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DETERMINING DIFFERENTIAL EFFECTS OF INTERLEUKIN-2 ON INNATE AND ADAPTIVE IMMUNE CELLS IN LYMPHOID ORGANS AND THE GASTROINTESTINAL TRACT

by

AYUSHI SINGH
B. Tech., Amity Institute of Biotechnology, 2014

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science
in the Burnett School of Biomedical Sciences
in the College of Medicine
at the University of Central Florida
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Major Professor: Karl Kai McKinstry

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ABSTRACT

Interleukin-2 (IL-2) is a pleiotropic cytokine demonstrated to be effective in treating cancer. However, clinical use of IL-2 can be associated with severe side effects including gastrointestinal toxicity (GT). Similar GT symptoms are observed in inflammatory diseases such as Crohn's Disease (CD). Interestingly mounting evidence indicates a role for IL-2 in CD, but the underlying mechanisms are unknown. Indeed, studies on the in-vivo activities of IL-2 have mostly focused on secondary lymphoid organs and immune cells associated with them. Very few studies have addressed how IL-2 signals impact populations of immune cells in the gut. Here, we aim to identify and compare the effects of systemic IL-2 administration on six major leukocyte population and their subsets in mice using multicolor flow cytometry. While we confirmed previously observed changes in specific immune cell populations in the spleen, very few changes were seen in the gut and gut associated lymphoid tissues. Unexpectedly, a sharp decline was seen in B cells, most notably in Peyer's Patches, in mice treated with IL-2. Our data furthermore indicates that B cells in IL-2 treated mice undergo enhanced apoptosis in Peyer's Patches. Some studies suggest that changes in B cells may contribute to development of CD. Thus, this study may aid in defining ways in which IL-2 can contribute to disease etiology, and lead to novel treatments for CD.

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LIST OF ACRONYMS/ABBREVIATIONS

APC's	Antigen Presenting Cells
CD	Crohn's Disease
DC's	Dendritic Cells
DSS	Dextran Sulphate Sodium
GALT	Gut associated Lymphoid Tissues
GvHD	Chronic Graft Versus Host Disease
HCV	Hepatitis Virus Infection Induced Vasculitis
HIV	Human Immunodeficiency Virus
IBD	Inflammatory Bowel Disease
IEC	Intraepithelial Cells (lymphocytes)
IFN γ	Interferon γ
Ig	Immunoglobulin
IL	Interleukin
IP	Intestinal Permeability
IL-2	Interleukin 2
IL-2R	Interleukin 2 receptor
IL-2C	Interleukin-2 complex
JAK	Janus Kinases
KO	Knock Out
LP	Lamina Propria

MLN	Mesenteric Lymph Node
MΦ	Macrophages
NK	Natural Killer Cells
PP’s	Peyer’s Patches
SCID	Severe Combined immunodeficiency Diseases
SLE	Systemic Lupus Erythematosus
STAT	Signal Transducer and Activator of Transcription Proteins
Th	Helper T cells, CD4
Tc	Cytotoxic T cells, CD8
TNF α	Tumor Necrosis Factor- α
Tregs	T Regulatory cells
T1D	Type 1 Diabetes
UC	Ulcerative Colitis
VLS	Vascular Leak Syndrome
WT	Wild Type
WT	Wild Type
μMT	Immunoglobulin heavy constant mu

CHAPTER ONE: - INTRODUCTION

IL-2 and IL-2 receptor system

Since the discovery of retroviruses, scientists have attempted to grow certain ones in laboratories. To do so, they needed to culture T cells. In the 1970s, a team of researchers led by Robert Hallo cultured T cells by stimulating lymphocytes with phytohemagglutinin, a protein found in plants, and then searching for the production of growth factors. As a result, the team identified interleukin-2 (IL-2). IL-2 was discovered in 1976 as a growth-promoting factor for T lymphocytes [1] and purified in 1980 [2]. IL-2 belongs to the γ chain family of cytokines [3]. This family also includes IL-4, IL-7, IL-9, IL-15, and IL-21. IL-2, an autocrine and paracrine growth factor, is secreted primarily by CD4 T cells also called as T helper cells (Th), but also by CD8 T cells also called as Cytotoxic T cells (Tc). Upon encountering an antigen, activated $CD4^+$ T cells begin producing IL-2, followed by the expression of the IL-2 receptor. This reaction allows the rapid and selective expansion of the antigen-specific effector T-cell population (CD8 and CD4).

Paradoxical to IL-2 stimulating an effector response, it is responsible also for downregulating immune responses to prevent autoimmunity. This effect was revealed when IL-2 knockout (KO) mice developed an autoimmune disease with robust T-cell involvement in the place of no expected T-cell growth. This is now known to be caused by the failure of T regulatory cells (Tregs) to develop in IL-2 KO mice. Tregs downregulate the immune response and require IL-2 for their survival and proliferation.

The IL-2 trimeric receptors consist of three chains: IL-2Rα (CD25), IL-2Rβ (CD122), and γc (CD132) [4]. The α chain (CD25) of the receptor does not participate in signalling, but is, instead, responsible for increasing the binding affinity by 10 to 100-fold. In addition to being in Tregs, this receptor is only found on activated CD8 and CD4.

The expression of CD25 is tightly regulated. T-cell receptor stimulation and IL-2 binding lead to the expression of CD25; thus, IL-2 acts as a positive feedback loop, leading to the production of more IL-2 and the sustained expression of high-affinity IL-2 receptors. However, IL-2 can also bind in the absence of CD25, except a greater number of receptors are expressed. The binding affinity of these receptors to IL-2 are less than the high affinity receptors. thus, the receptor is termed an intermediate affinity-binding receptor. Although intermediate affinity binding receptors are mostly present on memory CD8 T cells and on natural killer (NK) cells, these receptors can also be found on naïve CD8 and memory CD4 T cells in low levels. After receptor binding has occurred, the newly produced IL-2 receptor is internalized and its components degraded, but CD25 is recycled back to the surface. CD122 and γ_c cause signal transduction through pathways such as JAK-STAT (Janus Kinases Signal Transducer and Activator of Transcription Proteins) , phosphoinositide 3-kinase (PI3-K), and the mitogen-activated protein kinase pathway (MAPK), resulting in greater production of IL-2. These pathways impact gene expression to regulate cellular growth, death, and immune function in IL-2R-bearing cells.

In addition, IL-2 functions as a paracrine growth factor for NK cells and enhances NK cytotoxic activity by binding to an intermediate affinity receptor. Overall, IL-2 stimulates the proliferation and differentiation of CD4 and CD8 cells and is produced chiefly by these cells. However, IL-2 is also produced in a minimal amount by NK cells, B cells, and Dendritic cells (DC's) in at least some situations [5].

IL-2 immunotherapy

Following the discovery of IL-2 in 1976, a use for it was sought in therapeutics. Its potent ability to stimulate Tc and NK cells, which are involved in responses against tumors, meant that high-dose IL-2 immunotherapy trials began in late 1980s. In 1986, a 33-year-old woman was the first person to be entirely healed by aggressive IL-2 therapy. In that trial, 25 patients with metastatic cancer were treated: 4 out of 7 patients with metastatic melanoma and 3 of 3 patients with metastatic renal cancer exhibited regression of cancer [6]. Many trials followed this breakthrough, as reviewed in [7], and the success rate achieved was between 10 and 20%. Thus, in 1998, IL-2 was approved by the U.S. Food & Drug Administration for the treatment of metastatic melanoma and in 1992 for renal cell carcinoma.

Many issues are associated with the effects of the therapy. Capillary leak syndrome, hypotension, neural symptoms, substantial weight gain, increased serum creatinine and bilirubin levels and gastrointestinal symptoms all posed many problems. Furthermore, gastrointestinal symptoms, including severe diarrhea, nausea, and vomiting, were reported in the majority of the patients.

Occasional cases of bowel haemorrhage, stomatitis, infarction, perforation, acute pancreatitis, and gastrointestinal bleeding occurred. Stress-induced gastric-acid secretion and the administration of nonsteroidal anti-inflammatory agents to control fever predispose patients receiving IL-2 to develop peptic ulcer disease. The use of anti-inflammatory drug impacts the immunomodulatory and antineoplastic effects of therapy. All these symptoms lead to early termination of the treatment, thus hampering the potential beneficial effects of the therapy [8-10].

Surprisingly, later, low-dose IL-2 therapy was utilised to stimulate the production of Treg cells to improve autoimmune conditions in animals. It is firmly established that Tregs are required to prevent autoimmune diseases, and their depletion, whether it is hereditary or induced because of certain conditions lead to a variety of autoimmune diseases and inflammatory responses. Some of these responses include type 1 diabetes (T1D), hepatitis virus infection induced vasculitis (HCV), chronic graft versus host disease (GvHD), systemic lupus erythematosus (SLE), alopecia areata, gastritis, and colitis. Mice lacking IL-2 or lacking functional IL-2 receptor developed severe ulcerative colitis (UC) and produced multiple autoantibodies , although colitis developed delayed mild focal intestinal inflammation when mice were housed in germ free conditions [11-13]

Low-dose IL-2 produced greater levels of Tregs and the treatment was well tolerated. This finding marked the beginning of utilising low-dose IL-2 to stimulate the production of Treg cells in humans to improve autoimmune and allo inflammatory conditions. Zorn *et al* (2006) treated chronic myelogenous leukaemia patients' after hematopoietic cell transplantation with low-dose

IL-2. $CD4^+$ T Regs and Foxp3 expression in $CD3^+$ peripheral T cells were found to be increased many folds Similarly, in treating T1 D, IL-2 therapy was found to be safe and dose dependent [14]. The same observations of an increase in Tregs was observed in other diseases. There are ongoing clinical trials testing the effect of low-dose IL-2.

Although low-dose IL-2 therapy led to a significant drop of toxicity, there were adverse effects. Toxicity remained a problem and clinical trials were discontinued due to intolerable side effects. According to Yang *et al* [15], the common reasons for this cessation were hypotension and patient refusal. In the trial for low-dose IL-2 therapy for chronic GvHD, bacterial infection caused the deaths of three patients. Therefore, further clinical trials in this area are required to resolve these problems.

