3CL1 with CX3CR1. Jurkat mCX3CR1 transfected cells incubated with anti mCX3CR1 clone IC11 and 2E8 in a two fold dilution series, CX3CL1 was added at 2 µg/ml. The secondary goat anti hIgG PE was used

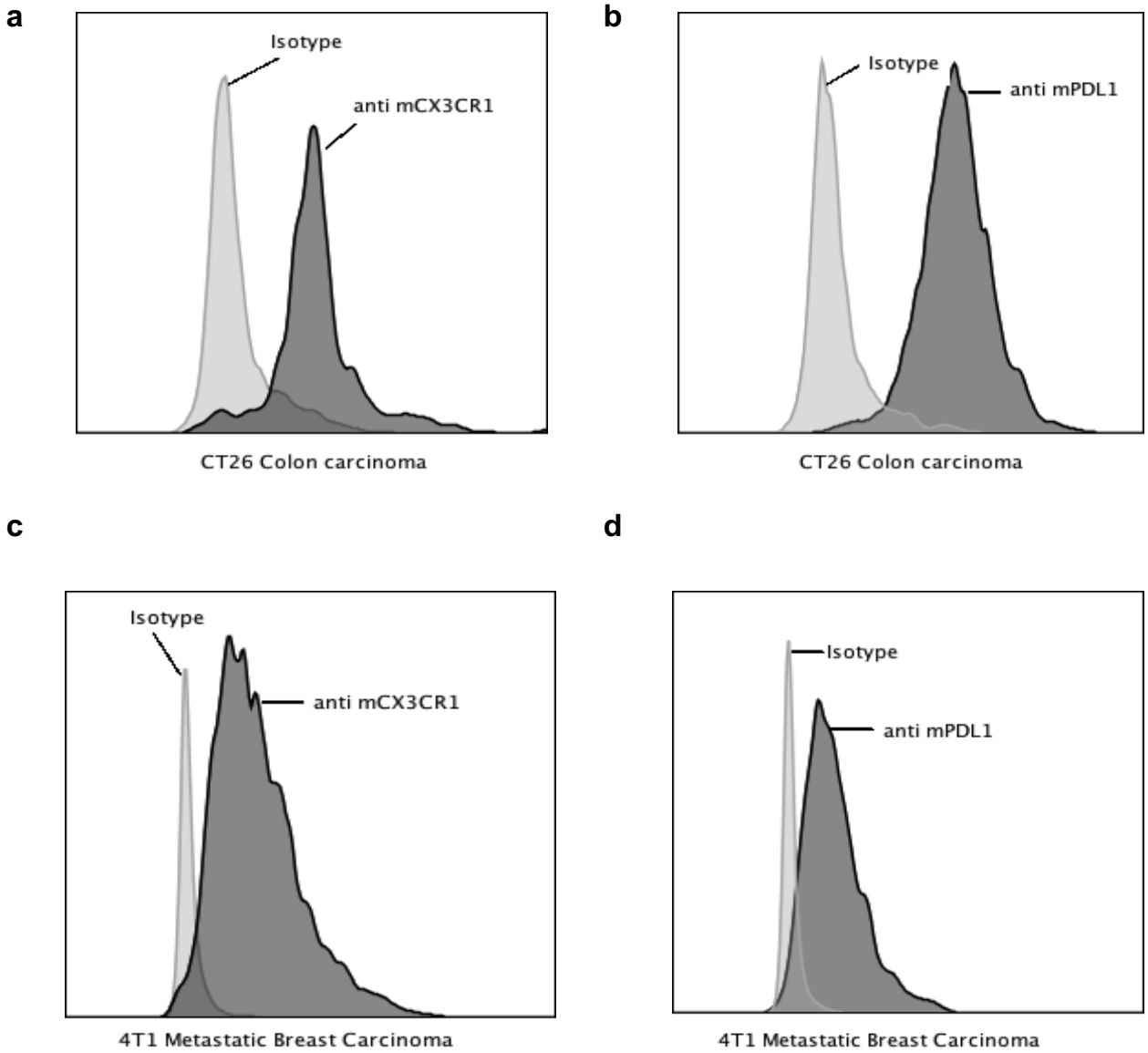


Figure 16. Cell surface expression of CX3CR1 and PDL1 on tumor cell lines by flow cytometry. Tumor cell lines were incubated with anti mCX3CR1, 1C11 and anti mPDL1, 8E2 at 10ug/ml.The secondary was Goat anti mIgG PE.

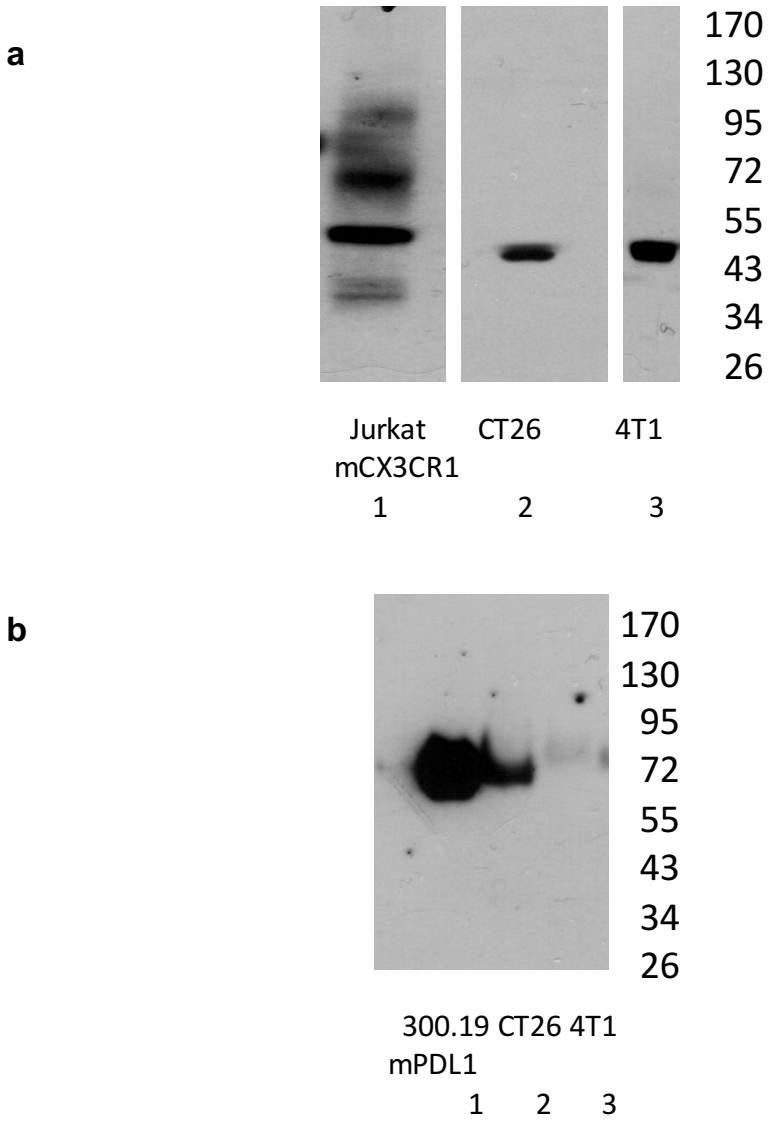
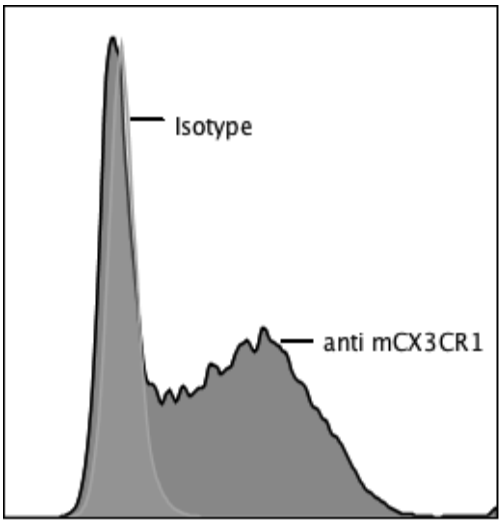


Figure 17. Expression of CX3CR1 and PD-L1 by western blot (a) Jurkat mCX3CR1 and CT26, 4T1 cells lysates were incubated with anti mCX3CR1 455.1C11 antibody at 1ug/ml. The secondary of goat anti mouse IgG HRP was used at 1 to 40,000; (b) 300-mPD-L1, CT26 and 4T1 cell lysates were incubated with Anti mPDL1 Antibody at 1ug/ml. The secondary was goat anti mouse IgG HRP at 1 to 40,000.

a



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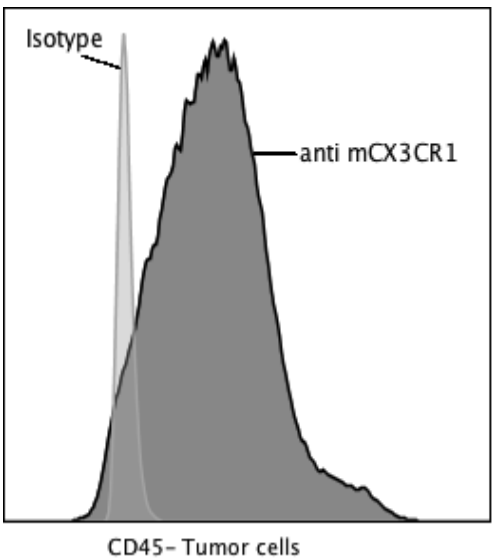

Figure 18. Ex vivo expression of CX3CR1 on mouse tumor and infiltrating immune cells. Immune infiltrates in the subcutaneous tumor (1 cm). Infiltrating immune cells as a percentage of live CD45 cells and tumor cells as a percentage of CD45- cells. Representative Histogram showing CX3CR1 expression.