<u>IL-2/anti-IL-2 monoclonal antibodies</u>

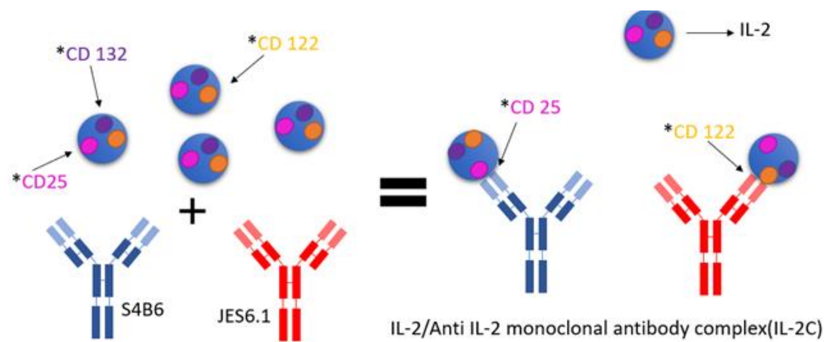


Figure 1:- Interaction of two different monoclonal antibodies with two different binding sites

Many groups have tried to find ways to curb the negative effects of IL-2 treatment. IL-2C was one of the ways of doing this, and is still being tested on mice. Boyman *et al*. (2006) demonstrated that IL-2-neutralising monoclonal antibodies skew the balance of responding lymphocyte population dramatically in a mouse[16]. Paradoxically, the antibodies upregulated the function of IL-2 instead of downregulating it. Memory CD8 cells and NK cells, which expressed only dimeric receptors, proliferated. The IL-2C made with S4B6 led to a 20 to 1000-fold increase of CD8 cell *in vivo*. Another monoclonal antibody clone JES6.1, stimulated the increase in $CD4^+$ Tregs and a low increase in CD8 cells but prevented the stimulation of other cells. The reason for these two very different results depends on the receptor: S4B6 worked by blocking the interaction between IL-2 and CD25; whereas, JES6.1 blocked CD122 [17].

The IL-2/S4B6 IL-2C has been proven to possess significant antitumor activity in mice. In a study , IL-2 expression plasmid was simultaneously injected with IL-2 in a B16 melanoma model [18]. The study found significant tumour removal in the lungs of experimental mice. Although published data by Tomala *et al*.[19] reveal that IL-2/JES6.1 also stimulates $CD8^+$ T cells in some of the tumour models, IL-2/S4B6-driven expansion is superior than the former. Two different studies focused on treating mice in conjugation with antibiotics and alone. Mice treated early after tumor inoculation or treated with antibiotics prior to treatment with anti-IL-2 displayed prolonged survival and, in some cases, significant improvement. As explained previously, IL-2-mediated toxicities are poorly understood, except vascular leak syndrome (VLS). In this context, Krieg *et al*.([20] compared the effect and toxicity of recombinant IL-2 and rIL-2 antibody complex (S4B6)

on VLS. They observed reduced toxicity with a greater increase of $CD8^+$ T cells. The reason VLS caused toxicity was found to be CD25 expressing lung epithelial cells via increased nitric oxide production.

With respect to IL-2/JES6.1 IL-2C, it has been majorly tested in terms of autoimmune disease and inducing immunosuppression, especially following organ transplantation. This testing is obviously because of the complex's ability to expand Tregs, which facilitates immunosuppression and initiates tolerance without affecting the $CD25^-$ cells. Injecting mice with IL-2/JES6.1 prevents the rejection of allogeneic pancreatic islets without the use of any other immunosuppression in the host mice [21]. In NOD (non-obese diabetic mice), IL-2/JES6.1 has been proven to impair several autoimmune diseases, including T1D and myasthenia gravis [21]. In conjunction with rapamycin, IL-2/JES6.1 has been demonstrated to be effective in experimental autoimmune encephalitis (EAE). Furthermore, IL-2/JES6.1 has been found to ameliorate type 2 diabetes by increasing Tregs in adipose tissues [22].

IL-2 Relation with inflammatory bowel disease (IBD)

IBD encompasses disorders that involve chronic inflammation of the gut. IBD primarily comprises two types – CD (Crohn's Disease) and ulcerative colitis (UC) – and their symptoms usually involve severe diarrhoea, abdominal pain, fatigue, and weight loss. As explained above, IL-2 also causes severe gastrointestinal toxicities, including diarrhoea and vomiting, which are very similar to the symptoms manifested in CD.

A number of existing studies in the literature have examined the amount of IL-2 present in CD patients. It has been widely adopted in the field that a marker for immune activation is the expression of soluble IL-2 receptor in the serum. A study of 30 patients suffering from CD reported that IL-2 or IL-2R was significantly found in patients with active CD. The concentration of soluble IL-2 was found to be correlated with the disease activity measured by the Harvey-Bradshaw index [23]. Furthermore, a report by Mahida *et al*. of 15 patients with active CD (six ileal, five ileo-colonic, and four colonic) demonstrated a high amount of IL-2 in the plasma [24, 25]. However, the levels in that study did not correlate with the disease activity index. In another study, in which the authors examined the intestines of 12 children with active CD, they found an abundance of $CD25^{+}$ cells in lamina propria (LP) in CD, which the authors concluded to be T cells [26]. Moreover, when sIL-2R beta (CD122) was measured, in a different study, in the serum of 31 CD patients, a positive and significant correlation existed between sIL-2R levels of alpha and beta chains, but not in UC patients or in healthy controls [27]. In yet another study, of 12 children suffering from CD, the levels of IL-2, along with other inflammatory cytokines, wwas increased 6 to 9-fold [28].

In addition to measuring IL-2 receptor expression, different studies have measured the levels of IL-2 messenger RNA (mRNA). Mullin *et al*. determined IL-2 mRNA levels using a quantitative reverse transcriptase polymerase chain reaction method. In CD, higher levels of IL-2 mRNA transcripts were detected in the mucosa from areas of active inflammation, but in areas that were

histologically normal, levels were similar to control subjects [29]. However, IL-2 mRNA levels were significantly ($P < 0.02$) increased, but only in active CD, not in UC or controls [30].

IL-2 caspase 3 chimeric protein was designed to target cells that express the high-affinity IL-2 receptors. In experiments performed by Shteingart *et al.*, in which they treated Dextran Sulphate sodium (DSS) colitis-induced mice with IL-2 caspase 3 chimeric protein for 3 to 5 days, they achieved up to 78% improvement in disease activity index with intravenous injections of 15 mg/mouse/day. Furthermore, IL-2 caspase 3 decreased neutrophil and macrophage infiltration to the inflamed tissue by up to 57%. Moreover, the Bcl2/Bax ratio, which is elevated in both experimental colitis and in human CD, decreased dramatically following treatment [31].

All the above mentioned studies indicate that IL-2 can be involved in the pathogenesis of CDD due to its pro-inflammatory nature.

Role of innate and adaptive immune cells in IBD

There are many different subsets of adaptive and immune cells which may be involved in CD. Continuing below are various experimental evidence that IL-2 can impact them

CD4 T cells

CD4 T-cell subsets and the cytokines produced by them (Th1-IFN- γ and TNF α, Th2-IL-4 and, Th17- IL-1 7 and Tregs) have long been recognised as being involved in IBD, as reviewed in [32-34].

There exists a considerable body of literature focusing on the involvement of Th cells (CD4 T cells) in CD. $CD4^{+}$ T lymphocytes, which commit to Th1 T-cell development, express a transcription factor called T-bet. A study has proven that T-bet-deficient T cells failed to induce colitis *in vivo*, suggesting that overexpression of T-bet is sufficient and essential to promote Th1-mediated colitis [35]. Th1 T cells produce IFN-γ and TNF-α. Ito *et al*. demonstrated the involvement of IFN-γ by treating an IFN-γ KO mouse versus a wild-type (WT) mouse with DSS and comparing the effects. They. found that the IFN-γ KO mouse manifested attenuated colitis; whereas, the WT mouse underwent a serious complication of colitis and died thereafter [36]. In addition to IFN-γ, TNF-α has been proven to be involved in the inflammatory reactions in CD. Many mouse models of colitis have been established. One better-established model, among many, is the SCID (Severe combined Immunodeficiency) model, in which the transfer of syngeneic splenic $CD4^{+}CD45RB^{high}$ T cells in SCID mice develops IBD. Studies have demonstrated that repeated treatments of SCID mice with neutralising antibodies against TNF attenuate or even prevention of colitis induction [37, 38]. In yet another mice model, in which TNF α is produced

systemically, the authors found that the mice displayed increased mucosal and systemic TNF-α levels compared with WT controls ($P<0.001$), as well as severe chronic ileitis [39].

Recently, IL-17 has been identified as an important player in IBD [40]. IL-17 is involved in the production of many pro-inflammatory factors, including TNF-α, IL-6, and IL-17, pointing to an important role of IL-17 in localizing and amplifying inflammation [41-44]. There is evidence that IL-17 produced by distinct lineage of CD4 T cells differentiates into inflammatory phenotype in the presence of IL-23 and IL-1β, produces IFN-γ, and suppresses FoxP3 expression; thus, not allowing cells to convert into Tregs. This plasticity of IL-17 and Treg cells may morph the regulatory response in IBD [45], which reveals that Th17 cells might be responsible for IBD.

Tregs produce cytokines such as IL-10 and TGF-β. In IL-10-deficient mice, most animals were growth retarded and anaemic and suffered from chronic enterocolitis [46]. Tregs have been found to be suppressed in various models of IBD [47], and the transfer of Tregs has been demonstrated to prevent colitis in many disease models of mice, as reviewed in [48].

In addition to the mouse studies, many clinical studies suggest the involvement of CD4 T cells and its subsets in CD. In a clinical study performed on 46 Chinese patients suffering from CD, the data demonstrate the increased levels of Th1 and Th17 subsets relative to the serum in the healthy donors. In contrast, the Th2 cells and serum IL-4 levels were similar to the controls (healthy donors) [49]. Additionally, another group confirmed the increased presence of circulating memory

(CD45RO $^{+}$ CD4^{+} T lymphocytes in peripheral blood from active CD patients. The cells lacked the receptor CD28 (null), which might be involved in a mark of disease activity and exhibited resistance to apoptosis [50]. Another viewpoint concerning the involvement of T cells in IBD comes from cases of patients with IBD and human immunodeficiency virus (HIV). Studies have revealed that, because CD4 cells are depleted in HIV patients, the remission of IBD occurs [37, 51]. However, other, controversial data exist and suggest no such possibility [37, 52].

Moreover, a different subset of Th cells expressing NKG2D – a molecule more prominently found in CD8^{+} T cells, γδ T cells, and NK cells and recognise mica (stress-induced histocompatibility complex) – was found in the LP in CD patients with a Th1 cytokine profile, compared with the controls [53-55]. Furthermore, human intraepithelial cells present in the gut endothelial lining do not activate T helper cells in the normal state, but activated Th cells with significant IFN- γ are found in IBD patients [56]. Moreover, activated Th1 cytokine IFN-γ is found significantly in IBD patients [36, 57]

TNF-α also increased in active CD. In organ cultures of involved IBD, mucosa was proven to spontaneously produce increased amounts of TNF-α than normal mucosa [58]. In addition, anti-TNF-α therapy has produced notable improvements in CD patients [59], implying that TNF-α might play an active role in CD. This possibility points out that Th1 cytokines contribute to inflammation in IBD. Thus, considering all the above cases, IBD patients display an increase in

CD4 T-cell activity. However, it is not yet clear if the changes in any of the T-cell subsets are the primary cause of disease or secondary to the inflammation response [60].

Overall, the literature pertaining to T cells, its subsets, and IBD strongly suggest the involvement of T cells in CD.

CD8 T cells

Many reports provide sets of data of either increased or decreased activity of $CD8^+$ T cells in CD [61]. In 1998, a colon biopsy of a CD patient revealed the infiltration of CD3 cells with a large proportion of CD8 cells. This patient was treated with immune replacement therapy – intravenous gamma globulin (IVIG) therapy – after which her health was regained. An *in-vitro* study attempting to explain the working of this treatment performed it on *in-vitro* lymphocytes, revealing that IVIG strongly reduces T-cell proliferation by suppressing antigen-induced IL-2 [62]. A recent study performed gene-expression profiling of $CD8^+$ T cells during CD and UC. The study found several pathways were upregulated including IL-2 signaling [63]. All these findings underscore that $CD8^+$ T cells may play a pathophysiological role in the occurrence of CD in the gut. In one experiment, the influence of intestinal epithelial cells on the cytokine expression of CD8 and Th cells from patients with IBD and HC was tested. It was found that IFN-γ-producing $CD8^+$ lymphocytes in patients with IBD significantly increased. In contrast, HC did not respond to the epithelial stimulus. Even in an inactive state of disease in IBD patients, exert an increased capacity for IFN-γ induction in $CD8^+$ lymphocytes. This finding demonstrates that IFN-γ could result in the

worsening of IBD [64]. In another study, $CD8^+$ IL-17 cells were found to be higher in patients with IBD. Double-expressing IFN-γ^+ IL-17^+ and Foxp3^+ IL-17^+ $CD8^+$ T cells were also identified, indicating possible $CD8^+$ plasticity [65]. In another experiment, the authors found that IFN-γ-producing specific $CD8^+$ T cells could induce acute and relapsing colitis [66]. Finally, CD8 T-cell subsets produce cytokine similar to CD4; thus, they are likely to play a role in IBD [67].

Gamma delta (γδ) T cells

Several animal [68, 69] and human studies [70-74] have pointed out that deficiency in γδ cells plays an important role in IBD, but this hypothesis is controversial. One of the studies involved CD patients with γδ deficiency in peripheral blood [71, 74, 75]. Treatments for IBD involving azathioprine and anti-TNF therapy increased the number of these cells in the patients, resulting in the reduction of inflammation symptoms [70, 73]. Conversely, another study found decreased numbers of γδ T cells in the colon, but the cells were activated and unusually located in the IBD patients, stimulating a Th1 response [75]. Very recently, IL-17-producing γδ T (γδT-17) cells have proved to be an important early source of IL-17 in many inflammatory settings and are emerging as an important participant in protumor immune responses. There is the expression of high-affinity IL-2R on γδT-17 cells. However, γδT-17 cells were found to be enriched, not depleted, in IL-2-deficient mice [76]. All these results suggest that IL-2 affects γδ T cells significantly and that γδ cells might be involved in causing CD.

B cells

B cells serve two major functions: first, they act as 'professional Antigen Presenting Cells' (APCs), presenting the antigen on the cell surface; and second, they secrete antibodies. The B-cell role in CD has not been greatly explored, and whether B cells are involved in the aetiology of CD remains a debatable topic.

In 1993, Yacyshyn *et al* found an increased presence of memory-cell B cells in CD patients, both in the gut and the serum [77]. They attributed this result as the cause of intestinal permeability (IP) [78]. IP is thought to be a key initiating event in CD [79, 80]. In a healthy human or mouse intestinal mucosa, immunoglobulin A(IgA) producing a B-cell system is prominent in the gut. IgA is present to provide non-inflammatory first-line defence functions – setting a state of tolerance, explained further in the review [81] by Brandtzaeg.

IgA's other function is to provide a barrier to pathogenic microbes and non-necessary antigens. The intestine becomes more permeable to these broad arrays of antigens due to disharmony among the immune system in the gut in IBD. Since, in the abovementioned study, the authors found an increased expression of B cells, they considered these cells to be responsible for disrupting the homeostasis. They tested this hypothesis through the inability of the intestine to exclude larger-sized molecules (antigens – lactulose and mannitol), causing intestinal inflammation, which was not the case in a healthy controls. This information states the importance of B cells in CD.

Various changes are observed in B cells in CD, as reviewed in [81]. Local IgA production downregulates in CD lesions, and excessive production of IgG has been found; this excessivity of IgG depends on the severity of the inflammatory disease. B cells suppress the action of neutrophils; thus, downregulating the action of pro-inflammatory cytokines [82]. Polymeric IgA B cells also block the non-specific biological amplification mechanism triggered by serum-derived IgG antibodies [83]. In IBD lesions, the chronicity of this change may result in the failure of antigen elimination. This result could be why 25% of colonic CD specimens exhibit mucosal invasion in contrast to normal intestine specimens. Furthermore, an increased number of CD patients were observed with selective IgA deficiencies (B cells). This change results in less-restricted leukocyte extravasation into the mucosal lesions [84] and, thus, an increase in pro-inflammatory cytokines such as TNF-α. Since B-cell functions are dysregulated in CD patients and IBD lesions, it could be possible that changes in B cells are involved in the pathogenesis of CD. However, due to a lack of data, this idea demands further attention.

Concerning IL-2, it has not been actively accepted that B cells proliferate in the presence of IL-2, although some research supports the notion that IL-2 is one of the growth factors for B cells, and that its receptor is found on B cells [85-89].

Macrophages (MΦ)

MΦ, or mononuclear phagocytes, also serve as APCs. The primary role of MΦ is to ingest and kill microbes and ingest dead host cells. In human non-inflamed intestinal mucosa, chemokines such as TGF β and IL-8 [90, 91] contribute to the recruitment of monocytes, which then turn into residential MΦ in the small intestine. Furthermore, normal human colonic and intestinal mucosa lack CD14, and CD14 is upregulated in the presence of bacterial lipopolysaccharide. In contrast, the inflamed intestinal endothelial cell lining in CD patients displays high levels of adherence factors that promote the migration of blood monocytes, as proved in murine models of mice [92]. Thus, there is a high level of recruitment of monocytes in gastrointestinal mucosa, meaning increased MΦ. MΦ in inflamed (CD) intestinal mucosa exhibit respiratory burst activity, which is absent in normal gut mucosa [91]. In addition, MΦ in the inflamed tissue release pro-inflammatory cytokines such as TNF-α [93], IL-1β, IL-6, and inducible nitric oxide synthase, contrary to normal mucosal MΦ producing IL-10 to maintain Treg function [92]. Thus, MΦ play a significant role in turning normal environment to an inflammatory surrounding.

Regarding the IL-2 receptor presence on MΦ, it was found that, in a tissue section of normal ileum, fewer IL-2 receptor MΦ were present; whereas, in inflamed mucosa, many IL-2 receptors were visible [94]. However, whether these receptors are functional is debatable. IL-2 has been found on activated monocytes, but not resting [95], and on alveolar MΦ in patients suffering from pulmonary sarcoidosis [96]. In another study on CD, $CD25^{+}$(IL-2 receptor) cells were found in

abundance in the LP mononuclear cells, especially in the submucosa. This result reveals the presence of IL-2 receptors on MΦ and may suggest their relation in IBD.

<u>Dendritic cells</u>

DC's are APCs, and are primarily of two types: classical DC's relocate themselves to lymph nodes for antigen presentation and respond to microbes; whereas, plasmacytoid DCs respond early to viral infections by providing type 1 interferons. DC's respond differently to different types of microbes, which signals the nature of the microbes [97, 98]. In the murine gut, DC's have the capability of upregulating IL-10 and IL-4 and less IFN-γ. Gut DC's can make TGF-β also [99-101]. Alternatively, DC's from inflamed CD patients express CD40 more than in non-inflamed CD patients or healthy controls. Production of CD40 causes IL-12 production, supporting Th1 cytokine production and TNF-α [97]. In the murine model of colitis, colonic LP DC's exhibit evidence of activation [102]. DC's either help in the activation of colitogenic T cells or aid Tregs in their suppressive function regarding IBD [103, 104].

Production of IL-2 by DCs has been demonstrated to impart crucial functions in both innate and adaptive leukocytes. Current research suggests that microbes are the most effective stimuli to trigger IL-2 production in DC's by activating the calcineurin/NFAT signalling pathway Moreover, it was found that deleting IL-2 in CD11c(high) MHCII (high) cells results in colitis that resembles IBD [105].

Neutrophils

Neutrophils, or polymorphonuclear leukocytes, are phagocytic cells that activate MΦ for their removal; their sustained existence leads to excess inflammation [106]. Neutrophils have been consistently associated with IBD. It has been established that leucocyte migration is significantly lower in CD, and that (mainly) neutrophils reduced the level of chemotaxis at the inflammatory sites [90, 107, 108]. It has been postulated that this limited infiltration of neutrophils can be attributed to the circulating inhibitors of chemotaxis or the altered MΦ in CD [109]. Furthermore, this defect in recruitment causes the incomplete clearance of microbes, inducing a strong secondary immune response and, thus, increased susceptibility to bacterial infections [110].

All the above mentioned defects and the even less expression of the Fc receptor have been associated with IL-2 therapy also, explaining the increased susceptibility to infections. Recent studies have proven that human neutrophils express both the β and γ chains of the IL-2 receptor, but have not found any trace of the IL-2Rα receptor. Furthermore, it has been indicated that IL-2 prevents the apoptosis of human neutrophils [111, 112]. Human PMNs also become activated by IL-2 after binding through the IL-2R beta present on the cell surface [113, 114]. NK and PMN cells play a central role in IL-2-induced VLS [115]. This finding indicates that IL-2 may indirectly affect the function of neutrophils during therapy.

NK cells

NK cells and CD8 cells share similarities regarding development, lytic mechanism, morphology, and cell-surface phenotype [116]. Both types of cell express receptors that are recognised by MHC class I glycoprotein. Concerning receptors, NK cells have, generally, two types – activating receptors (e.g. for NKG2D) and inhibitory receptors (e.g. for NKG2A) [117]. Furthermore, these cells express the IL-2/15Rb-chain (CD122) and the common g-chain (CD132) that are used as receptor components for multiple cytokines and are sufficient to stimulate proliferation in response to high concentrations of IL-2 [118, 119]. It has been demonstrated that human NK cells can be successfully expanded from PBMC fractions of peripheral blood in the presence of IL-2 [120], and that expanded cells are highly toxic. Thus, NK cells recognise and kill foreign antigens and secrete several cytokines such as IFN- γ, playing an immunoregulatory role.