I confirmed the expression by western blot technique. Jurkat m CX3CR1 and 300.19 m PDL1 transfected cells were used as controls, with 455.1C11 at 1 µg/ml as the primary antibody. The 4T1 and CT26 lysates showed a band at around 43 KDa and 55KDa corresponding to CX3CR1 and PDL1 respectively (Figure 17). The expected molecular weight of mouse CX3CR1 based on the amino acid sequence is 40.3 KD, in good agreement with my data of 43 KD. CT26 seemed to have a lower level of expression than 4T1 for CX3CR1 in western blot though it was higher in FACS. This may be due to a difference in the cytoplasmic expression of the protein or differences in cell size.

Expression of CX3CR1 in the Tumor Immune Infiltrate

I checked the tumor immune infiltrate for the expression of CX3CR1. Both the immune infiltrate and tumor cells expressed CX3CR1 (Figure 18a, 18b) I observed a variety of immune cell types - B, NK, CD4, CD8, Treg, M1, M2, MMDSC, GMDSC, DC to express CX3CR1. M1, M2, and MMDSC expressed the highest percentage of CX3CR1 amongst the individual cell types. When calculated as a percent of CD45+ cells, the M2s and MMDSCs were high expressors (Figure 19a, 19b). I also examined coexpression of CX3CR1 and PD1 on CD4 and CD8 T cells. Interestingly, 88 percent of CX3CR1 positive cells were PD1 positive in the CD8 group while only 24 percent CX3CR1 positive cells were PD1 positive in the CD4 group (Figure 20, 21). The gating strategy is illustrated in Supplementary Figure 1.

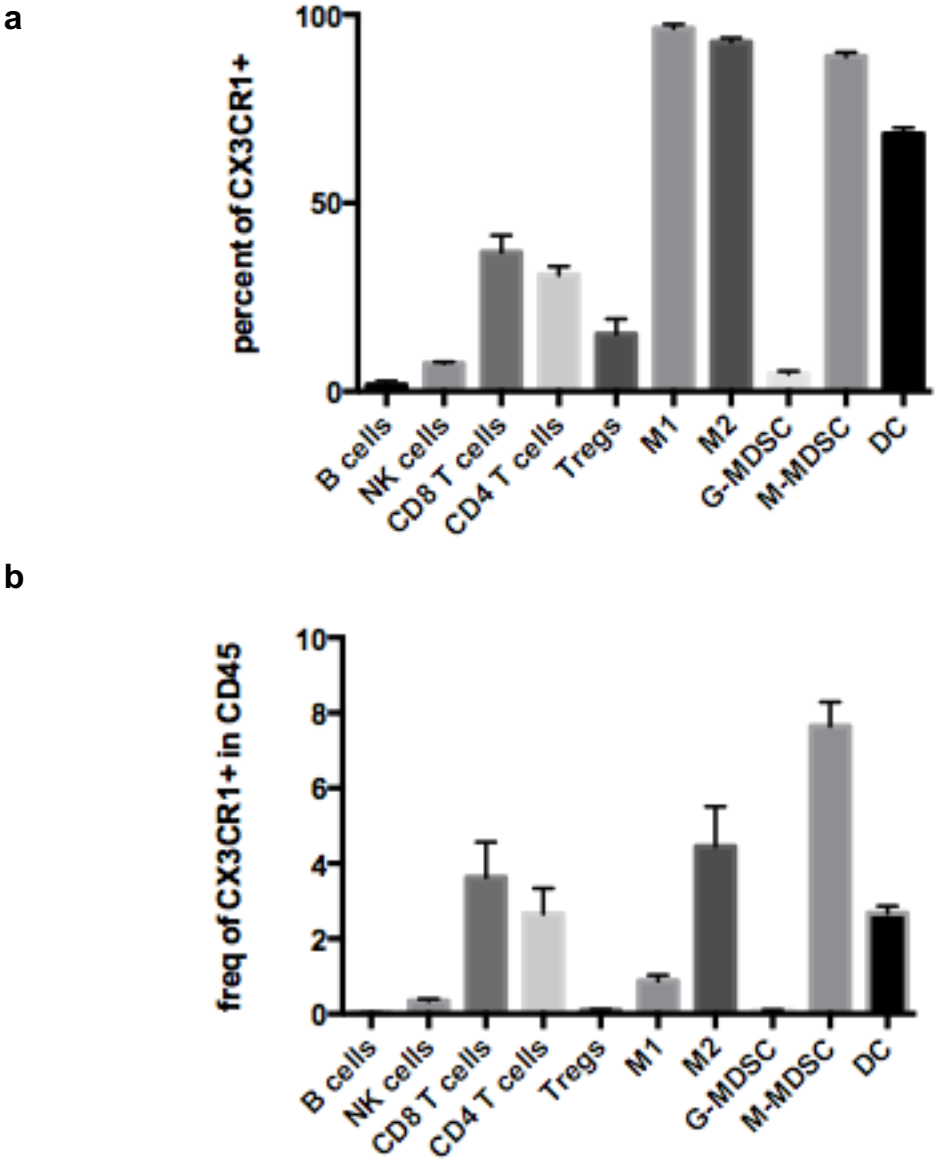

Figure 19. CX3CR1 on tumor infiltrating immune subsets. Immune infiltrates in the subcutaneous CT26 tumor (1 cm). CD4 cells as a percentage of live CD45 CD3,CD4,CX3CR1 positive cells. CD8 cells as a percentage of live CD45 CD3,CD8,CX3CR1 positive cells. Tregs as live CD45 CD3,CD4,FOXP3,CX3CR1 positive cells, B cells as CD45 live,CD19+,CX3CR1 positive cells, B cells, NK cells as live CD45,NKp46, CX3CR1 positive cells, M2 macrophages as live CD45,CD11b, Arg1, CX3CR1 positive cells, M1 macrophages as live CD45,CD11b,iNOS+, CX3CR1 positive cells. Graphs of mean±SEM for n=5 per group

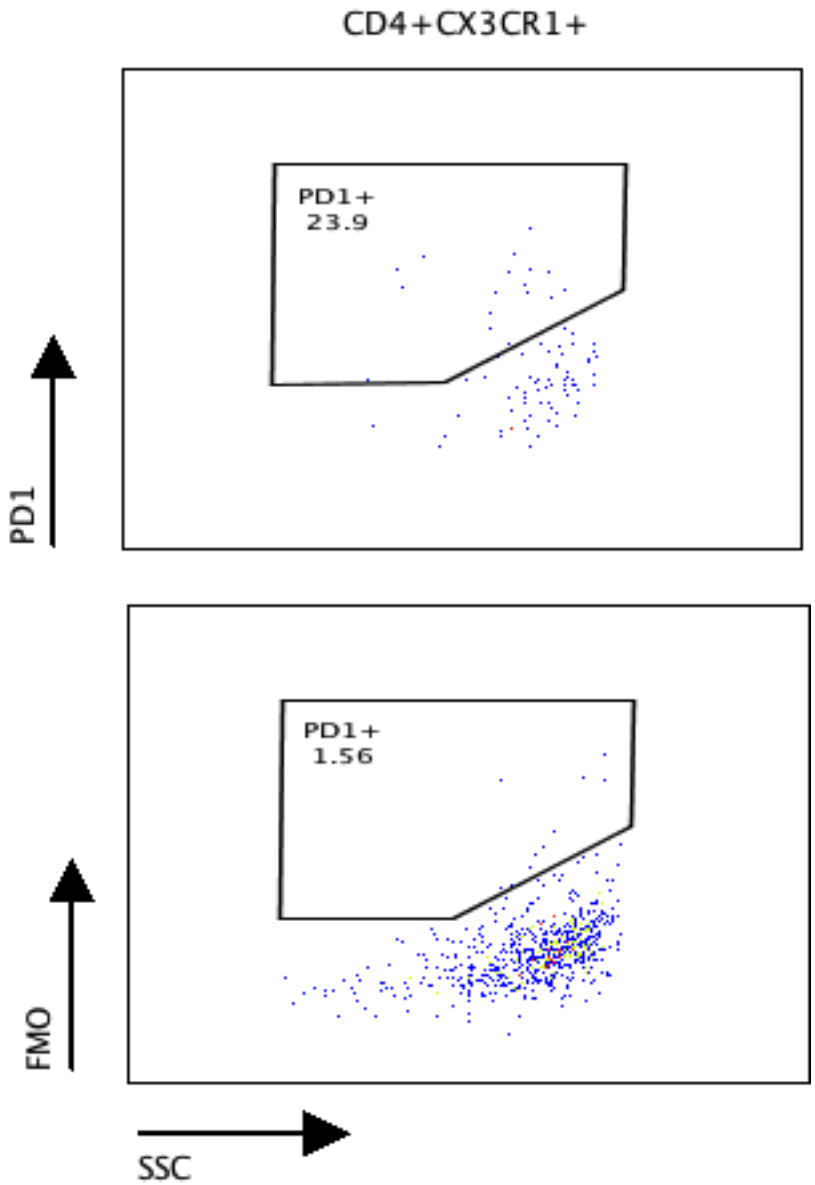

Figure 20. Low percentage of CX3CR1 positive CD4 T cells expressing PD1. Immune infiltrates in the subcutaneous CT26 tumor(1 cm). PD1 positive cells as a percentage of live CD45 CD3,CD4,CX3CR1 positive cells. Representative flow cytometric data of CX3CR1+PD1+ population.