According to old research, these cells tend to decrease in CD [121, 122], but recent research suggests that $CD16^{+}$ NK cells increase in the colonic LP of IBD patients [123]. In addition, $NKp44^{(+)}$/$NKp46^{(+)}$ NK cells are disrupted in the intestinal mucosa of patients [124]. Furthermore, there are mixed reports concerning whether mRNA levels of IL-2 are increased or reduced in inflamed mucosa of active CD [125-127]. A recent paper describes NK cells as saving mice from DSS-induced colitis by regulating the neutrophil function by using its NKG2A receptor. There have been many advancements regarding the NKG2D receptor as well. A recent phase II clinical trial revealed that an antibody against NKG2D induced clinical remission of CD in some patients,

suggesting that NKG2D and its ligands are of importance in the pathogenesis of CD [128]. This receptor has been found on inflamed intestinal cells [128-130]; thus, it somehow contributes to the pathogenesis of IBD.

Gastrointestinal immune system (GIS)

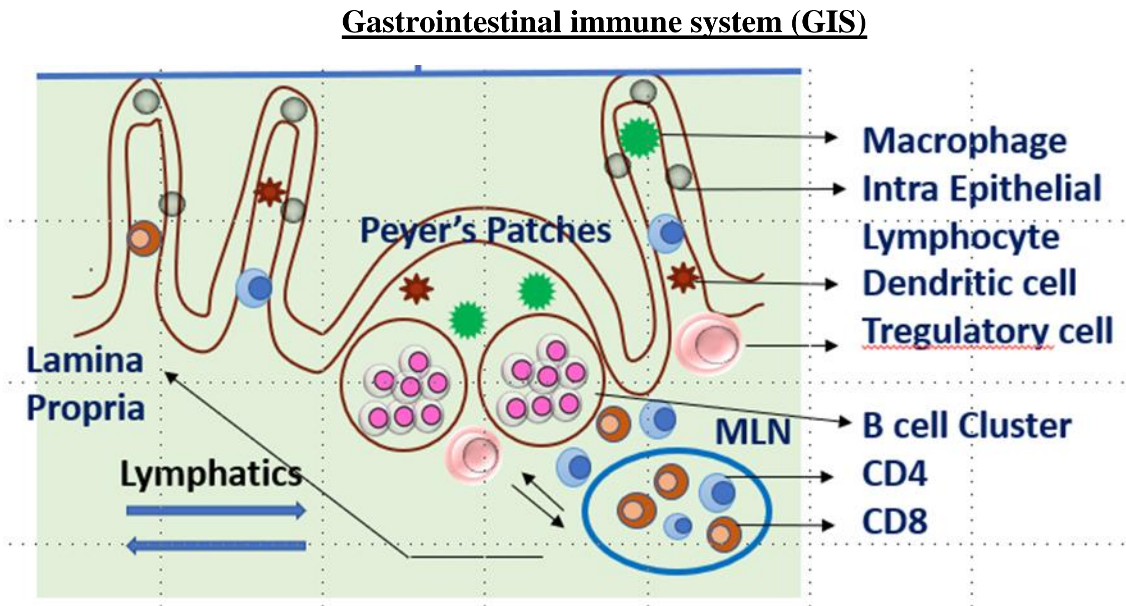


Figure 2:- Immune cells in the GALT (gut associated lymphoid tissue)

The GIS is a complex and sophisticated structure that not only saves the organism from pathogenic insults, but also makes it hospitable for all the gut commensal organisms (microbiota) that reside in the body. The GIS plays a pivotal role in maintaining the host relationship with food antigens and sustaining permeability for nutrient absorption.

The GIS consists of four major lymphoid areas:Peyer's patches (PPs), mesenteric lymph nodes (MLNs), LP, and intraepithelial lymphocytes (IELs). This system harbours the most complex

populations of immune cells in the body, and various subsets of populations change during the steady state and the state of inflammation.

The gastrointestinal system has many layers of different tissues specialised in different functions. A basement membrane is a thin membranous layer of connective tissue in the intestinal villi that separates a layer of epithelial cells from the underlying LP. The IEL's are located above the basement membrane, lying between the columnar epithelial cells. PPs are considered secondary lymphoid tissues that are embedded on the gut wall of the small intestine. PPs resemble lymph nodes in shape and there are 7 to 10 in the small intestine of each mouse. The MLNs are present in the mesentery and are usually 1 to 4 in number. All these organs are separated from each other, but the immune populations in them form a complex, interconnected network that responds differently in the steady state and the infected state. Therefore, these lymphocyte populations should be analysed when studying the immunological status of the intestine, for example, in oral immunisation or in intestinal disease (including infectious diseases and tumours). As there are different immune compartments, every compartment inhabit different compositions of cells and perform different functions.

<u>Lamina propria (LP)</u>

LP comprises mostly MΦ (132), $CD4^+$ T cells (128), and relatively fewer DC's [131-133], as well as containing $CD8^+$ T cells (128), $FoxP3^+$ regulated T cells, B cells – mostly IgA-producing B cells [134, 135], eosinophils, and some neutrophils [136-138]. Typical antibody markers can be

used to help search for T cells, B cells, etc. in gut LP, but there is a great debate among the scientific community concerning the characterisation of gut MΦ and DC's.

Both types of cell arise from a common precursor in bone marrow and belong to a mononuclear phagocyte system (MSP) network [139]. MΦ are tissue-resident cells and do not move farther than their tissue destination [140]. The common classical marker used for describing MΦ is $CD11b^+$. Above, MΦ were identified as the largest population in the gut than the other organs using marker F4/80 [141, 142]. $F4/80^+$, intermediate $MHCII^+$, and $CD11b^+$ MΦ are known to be found in close contact with the epithelium of the gut [142].

DCs when reside sessile in the cells, display an immature phenotype (i.e. low expression of MHCII) and high endocytic activity. However, as DC's are stimulated, MHCII expression increases and costimulatory molecules expression is upregulated. Classical DC's are then called $MHCII^+$ $CD11c^+$ DC's.

However, over the years, this classification has been challenged with more markers, such as $CX3CR1^+$, and by the fact that CD11c and CD11b are used interchangeably for populations expressing these markers, depending on the immunological state of the surroundings. $CX3CR1^+$ cells were previously called DC's, but more recently are used for MΦ. $CD103^+$ $CD11b^+$ $CD11c^+$ cells are now the markers attributed to DC's, and all the MΦ are considered $CX3CR1^+$ [143].

Altogether, there are studies that refer to $CD11b^+$ $CD11c^-$ as MΦ, and $CD11b^-$ $CD11c^+$ as DC's [144, 145]. Therefore, we, in this project, follow the classical classification of DCs and MΦ.

Peyer's patches (PPs)

PPs exhibit about 60-70% of B cells ($B220^+$) [146]. B cells further comprise various subsets – 36-40% naïve B cells, and approximately 20% activated cells. Among the activated B cells, approximately 10-12% are IgA^+ B cells. Furthermore, PPs contain 25% T cells ($CD3^+$), 10% DC's ($CD11c^+$), and less than 5% MΦ ($F4/80^+$) or polymorphonuclear neutrophil ($Ly\text{-}6G^+$). Among the T cells, almost half are $CD4^+$ T cells [147]. Among the $CD4^+$ T cells, 85% are memory T cells ($CD25^-CD45RB^{lo}$), 10% are naïve ($CD25^-CD45RB^{hi}$) and 5% are regulatory T cells ($CD25^+CD45RB^{lo}$) [148].

Distinct subsets of DC's, based on their cell-surface marker expression, together with their location, have been identified in PPs [149, 150]. All the subsets express CD11c and major histocompatibility complex class II antigens but differ regarding their expression of CD8α (lymphoid) and CD11b (myeloid) molecules. Lymphoid $CD11c^+$ $CD8\alpha^+CD11b^-$ DC's are preferentially localised within the extrafollicular T-cell-rich area of the PPs. Myeloid $CD11c^+$ $CD8\alpha^-CD11b^+$ DC's are present exclusively in the subepithelial dome region. Finally, the 'double negative' $CD8\alpha^-CD11b^-$ DC's are found in the Sub epithelial dome, the interfollicular region, and within the Follicular associated epithelium[149]. For more information on DCs in the PPs, the reader is referred to reviews by Jung *et al* [146] and Schenk *et al* [151].

Mesenteric Lymph Nodes (MLN)

MLN are small, bean shaped structures lying along channels of the lymphatic system in the body. The major contributor to the lymphocytes presents in mesenteric lymph comes from the GALT, specially Peyer's Patches. A study published in 1995 by Rothkotter HJ *et al* [152], demonstrated that the output of lymphocytes in mesenteric lymph draining regions with Peyer patches is higher than that of lymphocytes draining areas without Peyer patches. In the study they cannulated the intestinal lymph duct in eight minipigs and collected the lymph, after removing the MLN, so that all the lymphocytes from GALT, including Peyer's Patches can be obtained. Also, the number of T cells were more than the B cells [153]. Mesenteric lymph contains lymphocytes as well as nonlymphoid cells such as DC's and MΦ. The lymphocytes consist of mainly $CD8^+$ and $CD4^+$, activated B cells and unstimulated lymphocytes. Almost 80% of the cells are T cells, with $CD4^+$ T helper cells (about 39.5%) outnumbering the $CD8^+$ T cytotoxic/suppressor cells (about 30.2%). IgM cells (about 9.2%) also outnumbered IgA cells (about 1.2%)[154]. Not many DC's are found in MLN, but a specific subset of DC's are responsible for transporting apoptotic intestinal epithelial cells to T cell areas of MLN.[155]

CHAPTER TWO: - MATERIALS AND METHODS

Mice

Populations of various cell types from different organs were obtained from female wild type C57BL/6J(B6.PL-*Thy1*^{a}/CyJ) mice. These mice carry the T lymphocyte specific *Thy1*^{a} (Thy1.1, CD90.1) and *Thy.1*^{b} (Thy1.2, CD90.2) allele. These alleles are pan T cell markers expressed on T cells. In addition to B6 Thy1.1/Thy1.2, syngeneic B6 CD45.1 (B6.SJL-*Ptprc*^{a} *Pepc*^{b}, Pepboy) female mice were also used. Allele CD45.1 is a leukocyte marker. All mice were in the range of 6-9 weeks of age and were originally obtained from Jackson Laboratories (Stock No: - 000664, 002014). They were then bred at the University of Central Florida Vivarium at Lake Nona. All experimental animal procedures were conducted in accordance with the University of Central Florida's Animal Care and Use Committee guidelines.

Cytokine complexes

Experiments were performed by injecting IL-2 complexes (IL-2C) intraperitoneally (i.p.) in 200 µL of PBS over a three-day period at the same time every day. Along with the treatment mice, control mice were injected with the same amount (200 µL) of PBS alone. Mice received 2 µg of recombinant IL-2 (eBioscience) premixed with 20 µg of anti-mouse IL-2 monoclonal (m) Abclone S4B6-1 (S4B6) (BD Pharmingen) per day. Complexes were incubated at room temperature for 20 minutes (min.) before intraperitoneal (i.p.) injection.