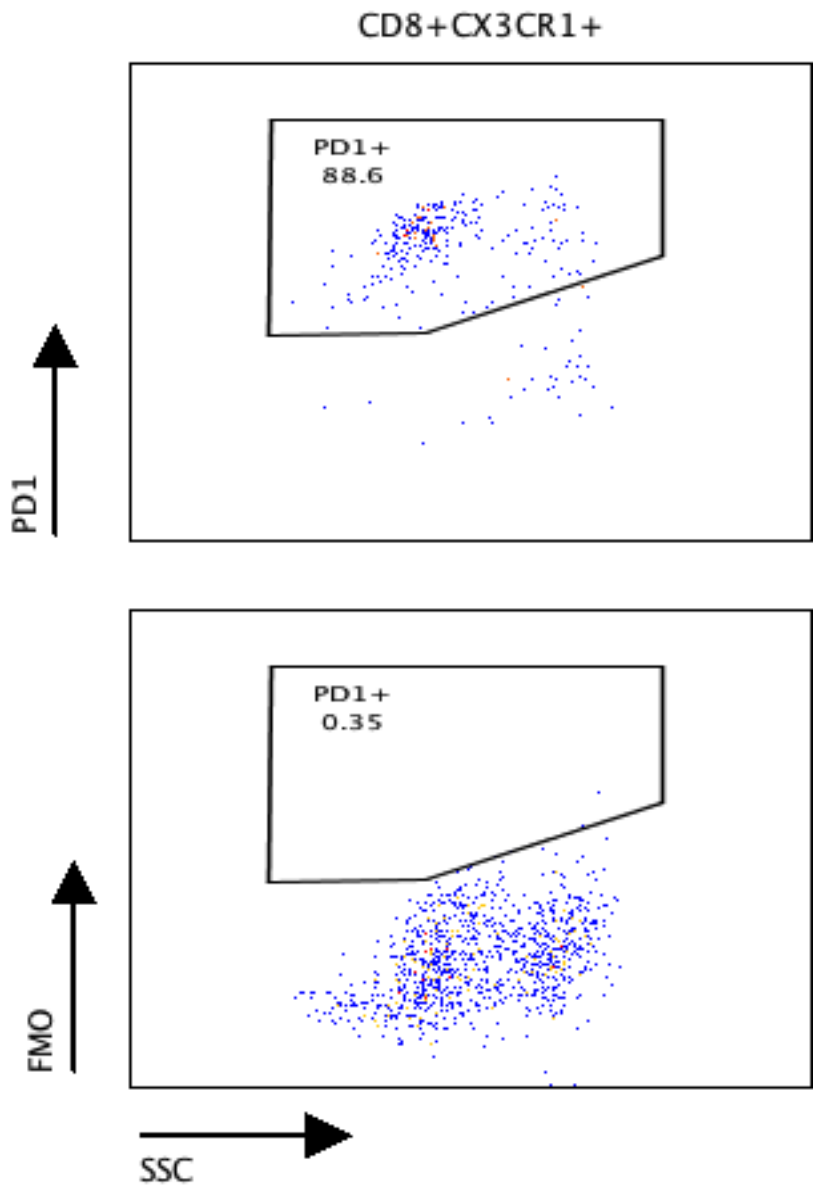

Figure 21. High percentage of CX3CR1 positive CD8 T cells expressing PD1. Immune infiltrates in the subcutaneous CT26 tumor (1 cm). PD1 positive cells as a percentage of live CD45 CD3, CD8, CX3CR1 positive cells. Representative flow cytometric data of CX3CR1+PD1+ population.

Tumor Immunotherapy with CX3CR1 monoclonal antibody

I performed a mouse CT26 tumor immunotherapy experiment with the antibody blockade therapy starting at day 7 with 200 µg mAb per mouse. I observed for the combination blockade of CX3CR1 and PD1 to enhance the survival in comparison to the single agent anti mPD1. As expected the IgG control mice died at early time points (Figure 22).

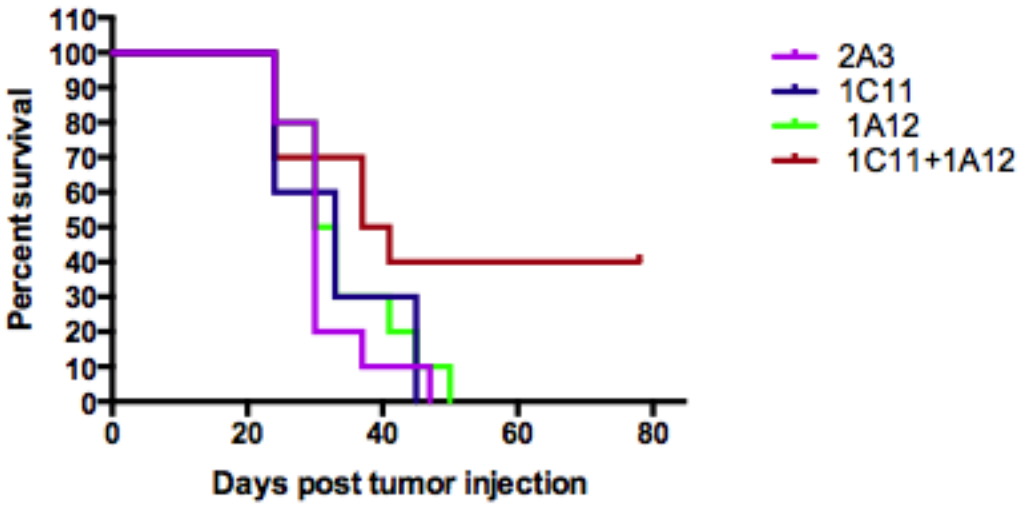

Figure 22. CX3CR1 mAb in combination with PD1 mAb shows survival benefit. CT26 tumor injections at 0.25million/mouse, Ab treatment at day 7 for 200ug/mouse, a total of 5 treatments. Treatment groups 1C11 anti mCX3CR1-mIgG2c, 1A12 anti mPD1-rat IgG2a, 2A3 rat IgG2a, combination of 1C11 and 1A12. Kaplan Meier survival curve for n=10

2.2.5 Discussion

CX3CR1 is a chemokine receptor important for homing of leukocytes, NK, B cells, effector T, and CD14+ monocytes. Previous studies have suggested the CX3CR1-CX3CL1 axis might be a good candidate for tumor immunotherapy but good agents were not available. Though this pathway is a chemoattractant for leukocyte migration, its function for the adhesion and migration of tumor cells has also been shown[70]. I along with others, report for CX3CR1 to be highly expressed on a wide variety of tumor cells types[48]. I confirm by both flow cytometry and western blot techniques for CX3CR1 to be expressed on cancer cell lines as well as ex vivo mouse tumors. The observation of CX3CR1 to be highly expressed on the tumor cells greater than of the immune infiltrate may suggest its dominance as a migration factor for the cancer cells.

My data is consistent with the previous results of CX3CR1 to be highly expressed on M2 macrophages within the tumor immune infiltrate[56]. In *Zheng et al, 2013*, mice lacking CX3CR1 showed a decrease in liver metastasis of colon carcinoma, with a decrease in the number of macrophages within the tumor microenvironment. They showed that CX3CR1 induced angiogenesis and enhanced macrophage survival by interfering with the pro-apoptopic pathway in macrophages. While my data does show a survival benefit, whether pro- angiogeneic macrophages are being decreased by CX3CR1 blockade will need to be answered by a mechanistic study.

I also show CX3CR1 to be highly expressed on the monocytic myeloid derived suppressor cells (MMDSC). My data is consistent with a previous study of the expression of CX3CR1 on MMDSCs in ovarian cancer[52]. This study suggested the CX3CR1+ subset of MMDSCs enhances tumor progression. The MMDSCs within the tumor microenvironment are highly immunosuppressive and release the immune dampening IL-10 cytokine[71]. The depletion of M2 macrophages and MMDSCs can be an effective anti tumor strategy.

We developed a novel anti mouse CX3CR1 antibody for use in tumor models. We also show our CXC3R1 antibody blocks the interaction of CX3CR1 with its ligand CX3CL1. The mechanisms of action of our CX3CR1 antibody may be by targeting the immune suppressive cell types- M2, MMDSCs and the tumor itself. I speculate the anti CX3CR1 antibody to be of a killer characteristic since it has IgG2c as its Fc domain. This study will be followed by a mechanistic study and ADCC in vitro assays to further understand the antibody characteristics.

Studies have shown the involvement of CX3CR1 across different tumors, for instance *Jamieson et al, 2011* show that malignant breast cancer cells overexpress CX3CR1 and this aids in the migration of cancer cells to bone and brain where the tissues release soluble CX3CL1[65]. It is important to test this blockade approach across different tumors to see if there is a therapeutic response.

We need more effective PD1 combination therapies to increase the number of responders to PD1 therapy in cancer[8]. My data supports the use of anti-CX3CR1 in combination with anti PD1 to enhance anti tumor immunity in a mouse colon carcinoma model. The study provides preliminary results suggesting that this combination therapy may prove to be clinically significant.

2.3.1 Abstract- Contributions of B7 blockade and CTLA-4 cell depletion to the mechanism of tumor immunotherapy by CTLA-4 mab

The human CTLA4 antibody Ipilimumab has shown effective results in treating patients with metastatic melanoma. While early studies highlighted the mechanism of action of CTLA-4 antibody as blocking B7-1 and B7-2 interaction with CTLA4, more recent studies have emphasized the depletion of CTLA-4 positive T regulatory cells. To determine whether depletion of CTLA-4 positive cells without B7 blockade was sufficient for tumor immunotherapy, I used a novel mouse anti mouse CTLA4 mAb 5E12. This clone should deplete CTLA-4 positive cells as it has an mIgG2a as its Fc domain; however, it does not block CTLA-4 binding to B7-1 or B7-2. On testing the 5E12 antibody in a mouse colon tumor model, I found that 5E12 was not an effective tumor immunotherapy whereas standard CTLA-4 mAbs such as 9D9 that both block and deplete were effective. Surprisingly, I observed that the vast majority of T cells are depleted by 5E12 mab as compared with the IgG control. To understand the mechanism behind the massive CD3 depletion in the 5E12 treated group, I use an apoptosis assay to test if the antibody is directly killing T cells. Rather surprisingly, there was no differences in vitro in the percentage of live CD3+ cells in 5E12 treated vs Isotype treated activated T cell cultures, suggesting that 5E12 does not directly kill T cells. Another possible mechanism for the depletion could be ADCC which is currently being studied in vitro. My data suggests that blocking B7-1 or B7-2 interaction with CTLA4 is a necessary attribute for a therapeutic CTLA4 monoclonal antibody. The mechanism by which the CTLA4 mAb 5E12 is depleting T cells is under investigation.

2.3.2 Introduction

CTLA4 is an inhibitory co-stimulatory molecule expressed on the surface of T cells following activation. It functions to dampen T cell responses. While it is highly expressed on immunosuppressive T regulatory cells[32], conventional CD4 and CD8 T cells also express CTLA4 in a modest amount. This is important to mediate T cell tolerance. CTLA4 signaling inhibits T cells responses by the action of PP2A. PP2A inhibits Akt preventing downstream signaling required for T cell survival and activation[11]. CTLA4 has both cell intrinsic and cell extrinsic mechanisms of action[31]. Blockade of CTLA4 is an effective strategy for tumor immunotherapy. Anti CTLA4 antibodies are therapeutically used for the treatment of metastatic melanoma. Though a proportion of the patients have had multiyear durable responses, several toxicities including autoimmune symptoms have been reported[72]. Previous studies have shown the mechanism of CTLA4 antibody action- the antibodies function as both CTLA4-B7 blockers[62] and depleters of Tregs within the tumor microenvironment[61, 63]. It has been concluded in different papers that one or the other mechanism of action is important for therapeutic efficacy. However each individual function has not been separately studied. We need to determine the dominant mechanism of action. Further it needs to be examined if only the depletion action of the antibody is enough for tumor immunotherapy. Here I use a novel anti mCTLA4 antibody 5E12 which is a non blocker of CTLA4–B7 interaction but is a depleter of CTLA4 expressing cells. I test for its therapeutic efficacy in a mouse colon tumor model and compare it with another anti mCTLA4 mAb which is both a blocker and depleter. Further I look into the tumor immune infiltrates of the 5E12 antibody and compare it with the isotype control treated mice. Next, I look into the depletion mechanisms of the 5E12 antibody. Together my results highlight the main mechanisms of antibody action of CTLA4 monotherapy.

2.3.3 Materials and Methods

Cells and cell culture

CT26 mouse tumor cell line was from ATCC. It was maintained in RPM1-1640 at 5% CO_2. 300 mCTLA4 cells were made in our laboratory and maintained in RPMI-1640 plus puromycin in 5% CO_2

Antibody

The antibodies for mCTLA4, clone 5E12 was made in our laboratory by immunizing CTLA4/B7 knockout mice (kindly provided by Dr.Arlene Sharpe) with CTLA4 protein. MPCII an IgG control antibody and 9D9, a mIgG2b mAb were purchased from BioXcell. For flow cytometry flourochrome conjugated antibodies and their isotype controls were purchased from Biolegend.

Tumor Infiltrating lymphocyte isolation

Tumors were disaggregated using collagenase and red blood cells were lysed using RBC lysis buffer. Cells were Fc blocked and stained with antibody for 30 min and permeabilised for intracellular staining. Acquisition was performed on an LSR Fortessa SORP HTS flow cytometer. Data analysis was performed using FlowJo X

Antibody Treatment

Tumor cells were injected at 0.5 million/mouse in Balb/c mice. At day 7 the antibody treatments were started for a series of 5 treatments once every 3 days. 200ug of antibodies were injected i.p. Tumor growth was monitored. For study of tumor immune infiltrate, mice were sacrificed when the tumors were about 1 cm.

2.3.4 Results

Tumor Immuno- therapy with anti mCTLA4 clone 5E12

To test for the efficacy of 5E12 (anti mCTLA4, mIgG2a isotype), I use a CT26 colon carcinoma model. The mouse injections were started at day 7 for 200ug antibody/mouse. I included 9D9, anti mCTLA4, mIgG2b and MPCII a IgG2b isotype as the two controls. There were 3 mice per group. I observed for the 9D9 to have great therapeutic efficacy for all the mice and these mice became tumor free after three antibody treatments. The 5E12 did not show any therapeutic efficacy, with the tumor growth being relatively faster than the IgG isotype control (Figure 23).

Tumor immune infiltrate of the antibody treated group

I performed a mechanistic study for the 5E12 and IgG isotype control treated mice. I looked for CD3, CD4, CD8, Tregs within the tumor immune infiltrating lymphocytes compartment. All CD3, including CD4, CD8, Tregs were reduced in number with very low percentages in the 5E12 treated group as compared to the isotype control. Within the CD45+ compartment the immune fraction was greatly reduced as compared to the Isotype treated group (Figure 24). The gating strategy is illustrated in Supplementary Figure 2.

In vitro Assay for Antibody mediated apoptosis

To determine the underlying mechanism for antibody mediated apoptosis, I used an in vitro activated T cell assay and checked for apoptosis using annexin-APC and PI. The method used is illustrated in Supplementary Figure 3. I also phenotyped to confirm that the T cells were activated. I observed for T cells to express CD25, CD69, and extracellular CTLA4 (Figure 25). My preliminary study of 12hr antibody

treated T cells showed similar live percentages for both the 5E12 treated and the Isotype control treated cells (Figure 26).

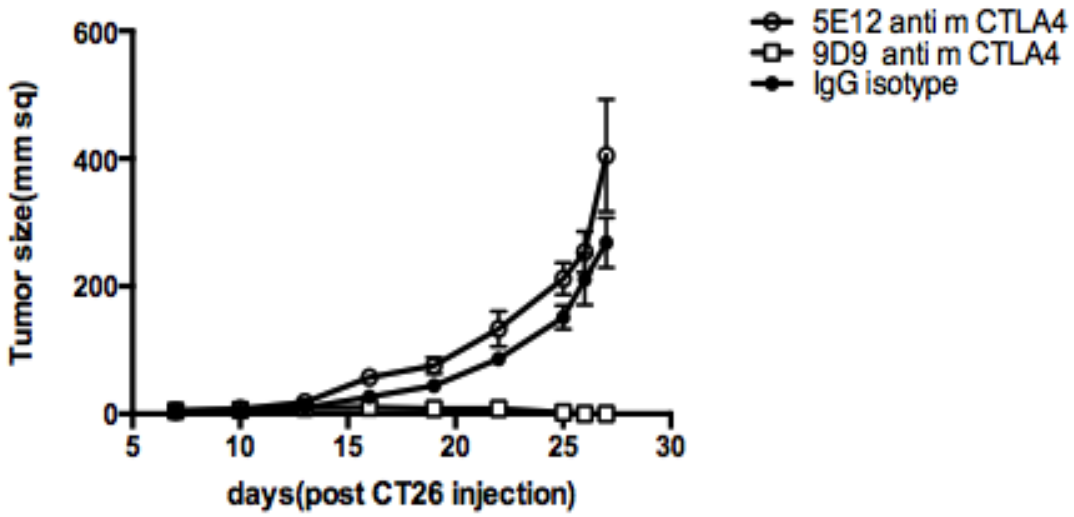

Figure 23. 5E12 CTLA4 mAb does not show survival benefit. CT26 tumor injections at 0.5million/mouse, Ab treatment at day 7 for 200ug/mouse, a total of 5 treatments. Treatment groups 5E12 anti mCTLA4 mIgG2a, 9D9 anti mCTLA4 mIgG2b, MPCII mIgG2b. Tumor growth as Mean ±SEM for n=3.