Preparation of mice systemic organs and Intestinal Lamina Propria

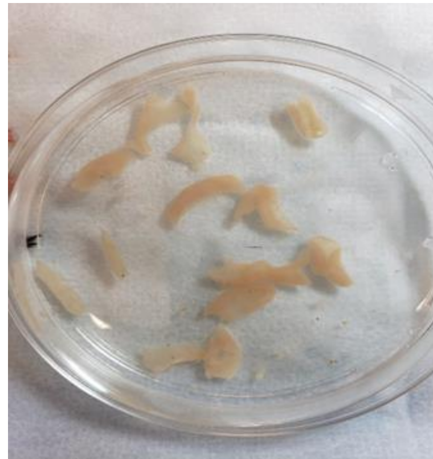


Figure 3:- ˜ 0.5 cm of cut pieces of small intestine

On Day 4 mice were sacrificed and organs were harvested sequentially. Using forceps and scissor, incision was made on the abdominal side of the mouse cutting away the skin and then the peritoneum. First the spleen is removed. Then to identify the colon it is traced to its distal end and is cut where it joins the anus, followed by a cut 1 cm away where the intestine joins the stomach. The intestine along with the associated fat is then transferred to a petri dish filled with chilled Dulbecco's phosphate buffered saline (DPBS) for further dissection. Continuing, peripheral lymph nodes and lungs are taken out as fast as possible It should be noted that further dissection of the intestine should be resumed immediately after the removal of all the organs from the mouse, otherwise recovery of cells is not well obtained. All the organs are then kept in 4mls of wash buffer (RPMI -500 ml, 1M Hepes - 4 ml, FBS -5 ml, PSG -10 ml) until mashed on ice.

Finally, MLN (2-3 in number) are excised carefully from the mesentery (mesh like structure), composed of fat and blood vessels, followed by removal of Peyer's patches from

the intestine in a dark room. Peyer's Patches look like a dome, lying along the epithelial lining of the small intestine. Since they are not visible clearly against the natural background of the small intestine, they can be very easily seen in a dark room in minimal light, under a lamp (Picture A). Once they are visible the area is cut with the scissors. In general, 6-9 PP's are obtained from a B6 mouse. All the organs are then mashed using a rubberized end of a 3 ml plunger in a 100 µm stainless steel screen cell strainer standard test sieve utilizing 10 ml wash media, then transferred into a 15 ml falcon tube using a transfer pipette. The falcon tubes are then centrifuged and resuspended in wash media, counted using haemocytometer and stained as required. However, with the lung, the resuspended solution is filtered through a 70 µm filter in a 50 ml conical flask, followed by a rinse of 10 ml media of the original tube and filtering again, so that cells are not left behind in the original tube and in the filter as well. The media with the cells is then centrifuged, resuspended in the required media and cells are counted.

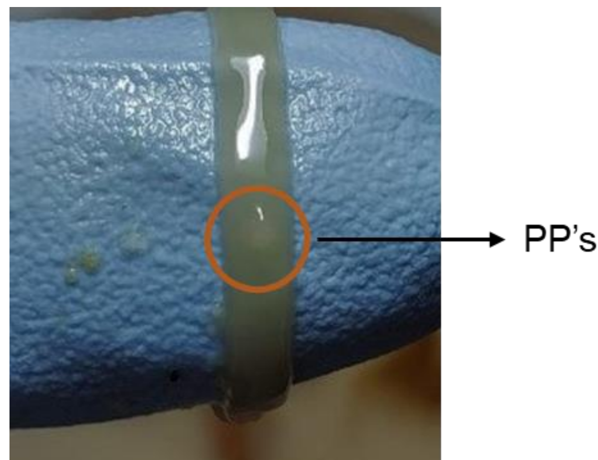


Figure 4:- A Peyer's Patch on a mouse small intestine

Simultaneously, the intestine is flushed with ice cold DPBS, cut longitudinally and washed thoroughly to remove lumen contents. This whole procedure should be done on ice to maximize cell viability. It was then cut into ~0.5-cm pieces (Picture B) and processed according to methods specified by Lamina Propria Dissociation kit with some modifications (Miltenyi biotec). It involves two major steps - in the first step, the intraepithelial lymphocytes (IELs) are disrupted from the mucosa by shaking the tissue in a pre-digestion solution (1× Hank's Balanced salt solution containing 5 mM EDTA, 5% fetal bovine serum (FBS), 1 mM DTT, 10 mM HEPES) twice at 37°C (15 mins). The lamina propria tissue is further treated enzymatically using the digestion solution (1× HBSS with Ca2+ and Mg2+ containing 10 mM HEPES) and enzymes provided in the kit.

It is then mechanically dissociated into a single-cell suspension by using the gentleMACS™ Octo Dissociator with Heaters by running the gentleMACS Program m_intestine_01. Single cell suspension is then centrifuged and resuspended in T cell buffer ((RPMI -500 ml, 1M Hepes - 4 ml, FBS -35 ml, PSG -10 ml, 250µl Mercaptoethanol), counted using hemocytometer and stained as required.

There were some modifications done in the protocol and reagent preparations specified by Lamina Propria dissociation kit [156].

In the pre-digestion solutions, 4.2mM sodium bicarbonate was also added. Once sodium bicarbonate is added the pH of the solution maintained should be 7.4. 1M stock of sodium carbonate was kept at room temperature and can be used for 2 months.

- FBS was not added in the digestion media as serum has been shown to inhibit collagenase.
- EDTA was always made a day prior to the treatment and the PH of the EDTA was maintained to be between 7.4-8.
- DTT is known to be very unstable compound. It should be always stored at -20ºC once it is reconstituted in water. For that reasons, fresh aliquots of DTT were made and stored at -20ºC freezer. DTT cannot be used after six months from the date of suspension.
- The buffer stock of HEPES was stored at room temperature always. Therefore, the required solutions were made fresh during the day of the experiment.
- Predigestion and digestion solution were kept at 37 ºC to acclimatize to the temperature before adding the tissue.
- Instead of keeping the solutions for 20 mins in pre-digestion solution and 30 minutes in the digestion solution, the solution was kept for 15 mins and 25 mins respectively.
- Resuspension media used for IEC and Lamina Propria cells was T cell media, instead of PBS.

Once the cells were obtained, they should be immediately stained with the required antibodies. The more the time is taken to stain, the more the chances are that cell will loose their viability and markers can get cleaved off.

Flow cytometry

Cytokine and surface staining were performed as previously described [157]. Briefly, cell suspensions were washed, resuspended in FACS buffer (PBS plus 0.5% BSA and 0.02% sodium azide), a cocktail of Fc block, antibodies suspended in FACS buffer is added (For 1 sample- 1 µl of Fc block + required diluted antibody+ FACS buffer = 100 µl) and then incubated-on ice(2-8°C) for 30 minutes with minimal exposure to light. The samples were treated with anti-FcR (2.4G2, BioXcell) because Fc receptors are found on monocytes, MΦ, DC's and B cells They bind antibodies via their constant Fc domain rather than the antigen specific Fab domain. This type of binding can lead to false positives and meaningless data. In order to prevent this type of binding, Fc blocking reagents are used which, when added to a staining protocol, can help minimize non-specific binding.

Also saturating concentrations of the fluorochrome-labeled antibodies were added for surface staining to look for the desired cells. After the incubation cells are washed again with 1 ml of FACS buffer and resuspended in 100 µL of FACS buffer and 100 µL of FACS fix

Ki67- Nuclear (Proliferation) Staining.

Ki67 is a cell proliferation marker.as it is expressed actively throughout all the stages of cell cycle. The staining is done according to eBioscience™ Foxp3 Transcription Factor Staining Buffer Set (Catalog number: - 00-5523-00). Proliferation of B cells and CD8 T cells were determined and the surface stains used are listed in Table (1). This protocol involves use of Permeabilization Buffer as it is necessary to permeabilize the cell with detergent or alcohol to allow antibodies against intracellular antigens access to stain intracellularly. After surface staining cells with the required markers, the cells are treated with 1X working solution of permeabilization buffer, incubated for 45 minutes, followed by washing twice and then stained with Ki67 for 30 minutes. After the incubation cells are washed twice with 1X permeabilization buffer and resuspended in Facs buffer. The samples were then run on the same day. All FACS analyses was performed using a FACS Canto flow cytometer (BD Biosciences) and FlowJo (Tree Star) analysis software.

Annexin V- 7AAD Staining

Cell death was measured by flow cytometry using Annexin V Apoptosis Detection Kit with 7-AAD as per manufacturer's instructions (FITC Annexin V Apoptosis Detection Kit with 7-AAD, catalog no -640922, BioLegend). This kit is designed for the identification of apoptotic and necrotic cells.

Annexin V (or Annexin A5) is a member of the annexin family of intracellular proteins that binds to phosphatidylserine (PS) in a calcium-dependent manner. PS is normally only found on the intracellular leaflet of the plasma membrane in healthy cells, but during early apoptosis, membrane asymmetry is lost and PS translocates to the external leaflet. Fluorochrome-labeled Annexin V can then be used to specifically target and identify apoptotic cells.

To help distinguish between the necrotic and apoptotic cells 7-amino-actinomycin D (7-AAD) solution is used. Early apoptotic cells will exclude 7-AAD, while late stage apoptotic cells will stain positively, due to the passage of these dyes into the nucleus where they bind to DNA. 7-AAD (7-amino-actinomycin D) has a high DNA binding constant and is efficiently excluded by intact cells.

The protocol starts with surface staining with the respective antibodies. The cells are washed twice with cold 1X Macs Buffer, and then resuspend cells in 100 µl Annexin V Binding Buffer. Then 5 µl of FITC Annexin V antibody is added, followed by 5 µl of 7-AAD Viability Staining Solution. The tube is then vortexed gently and incubate for 10 min at room temperature (25°C) in the dark. Then 200 µl of Annexin V Binding Buffer is added to each tube. The samples were then run on the same day immediately after the last step. All FACS analyses was performed using a FACS Canto flow cytometer (BD Biosciences) and FlowJo (Tree Star) analysis software.

Statistical analysis

All statistics were analyzed by GraphPad Prism. Unpaired two-tailed student t test was utilized to determine the significance by which two normally distributed groups differed. A P value of <0.05 was deemed significant. All error bars represent the standard deviation. In all figures, significance is indicated as *P < 0.05, **P < .005, and ***P < .0005

Table 1:- Antibodies required to stain different immune cell subtypes

S.No	Surface, Transcription factor and Nuclear stain	Antibodies used	Flurophore	Clone	Company
1.	T cells	Anti- CD90.2+	Pacific Blue	53-2.1	eBioscience
	CD4	Anti- CD4	PE	GR 1.5	eBioscience
	CD8	Anti- CD8	PERCP	53-6.7	BD-Pharmigen
	γδ	Anti- γδ	APC	GL3	Biolegend
		Anti-CD44	FIT-C	IM7	eBioscience
		Anti-CD45.2	FIT-C	104(RUO)	BD Biosciences
2.	Antigen Presenting cells	Anti-MHC II	Pacific Blue	M5/114.15.2	eBioscience
		Anti-MHC II	PERCP	M5/114.15.2	Biolegend
		Anti-CD11C	FITC	N418	eBioscience
		Anti-CD11B	APC	M1/70	eBioscience
3.	B cells	Anti-B220	PERCP	RA3-682	eBioscience
		Anti-CD19	PERCP	1D3	Biolegend
	Mature B cells	Anti-IGM	FIT-C	RMM-1	Biolegend
		Anti-IGD	Pacific Blue	11-26c.2a	Biolegend
	IgA+ B cells	Anti-IGA	FIT-C	C10-3	BD Biosciences
4.	NK cells	Anti-CD3	Pacific Blue	71A2	eBioscience
		Anti-NK1.1	PE	553165	BD Pharmigen
5.	Neutrophils	Anti-CD11B	APC	M1/70	eBioscience
		Anti-Gr-1	FIT-C	RB6-8C5	BD Biosciences
6.	IL-2 receptor	Anti-CD25	PE	7D4	BD Biosciences
		Anti-CD122	FIT-C	TM Beta-1(RUO)	BD Biosciences
7.	Cell Death Staining	Annexin V	FIT-C	NA	Biolegend
		7 AAD	PERCP	NA	Biolegend
8.	Ki67 staining	Anti-Ki67	PE	SolA15	eBioscience

CHAPTER 3: - SIGNIFICANCE AND INNOVATION

Immunotherapy with IL-2 was the first immunotherapy approved by the FDA (Food and Drug Administration) to treat cancer. Although the therapy was successful in 15-20% of the patients undergoing the treatments, clinical trials, often had to be stopped during the course of the procedure. The reasons, detailed in section (1.1) are the manifestation of severe toxicities in most organ systems, one of which are gastrointestinal toxicities. Currently, through literature, it is evident how IL-2 affects the immunology of the systemic organs such as spleen and lymph nodes, i.e., by changing the expression of the immune cell populations (CD8 T cells and NK cells). There are many clinical and mouse studies demonstrating the effect of IL-2 delivery on the said organs. In comparison to the systemic organs, a few groups have addressed the issue of IL-2 effect on lungs (vascular leak syndrome), which is one of the mucosal organs. However, in the studies, they explained the impact of IL-2 on endothelial cells, which also possess IL-2 receptor, but little is still known about the changes in numbers of immune cells. Contrary to systemic organs and the lungs, not many studies describe what effect IL-2 causes in the immune cell populations in the gut and how it results in such drastic toxicities.

The gut encompasses the largest population of immune cells. It is the organ which deals with the commensal bacteria and creates an environment of tolerance to let the needed commensal organism survive without causing an immune response against them. But it also must be able to respond to potentially harmful pathogens and toxins. Thus, the local immune environment in the gut is significantly different from the systemic immune system in terms of both its inductive and

effective sites and the composition of various immune cell populations such as intra-epithelial lymphocytes, different subsets of APC's, etc. Chronic Inflammation in the gut is sometimes a sign of IBD. Causes for IBD are thought to be genetic, environmental, dietary and infection caused due to *Mycobacterium avium subspecies Paratuberculosis [158]* .Despite significant research in the field, the etiology of IBD is not clear. Some clinical trials show the presence of the substantial amount of IL-2 present in Crohn's patients not only inactive CD patients but also during inactive disease states. The similarity of symptoms shown by a CD patient and similar gastrointestinal issues observed during the therapy demands to look if IL-2 is somewhere involved in the etiology of CD.

The first aim of this project in its broader scope will characterize the effect of IL-2 immunologically on the cells present in the GALT - Peyer's Patches (PP), MLN (MLN, Lamina Propria (LP) and Intraepithelial lymphocytes (IEL's) of the intestine. The effects will be observed in an immunologically unaltered state. This contribution is significant since it may establish specific unknown changes of the immune cell's populations of the GALT, caused by IL-2, which might not be present in the spleen. We hypothesize that such changes can be responsible for disrupting the homeostasis of the gut immune system. This, in turn, could contribute to effects observed during the IL-2 therapy in the setting of cancer and the pathogenesis of some of the causes of CD. We also want to bring attention to the point that the observations might be helpful for future studies directly addressing the role of IL-2 in CD and IBD.

The second aim of this project is to determine whether IL-2 will similarly or differentially impact immune cell subsets at distinct mucosal sites. We chose the lungs because it is the prototypical and vital mucosal organ. Although both the gut and lungs are mucosal organs, they are distinct in many ways from each other. A significant difference is the - commensal microorganisms at each site. As the source of antigens is distinct, it could be that these antigens might be perceived differently through different TLR's or other mechanisms by the cells of these organs. This difference can generate a very different environment in both these organs. Thus, this aim seeks to determine whether IL-2 might differentially regulate immune cell subsets at various mucosal sites.

IL-2 has been combined with other therapies like chemotherapy to bring down the toxic effects in cancer patients. Despite significant advances, clinical trial data demonstrate remission rates at best 15-30%. Thus, there remains a need to find a safer way to use IL-2 in therapeutics potentially. The clinical significance of our study will be to shed light on various considerations that should be taken into account while using IL-2 in therapeutics. The consideration will be related to the point that the regulation of IL-2 is not similar to different organs. While IL-2 can be effective in treating melanoma but can also disrupt the immune system at a different organ-like gut. Therefore, researchers may need to think in terms of the organ they are targeting IL-2 with, regarding therapy.

One easy way to test the effect of IL-2 on gut cells would be to perform experiments in a petri dish. However, in-vitro experiments introduce to two problems. First, the differential way IL-2 reacts in a petri dish versus the way IL-2 will respond in-vivo. As mentioned, and explained, IL-2

is a pleiotropic cytokine. Certain conditions and presence of cells determine its reaction to a particular stimulus. Second, it is impossible to create the gut- microbiome environment in a petri dish. The amalgamation of different kinds of food antigen, microbiota, complex immune networks could change the way a cytokine will response in a petri dish vs. the in-vivo model.

Therefore, we chose to perform experiments in a steady-state (immunologically unaltered state, “not ill”) mouse, by treating the mouse with IL-2C treatment and look at the effects in the gut versus in systemic lymphoid tissues.

HYPOTHESIS I

We hypothesize that acute treatment with IL-2C elicits differential quantitative changes in leukocytes in the spleen versus the GALT.

CHAPTER 4: - RESULTS

A)

B)

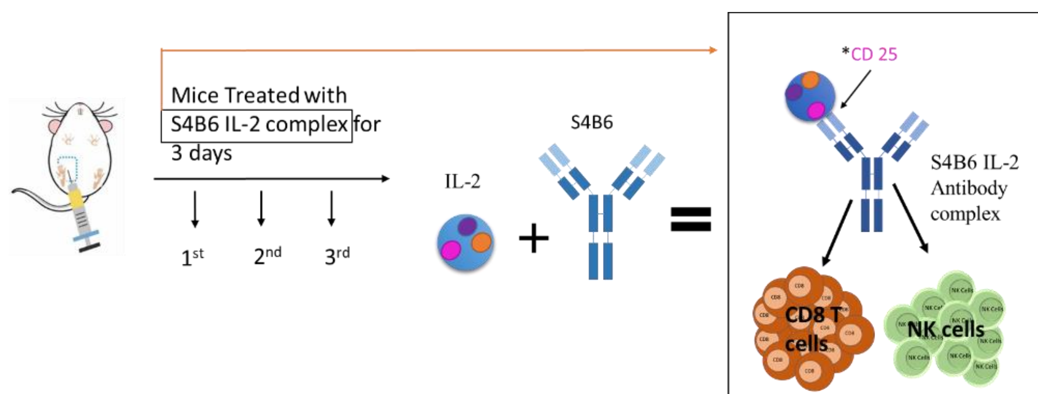


Figure 5:- IL-2C selectively increases $CD8^{+}$ T cell and NK cell population in spleen and lymph nodes in vivo.

Naïve C57BL/6 Thy1.1/1.2 mice received 2 µg per day of recombinant IL-2 premixed with 20 µg of anti-mouse IL-2 monoclonal (m) Ab- S4B6-1 for three days. On the fourth day, cells were harvested and analyzed (A) C57BL/6 mice injected with PBS serving as control. (B) C57BL/6 mice injected with IL-2C S4B6.

A)

B)