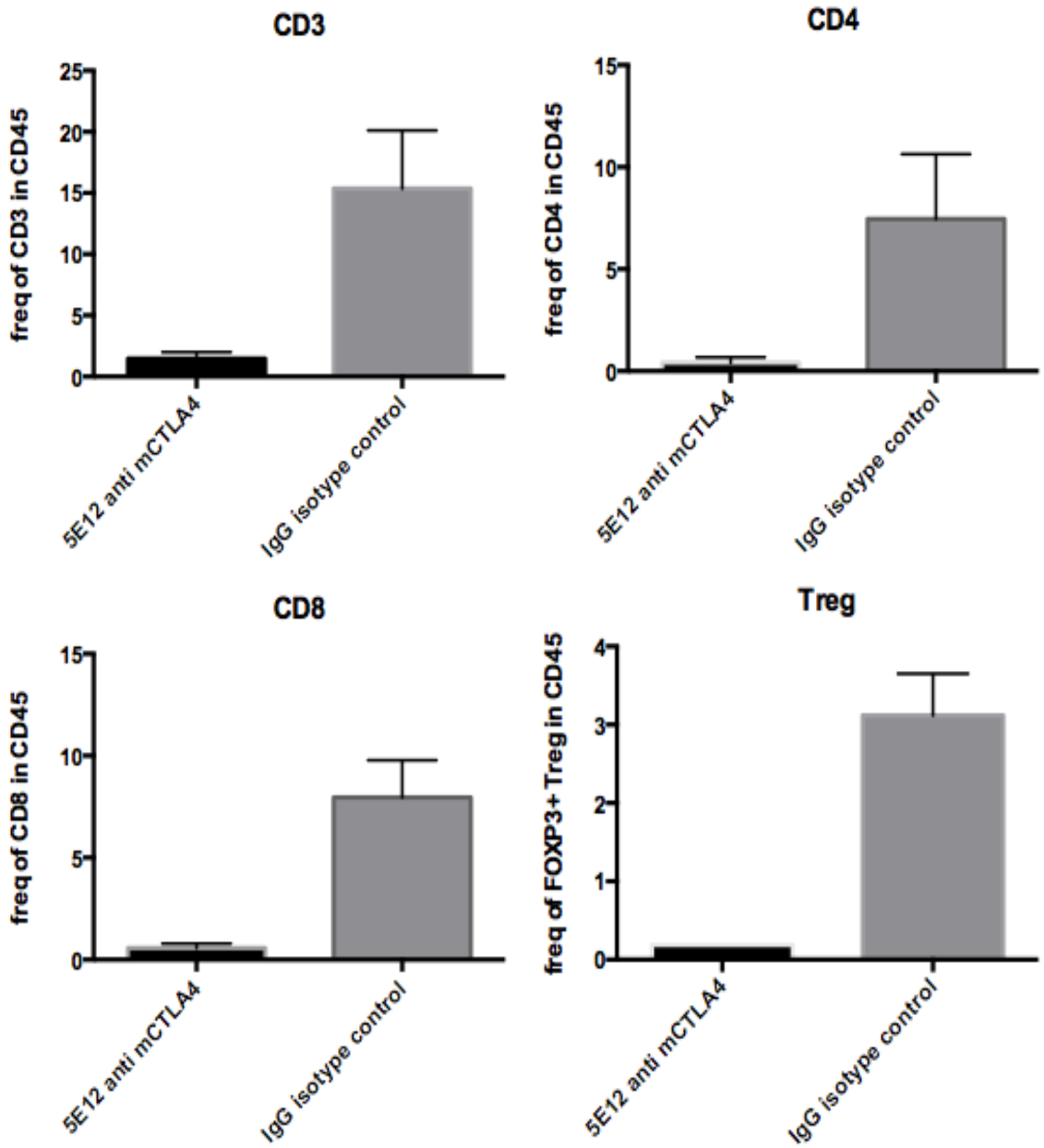

Figure 24. Tumor Immune infiltrate after 5E12 CTLA4 mAb treatment. Immune Infiltrate in the subcutaneous CT26 tumor. CD3 cells as a percentage of live CD45. CD4 cells as a percentage of live CD45, CD3 positive cells. CD8 cells as a percentage of live CD45, CD3 positive cells. Treg cells as a percentage of live CD45, CD3, CD4, FOXP3 positive cells. Graphs of mean±SEM for n=3 per group.

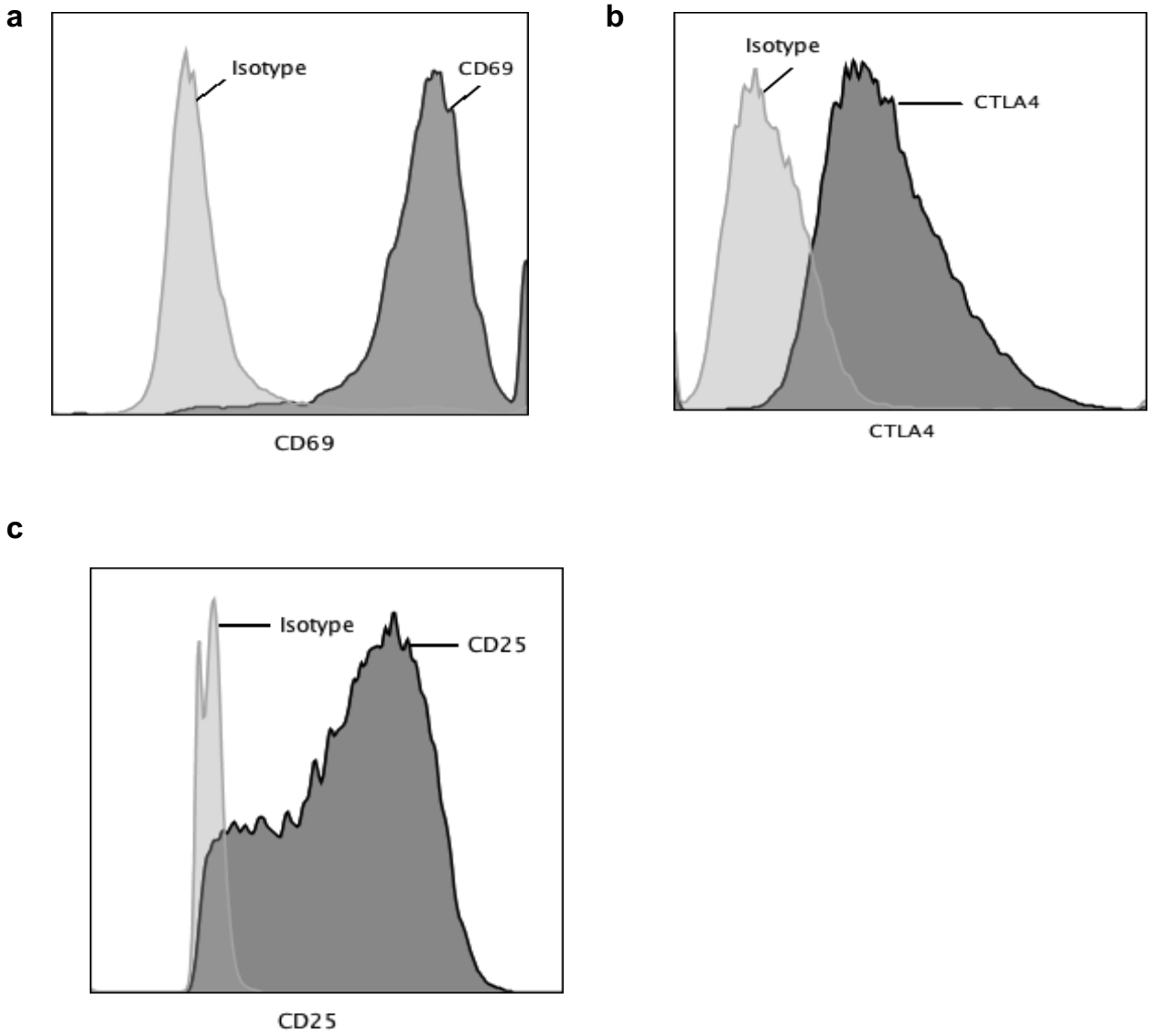

Figure 25. Activation markers on the T cell surface after CD3, CD28 co stimulation. 48 hours after CD3-CD28 co stimulation T cells were analysed for activation markers of CD25, CD69 and for CTLA4 by direct antibody staining