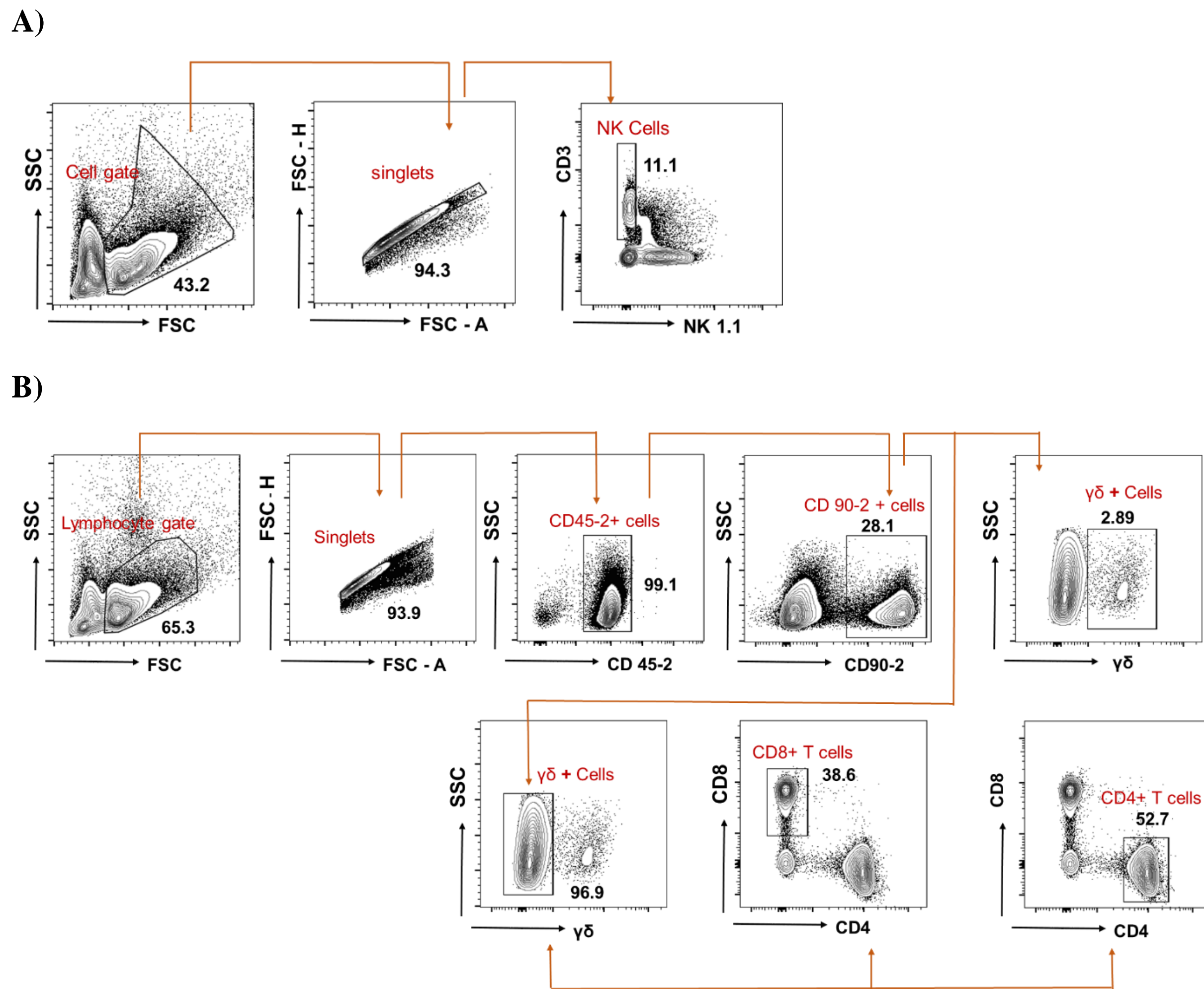


Figure 6:- IL-2C selectively increases CD8$^+$ T cell and NK cell population in spleen and lymph nodes in vivo.

(A) Gating strategy to segregate NK cells using CD3 and NK 1.1. (B) Gating strategy to segregate T cells and its subsets using CD45-2, CD90-2, CD4, CD8, and TCR antibodies.

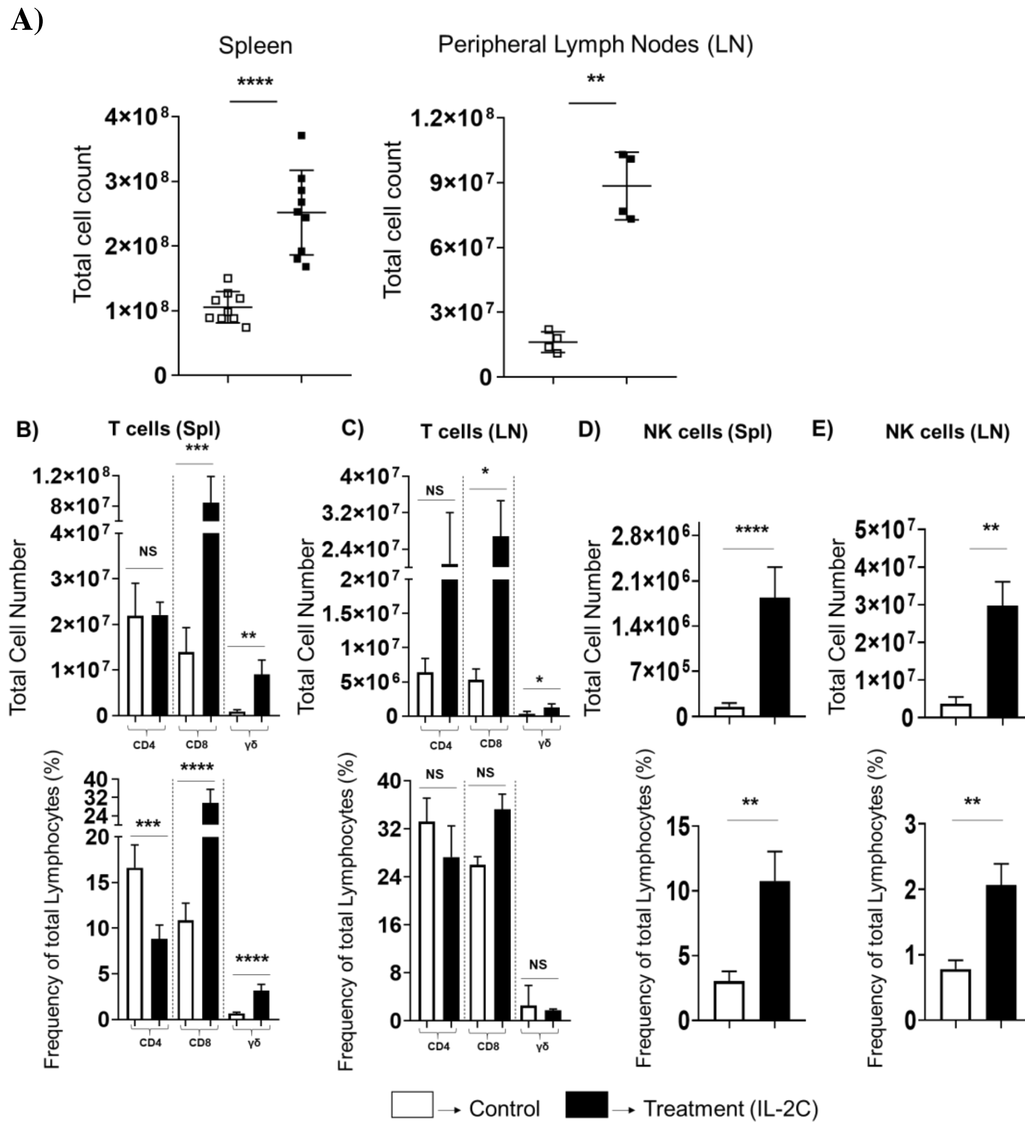


Figure 7:- IL-2C selectively increases CD8⁺ T cell and NK cell population in spleen and lymph nodes in vivo.

(A) Increase in absolute cell numbers of splenocytes and immune cell populations in the lymph nodes after IL-2C treatment. Effect of IL-2 treatment on total cell numbers and frequency of (B) Spleen T cells (5 fold) (C) LN T cells (2 fold) (D) Spleen - NK cells (4 fold) (E) LN – NK cells (2 fold) (Summary of at least 4 - 9 experiments with at 2 mice per group). All error bars represent the standard deviation and significant differences determined with unpaired, two-tailed, students *t*-tests ($\alpha = 0.05$, and * $P< 0.05$, ** $P< 0.005$, *** $P< 0.001$, **** $P< 0.0001$). NS represents non-significant.

Result 1: - IL-2C treatment cause increased expansion of CD8+ T cells and NK cells in systemic lymphoid organs – Spleen and Lymph Nodes.

S4B6 IL-2 Complex

To study the effects of IL-2 treatment on the total number of immune cells of the systemic lymphoid organs, we treated non-infected (steady state) mice intraperitoneally with 2 µg per day of recombinant IL-2 (eBioscience) premixed with 20 µg of anti-mouse IL-2 monoclonal (m) Abclone S4B6-1 (S4B6) (BD Pharmingen) to form IL-2 complexes (S4B6).

IL-2 complex (S4B6) exhibited potent biological activity and imparts target cell specificity which made its use widespread among the scientific community in mouse models. Number of reasons such as increased half-life and lower toxicity can be attributed to increased biological activity of this complex.

Our procedure involves injecting IL-2C complex for three days as this dose has been employed and proven effective as by many labs as one of the optimal physiological doses to obtain a IL-2 response [5, 159]. The dose is not toxic enough that it would cause death, but it can promote a lot of inflammation [159]

Panel and Gating Strategy

To look for the effect of the complex on immune cells i.e. ($CD8^+$ T cells and NK cells) we needed a panel and a gating strategy. The panel as shown in table 1 comprises 5 antibodies in 5 different colors to explore the presence of T cells subsets, whereas panel 2 utilizes only 2 antibodies to look for NK cells.

In addition to $CD8^+$ T cells there are other subsets of T cells – $CD4^+$ T cells and Gamma delta (γδ) T cells. The complex is known to not cause a major expansion of $CD4^+$ T cells. The said populations of T cells - $CD4^+$ T cells and $\gamma\delta^+$ T cells have been shown to be involved in CD as explained in the introduction [49, 76, 160, 161] . Apart from being involved in the mentioned disease, γδ T cells have been also been shown to get affected by IL-2 [76]. For these reasons we also investigated these subsets of T cells.

We first optimized a 5 colour panel (Table 1) to detect distinct T cell subsets. After gating on the live cells based on FSC- SSC lymphocyte gate, the next step was double cell exclusion analysis. A doublet is a single event that consists of 2 or more independent particles. The particles are bonded while they pass through the laser in the FACS machine, such that the instrument is incapable of distinguishing them as individual events. As shown in the figure 6 (A) doublets need to be identified and excluded, so that cells can be further analyzed and to avoid artifacts to make staining clearer. To analyze the doublets, we are required to select a channel to compare area, height, and

width. We attained this by choosing FSC-A and FSC-H. The only requirement for this channel is that it should be scaled linearly. This strategy is used throughout this study

Further on, we distinguished all leukocytes using the marker CD45-2 or CD45-1 depending on the mouse genotype and then gate the T cells using pan T cell marker CD90-2, found on all T cells originating from the thymus. We then mark
distinct subsets of T cells i.e γδ, CD4 and CD8, distinguished according to the surface molecules they express. So, using the antibody for γδ we first gated on γδ positive cells, revealing a separate population of γδ negative cells, then through further gating on γδ negative cells we isolate CD4 and CD8 T cells separately. The gating strategy is shown in fig 6 (B). The graphs are then plotted on Prism comparing the frequency and total cell numbers obtained after the gating. The gating strategy required to gate NK cells is shown in fig 6 (A).