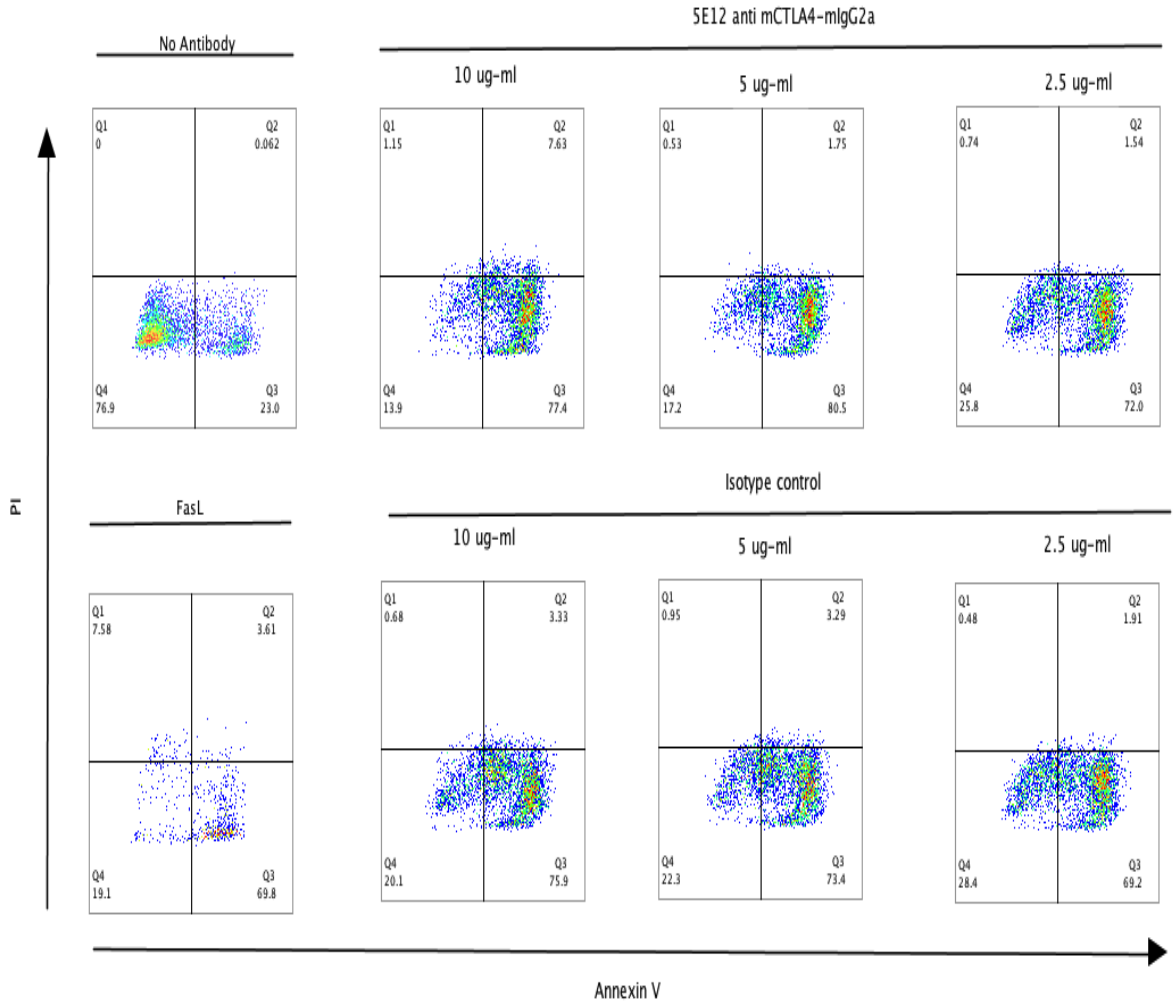

Figure 26. 5E12 treatment does not mediate direct apoptosis of T cells. Activated T cells were incubated with antibody 5E12 or Isotype control MPCII at the indicated concentration for 12 hours at a two fold dilution series. Following apoptosis analysis by annexin vs PI staining. FasL was used at 1%. Flow cytometric data showing apoptosis percentage.

2.3.5 Discussion

CTLA4 monoclonal antibody, Ipilimumab anti CTLA4 (human IgG1 isotype) is an effective treatment strategy for metastatic melanoma[72]. Studies have highlighted the role of CTLA4 antibody action as both preventing the interaction of CTLA4 with B7-1, B7-2 and depleting the immunosuppressive regulatory T cells within the tumor microenvironment[63]. Another CTLA4 mAb in clinical trials, Tremilumumab (human IgG2), is a blocker but non depleter in humans but does not seem to be therapeutucally effective in patients as a single agent. It is thus worthwhile to check if an antibody that is a depleter and a non blocker will be therapeutically effective. This study is to determine the effective mechanism of CTLA4 antibody action. We need to understand each mechanism separately so as to make antibodies that cause fewer adverse effects in patients.

Our data suggest the mechanism of action of an effective anti CTLA4 antibody to be both depletion of Tregs in the tumor microenvironment and blocking the interaction between CTLA4 and its ligands, B7-1 and B7-2. I use our novel anti mCTLA4 antibody clone 5E12 which has a specific characteristic of being a depleter but a non blocker that is not inhibiting CTLA4 from binding with its ligands B7-1 and B7-2. I test this in a CT26 colon tumor mouse model and observe that there is no therapeutic efficacy or survival benefit of our anti mCTLA4 antibody. On the other hand our control anti CTLA4 clone 9D9, that is both a blocker and depleter shows great survival benefit with tumor regression in all four mice. This observation of no survival with the 5E12 clone might be due to an enhanced killing by the antibody of all T cell types. It may somehow linked to its property of being a non blocker as other killer antibodies that are blockers do not exhibit this characteristic.

Previous studies have also pointed out that anti hCTLA4 antibodies that are non blockers may mediate T cell apoptosis. In a study published by *Gribben et al*, *1995* anti CTLA4 antibodies induced antigen

specific apoptosis of activated human T cells[73]. The mechanism of antibody action remained elusive and it was thought that CTLA4 might be involved in direct T cell apoptosis; however, this idea has received little experimental support.

To further understand the mechanism of antibody mediated direct T cell apoptosis, I use an in vitro T cell mediated apoptosis assay. I however do not observe our antibody to directly kill activated T cells in vitro. Thus the possibility of a non blocking CTLA4 antibody to mediate T cell killing directly as observed in previous literature[73] is not supported in our study. Another possibility for T cell mediated apoptosis might be sequestration of B7 by cell surface CTLA4, that prevents CD28-B7 co stimulation required for cell survival.

I will further extend our study with ADCC experiments in vitro to answer whether the antibody is an enhanced depleter.

Chapter 3 Discussion and Perspectives

3.1 The interaction of PDL1-B7-1

The PDL1-B7-1 pathway was discovered in 2007[15]. It was proposed for B7-1 on APCs to interact with PDL1 on T cells. The functional role of this pathway has been shown to be involved in maintaining peripheral T cell tolerance[41, 43]. However there have been conflicting reports in the field. For instance, *Haile et al, 2011* showed that the soluble CD80 restored T cell activation by preventing PDL1 from interacting with the inhibitory PD1[45]. There have also been reports that PDL1 does not bind to B7-1 with biologically significant affinity, *Davis et al, 2001* showed using biacore that though PDL1-PD1 had a strong interaction, PDL1-B7-1 had a very weak interaction and that it therefore may not have a functional relevance[47].

The major questions still remaining that need to be addressed are the functional role of the pathway, does it inhibit or activate a T cell, and most importantly if the two molecules bind to each other in cis on a real cell surface. No literature describes the downstream signaling pathways. While extensive studies have looked into PD1-PDL1, not much research has been done on the PDL1-B7-1 pathway. My study highlights the mechanistic constraints for an interaction to occur between PDL1 and B7-1. I propose a model in terms of the binding interactions of the pathway, for it to function from a within the cell interaction rather than a cell to cell interaction.

Knowing the exact particulars of the two interacting molecules and pathways may help us target and apply it more effectively in a disease setting. Its ability to affect the other co stimulatory interactions of CTLA4-B7 and PDL1-PD1 can offer insight to better enhance and target these clinically relevant pathways.

My experiments demonstrate the PDL1-B7-1 interaction takes place only when either of the molecules is flexible and accessible. I also confirm this pathway competes for the PD1-PDL1 interaction. My experiments with 29E.2A3 PDL1 antibody demonstrate the binding regions of both PD1 and B7-1 to be overlapping in the IgV domain of PDL1. Further, our cell conjugation assay showed PDL1 does not bind to B7-1 in trans. I therefore suggest the two molecules interact on the same cell in cis as opposed to a cell to cell trans interaction.

We cannot determine in the inflexible ELISA format whether all our PDL1 molecules in the Ig fusion protein dimer are accessible or if the molecule is substantially masked by binding to the plate. Since both B7-1 and PD1 are treated with the same conditions, our conclusions are made on comparing the interaction of B7-1 to PD1 with PDL1. Our observation of a difference in the maximal mean fluorescence intensity for PD1 binding to PDL1 when using the two different transfected cell types- 300.19 hPDL1 and 300.19 hPDL1-Tim3 can be due to a difference in the number of PDL1 transfected molecules on each cell. However, the difference in the MFI of B7-1 binding with PDL1 relative to PD1 is decreased when using a flexible and accessible PDL1. We can extend our study to using a flexible and accessible B7-1 to test whether this will also result in a stronger binding interaction with PDL1. In Figure 6 with flexible PD-L1, the lower EC-50 value for B7-1 in comparison to PD1 indicates B7-1 can have a higher affinity binding for PD-L1 than PD-1. The explanation for this needs further physical and structural investigation.

Future experiments that I can do are to cotransfect both PDL1 and B7-1 on the same cell. Using techniques such as CO-IP and FRET we can test our model of the interaction being on the same cell. We need to expand our observation of B7-1 ability to prevent PD1 from binding with PDL1 to a functional study. The significance of this observation should be extended to a disease study. This can help us understand whether the pathway is involved in T cell exhaustion or is rather for T cell proliferation. Two

different possibilities exist, either the B7-PDL1 pathway has evolved as a mechanism of exhaustion or that the pathway prevents the inhibitory PDL1-PD1 and promotes T cell proliferation.

Creating knockout mice lacking either B7-1 or PDL1 is not a solution for determining the pathway's functional role. The reason being both PDL1 and B7-1 have multiple binding partners- PD1, CD28, CTLA4. Due to this complex system of multiple partners for the same molecules we cannot confidently interpret gene specific deletion of the molecules. A gene deletion approach will affect more than one pathway making it very difficult to intepret the changes in T cell function. Generating antibodies that specifically block either one of the pathways, PDL1-PD1 or PDL1-B7-1 without affecting the other can offer insights into the functional aspects of the pathway. This can be achieved by creating antibodies that target only the non overlapping, unique, region of interaction. This will also offer insights into the exact binding region of PDL1-B7-1. To determine the binding regions I can also extend my study to spectroscopy and crystallography that will help us understand the exact binding regions of B7-1 on PDL1.

The paper by *Paterson et al, 2011* used a novel 10F.2H11 anti mouse PDL1 antibody that specifically blocked B7 from binding with PDL1 but not PD-L1 with PD-1[43]. Using this antibody it was shown that there was an enhancement of diabetes in a NOD mouse model. It was not characterized clearly if the PDL1-PD1 pathway was playing a role in the observations. However antibodies that only block PD1 from binding with PDL1 but allow B7-1 to bind to PD-L1 are not known. It will be worthwhile to generate them and then test them in an in vivo disease setting to better understand the function of B7 and PDL1.

My study also does not address whether this is a chance binding between PDL1 and B7-1 or if it does play a significant role; however, it is conserved in mouse and human. I believe based on the previous literature that the pathway exists for a specific function that needs to be studied in more detail.

3.2 Combination blockade of CX3CR1 with PD1

The PD1 blockade therapy has shown response in a wide range of tumors. It is now FDA approved for advanced melanoma, non small lung carcinoma, and kidney cancer. It has also shown efficacy in multiple other tumor types including Hodgkin lymphoma, triple negative breast cancer, and ovarian cancer[24, 74]. However only about 30 percent of patients respond to this therapy. Thus there is an urgent need for more effective combination therapies. Many combination therapies are being tested for better efficacy. Effective regimens include combining PD1 therapy with CTLA4 therapy, chemotherapy[75] or radiation therapy with PD1[76], PD1 with immunostimulants, PD1 with CAR-T therapy[77] and cancer vaccines. Strategies such as targeting other inhibitory receptors LAG-3, TIM3, CD160, TIGIT are in progress[8]. However besides the PD1 and CTLA4 combination therapy, other combination therapies have not reached the clinic yet and are still under investigation for a prospective efficacy and safety.

Studies have highlighted the role of CX3CR1 in tumor progression and metastasis. Previous research using mice lacking CX3CR1 show a decrease in metastasis[56]. Because of the expression of this molecule in a variety of immune cells it remains unclear whether it can be effective for tumor immunotherapy.

I test the anti CX3CR1 antibody for the blockade of CX3CR1 binding with its ligand CX3CL1 as well as depletion of immunosuppressive cells expressing CX3CR1. Repeat experiments of tumor immunotherapy using increased numbers of mice are required. The expression of CX3CR1 on tumor cell lines was assessed by both flow cytometry and western blot analysis. For western blot, additional control blotting with a housekeeping gene (Beta actin) are required. The difference in relative expression of CX3CR1 between CT26 and 4T1 between the two techniques of flow cytometry and western blot analysis remains unanswered.

My study focuses on only two tumor cell lines- colon carcinoma and metastatic breast carcinoma. Since CX3CR1's functionality varies across tumors[48], applying this combination therapy to additional tumor types may provide insights into the tumor types where in the combination can effectively work. It will be worthwhile to test this combination in mouse tumor models that have an effective response to PD1 therapy and for tumors where tumor associated macrophages play a dominant immunosuppressive role.

I observed that ex vivo tumor cells have very high levels of CX3CR1, higher than the immune infiltrate. The main mechanism has not been explored for this induced over expression of CX3CR1 on tumor cells in vivo. The levels on tumor cells in vitro and in vivo should be compared in the same experment. The exact mechanisms for this induced expression are not known.

Further the mechanism of resistance by the tumor to immunotherapy has not been explored as yet. The main reason for the tumor to express CX3CR1 is not known. So far studies have reported that CX3CR1 ligation by its ligand CX3CL1 caused upregulation of survival genes via MAPK, JAK, kinases[60]. My future experiments would be to test the downstream signaling consequences of CX3CR1 on tumor cells.

I further show that CX3CR1 is expressed on both M1 and M2 macrophages. Further the number of M2 macrophages within the CD45 immune infiltrate is greater than the M1 macrophages. It has been shown that CX3CR1 may cause repolarization of M1 macrophages to the M2 phenotype. The mechanism behind the repolarization of macrophages by signaling via the CX3CR1-CX3CL1 pathway is not known. Our future experiments would be to understand the phenotypic switch to the M2 phenotype by CX3CR1 ligation; I would identify the genes downstream of CX3CR1 that cause this phenotypic switch. Understanding this may offer additional tools to directly target genes causing the change to a M2

phenotype and making drugs against these genes, which may be an additional therapeutic strategy for cancer treatment.

A major question yet to be answered in my study is the mechanism of action of the CX3CR1 antibody. I will follow with a study of the tumor immune infiltrate on antibody treatment. A comparison between the groups of single agent or combination blockade will give insight to the immune subsets and the cell types being depleted by the antibody blockade. I assume this CX3CR1 antibody with an IgG2c Fc region to be a killer antibody; however, there is very little published literature on IgG2c effector functions. Most are by analogy to the related IgG2a. The best way our antibody could work is if it depletes the tumor, M2 macrophages and MMDSCs. The expression of CX3CR1 on most immune cell types raises the possibility the antibody might kill too wide variety of immune cell types and thus dampen an effective anti tumor immune response. A moderate killer antibody might target only the highest expressors of CX3CR1: tumor, M2 and MMDSCs. Further experiments might include on target antibody injection into the tumor microenvironment rather than a systemic injection to prevent the depletion of various immune cells.

I will follow our study with ADCC (antibody dependent cellular cytotoxicity) and CDCC (complement dependent cellular cytotoxicity) in vitro assays to understand the depletion mechanism of this antibody. Not much is reported in the literature of the depletion mechanism of the IgG2c Isotype. The in vitro assays will help us understand whether the antibody targets tumor cells and/or immune cells.

Lastly, this study presents a preliminary survival curve of this immunotherapy. Additional repeat experiments are required for statistical confidence.

3.3 Contributions of B7 blockade and CTLA-4 cell depletion to the mechanism of tumor immunotherapy by CTLA-4 mab

CTLA4 monotherapy was FDA approved for the treatment of metastatic melanoma[72]. Severe toxicities are associated with treatment in about 30% of patients[72]. In mouse models, the mechanism of antibody action was first described as blocking the interaction between CTLA4 and its ligands B7-1, B7-2[62]. Follow up studies showed depletion of tumor infiltrating regulatory cells that express high levels of cell surface CTLA4 as the major mechanism of action[61, 63].

Questions that remain in the field are which mechanism plays a more dominant role and whether either mechanism alone, blockade or depletion, is sufficient for treatment efficacy. The role of each mechanism needs to be studied separately. Further it is not known if a CTLA4 antibody with only depletion activity is enough for tumor immunotherapy. In order to identify the best antibodies with decreased toxicity and lower side effects we will need to answer the above questions. Though antibodies against CTLA4 have worked for metastatic melanoma, they have not been successful in other tumor types in Phase 3 studies. For us to make CTLA4 therapy work in other tumor types, we need to better understand the mechanisms of antibody action.

9D9 is a CTLA4 mAb that blocks and is a moderate depleter. The ideal positive control for our experiment would be 9D9 with an IgG2a isotype. The 9D9 variant that I use is of IgG2b Isotype. Thus the experiment will have to be repeated with a 9D9 positive control that is of IgG2a Isotype.

This study presents preliminary data of the tumor infiltrating lymphocytes. Repeat experiments of the mechanistic study should be performed to better validate the results. Further a mechanistic study of the

9D9 treatment group was not performed as the tumors regressed rapidly. A repeat of the mechanistic study including the 9D9 positive control needs to be performed. In our apoptosis assay, all the cells were negative for PI, which is technically unexpected. The assay should be repeated with higher voltages so as to have more separation for PI.

Our future experiments will mainly focus on the reasons behind the massive CD3+ depletion in the 5E12 treated group. I will focus on the main mechanism behind CD3 cell depletion since our direct antibody mediated apoptosis assay did not show any apoptosis in the antibody treated group. I will extend our study to ADCC, to understand if the antibody is too strong a depleter and is killing cells even with very low CTLA4 expression. Further our proposed hypothesis of sequestration of B7-1 by CTLA4 will be tested by including APCs in our direct antibody mediated apoptosis assay. I will also extend our study to epitope mapping of our 5E12 clone to determine the binding region of this antibody on CTLA4.

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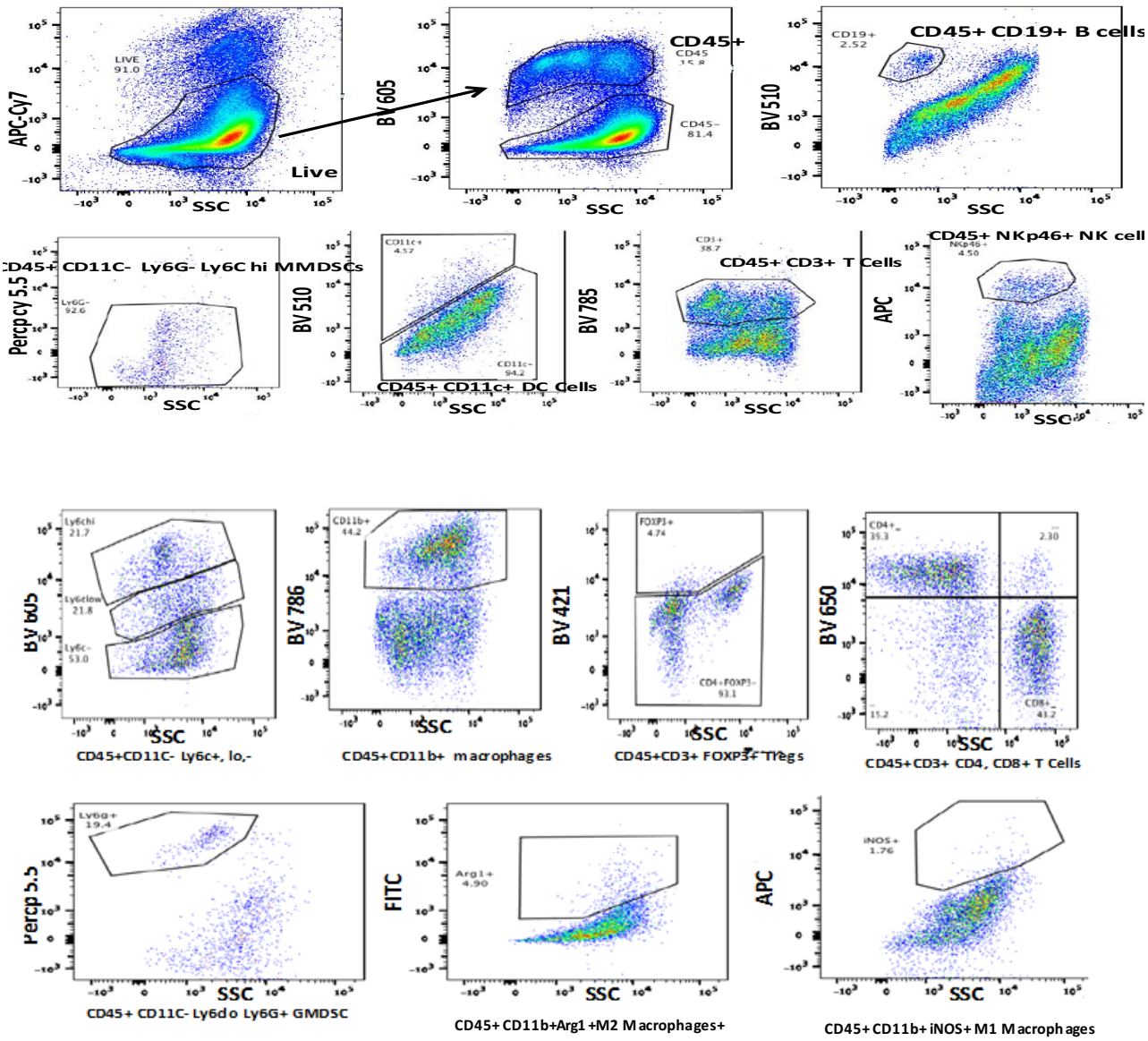
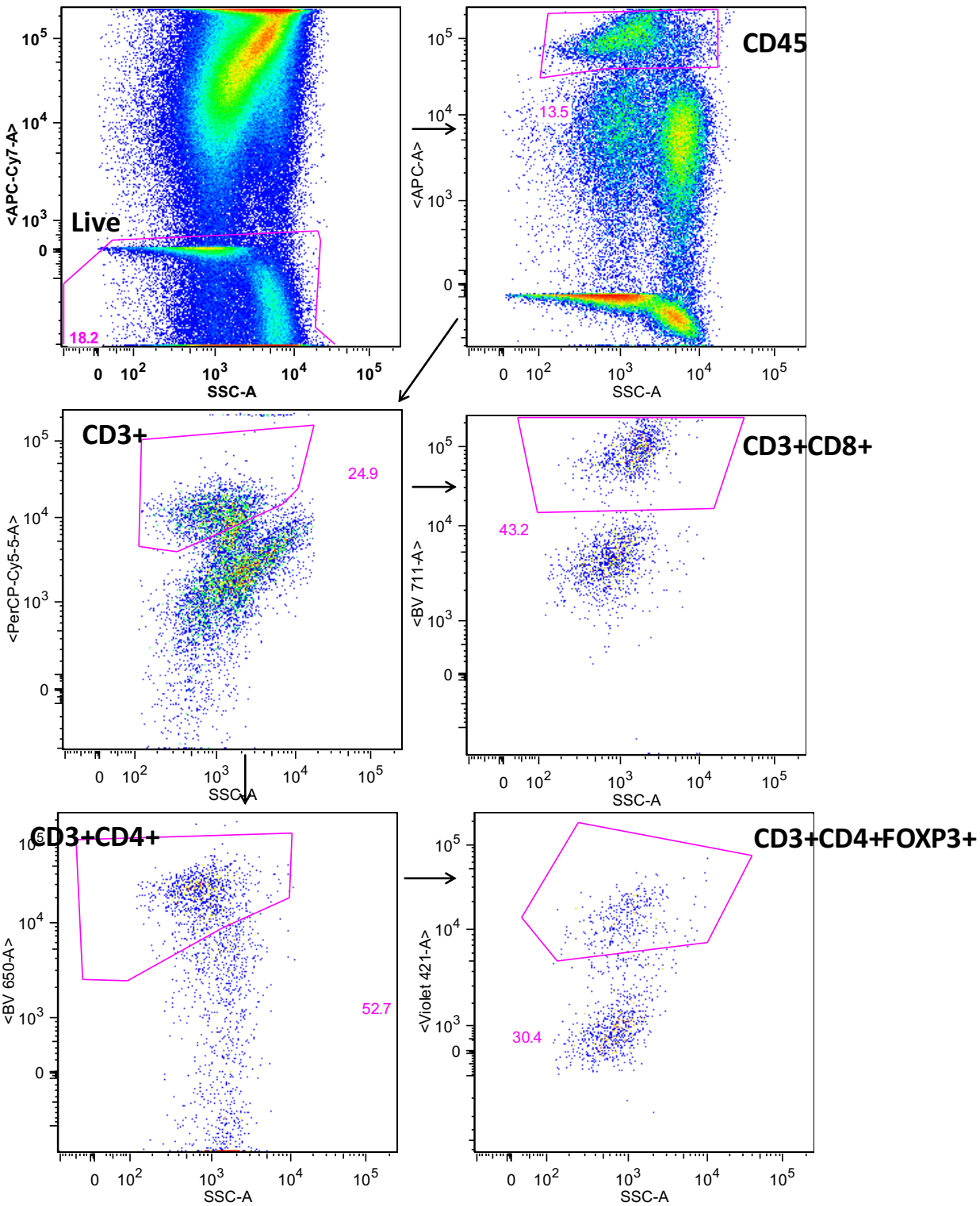
Appendices

1. Gating strategy for Tumor Infiltrating Lymphocytes for CX3CR1



2. Gating Strategy for Tumor Immune Infiltrating Lymphocytes in the CTLA4 5E12 anti mCTLA4 antibody treated experiment



3. In vitro assay for CTLA4 Antibody mediated Apoptosis