Effects on T cells and NK cells

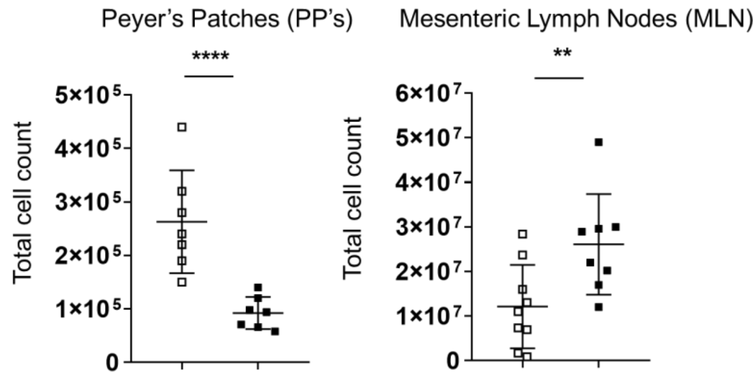
Earlier research has shown that IL-2 complexes cause expansion in the number of memory CD8 T cells and NK cells from 10-100 fold in the spleen and the lymph nodes [16]. Our results confirm the significant in-vivo expansion of these immune cells in the same organs. The absolute numbers of CD8+ T cells and NK cells remained significantly higher than at the baseline after treatment in both spleen and lymph nodes (Fig 7 A). The frequency increased by 20% and total cell numbers of CD8 T cells quadrupled (Fig 7 B, C). The total cell numbers doubled for NK cells with a 10% increase in the frequency (Fig 7 D, E). Hence, the expansion was driven by enhanced in-vivo

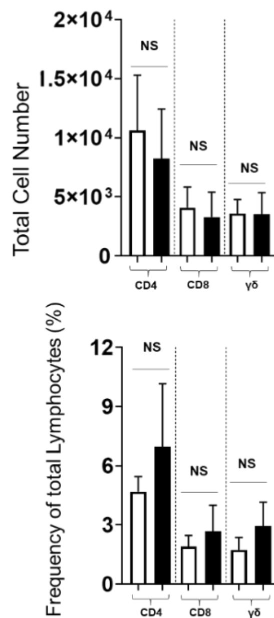
proliferation of CD8 T cells and NK cells. We found a significant 2-fold total cell increase of Gamma delta (γδ) T cells in spleen but no significant changes in CD4 T cells. (Fig 7 B, C).

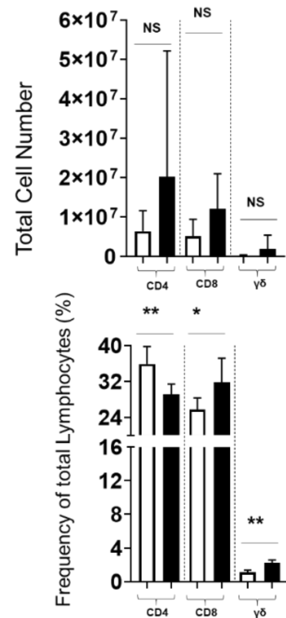
Each dot in the graphs (Fig 7 A) represent the absolute immune cells population of that organ in an individual mouse with or without treatment on a given day. Thus, the graph is a cumulation of data sets collected over a span of experiments with different mice. Over the duration of these experiments, the results show the counts to be relatively similar and present a repetitive pattern each time, clearly indicating the expansion of CD8 T cells and NK cells in systemic lymphoid tissues. These results are reproducible providing relevant information which can be statistically analyzed.

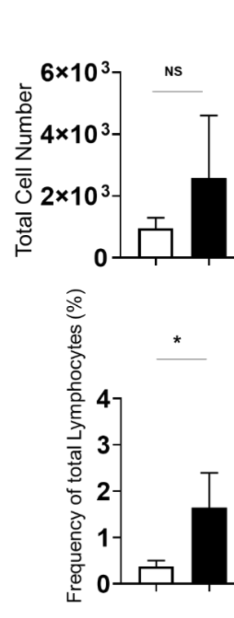
Therefore, having validated our experiments data against known impacts of IL-2C treatment in secondary lymphoid organs (i.e. the increase in $CD8^+$ T cells and NK cells) we went on to test the effects of IL-2C on the gut tissues (Gastrointestinal Associated Lymphoid Tissues).

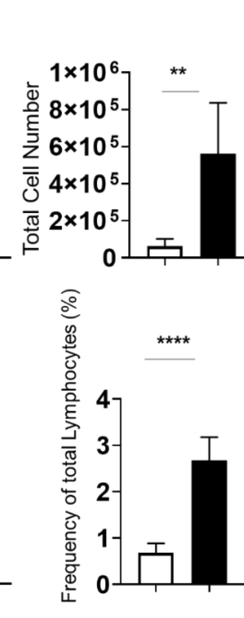
A)











→ Control → Treatment (IL-2C)

Figure 8:- IL-2C treatment cause non- significant quantitative changes in CD8+ T cells and NK cells in the gut tissues (GALT).
(A) Decrease in absolute immune cell populations in PP's and increase observed in MLN after IL-2C treatment. No significant effect of IL-2 treatment on total cell numbers and frequency of (B) PP's T cells (C)M LN T cells (D) PP's - NK cells (E) 2-fold increase of total NK cells numbers is observed in MLN. (Summary of 5-10 experiments with 2 mice per group). All error bars represent the standard deviation and significant differences determined with unpaired, two-tailed, students *t*-tests ($\alpha = 0.05$, and * $P< 0.05$, ** $P< 0.005$, *** $P< 0.001$, **** $P< 0.0001$). NS represents non-significant.

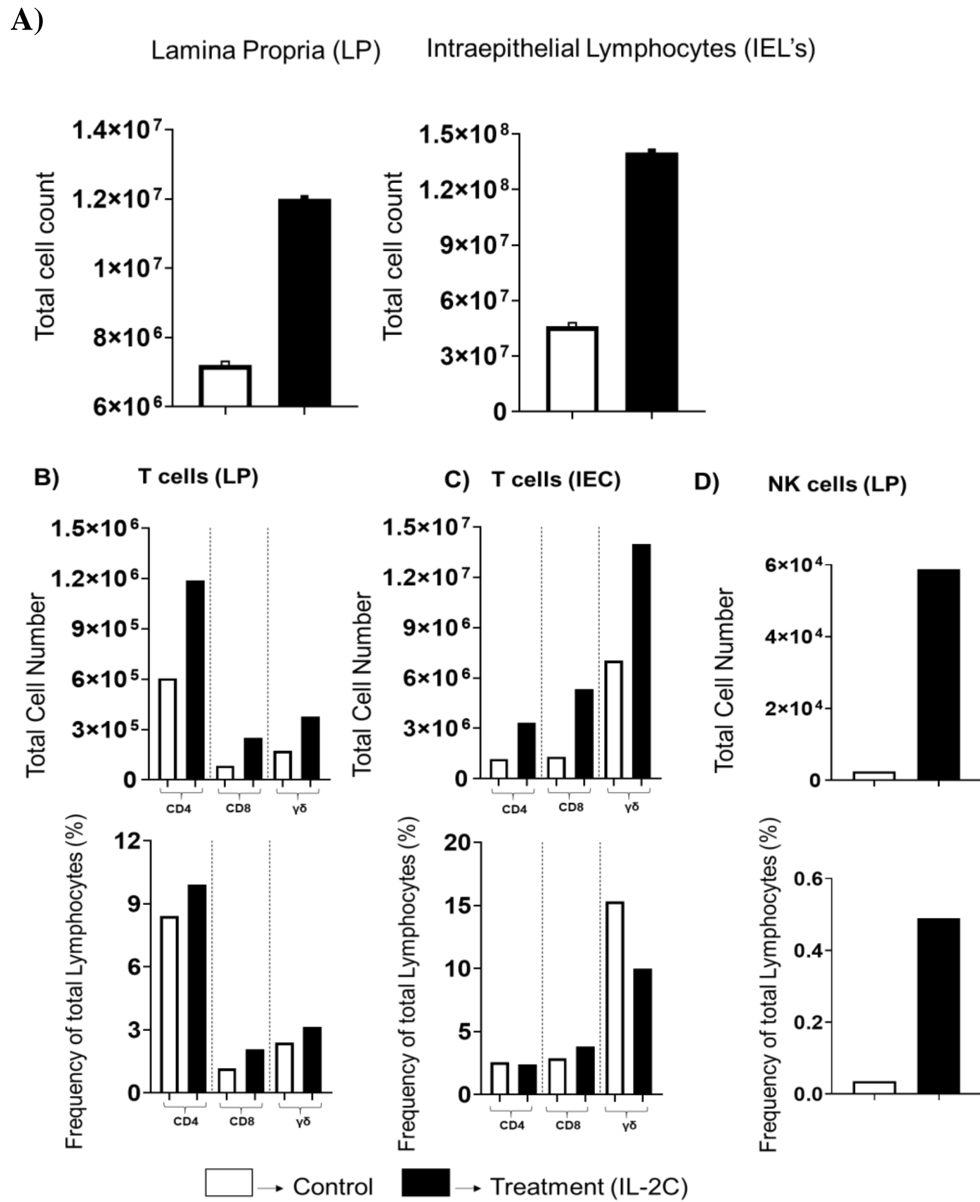


Figure 9:- IL-2C treatment cause non- significant quantitative changes in CD8+ T cells and NK cells in the gut tissues (GALT).

(A) Increase in absolute immune cell populations in LP and IEC after IL-2C treatment. 2-3-fold increase can be observed in (B) LP T cells (C) IEC T cells (D) LP - NK cells. The graphs are from only one experiment with a mouse per group. The graphs only give a hint towards the increase number of cells, but not infer any of the changes. More experiments are needed to observe a significant difference, if any.

Result 2: - IL-2C treatment cause non- significant quantitative changes in CD8+ T cells and NK cells in the gut tissues (GALT).

We next tested the effects of IL-2C on the GALT (PP's, MLN, IEC, LP). We hypothesize that changes will be observed in the gut tissues that are not observed in the spleen and vice versa. We base our prediction on different inductive and effective sites of gut than the spleen (systemic organ) where different cytokine environment and populations of immune cells exist [162]. Hence, we checked the effect on total numbers of immune cells focusing on CD8 T cells and NK cells in the gut tissues, as changes in these first populations induce by IL-2 in systemic organs in the literature are well described. In accordance with our first hypothesis, gut tissues showed disparate changes at different sites. To begin with, no significant changes were observed in absolute cell numbers in MLN (Fig 8A). But surprisingly, a decrease is observed in total cell number of Peyer's Patches (Fig 8A). With regards to the CD8 T cells, both Peyer's Patches and MLN exhibited no significant differences as shown in (Fig 8B and (Fig 8C) respectively. However, with respect to NK cells, MLN displayed significant difference of 5-fold after the treatment (Fig 8E). No significant difference was observed in total cell numbers of NK cells in PP's (Fig 8D). Overall the results concur that decrease in PP's is not due to dropping down of CD8 T cells and NK cells numbers, and thus warranted further testing of other cell types.

The other two gut organs LP and IEC showed 2 to 3-fold changes in total cell numbers of $CD8^+$ T cells (Fig 9B, 9C). But the results mentioned are representative of only one experiment. This is because of extended amount of time it took to optimize LP and IEC dissociation protocol to isolate

these cells. Therefore, from now on LP and IEC will be discussed in relation to one experiment just to shed light on potentially expected results. More experiments are needed to understand if the changes are significant or not.

Taken together the data suggest no significant changes in total number of CD8 T cells in gut organs in comparison with the spleen. Therefore, based on these findings, the plausible next step was to examine other major cell types, especially in Peyer's Patches, to find out the cause for reduction in absolute cell numbers. For this reason, we looked for B cells and other APC's as B cells constitute almost 80 % of PP's [146] and other APC make this organ an important inductive site.

A)

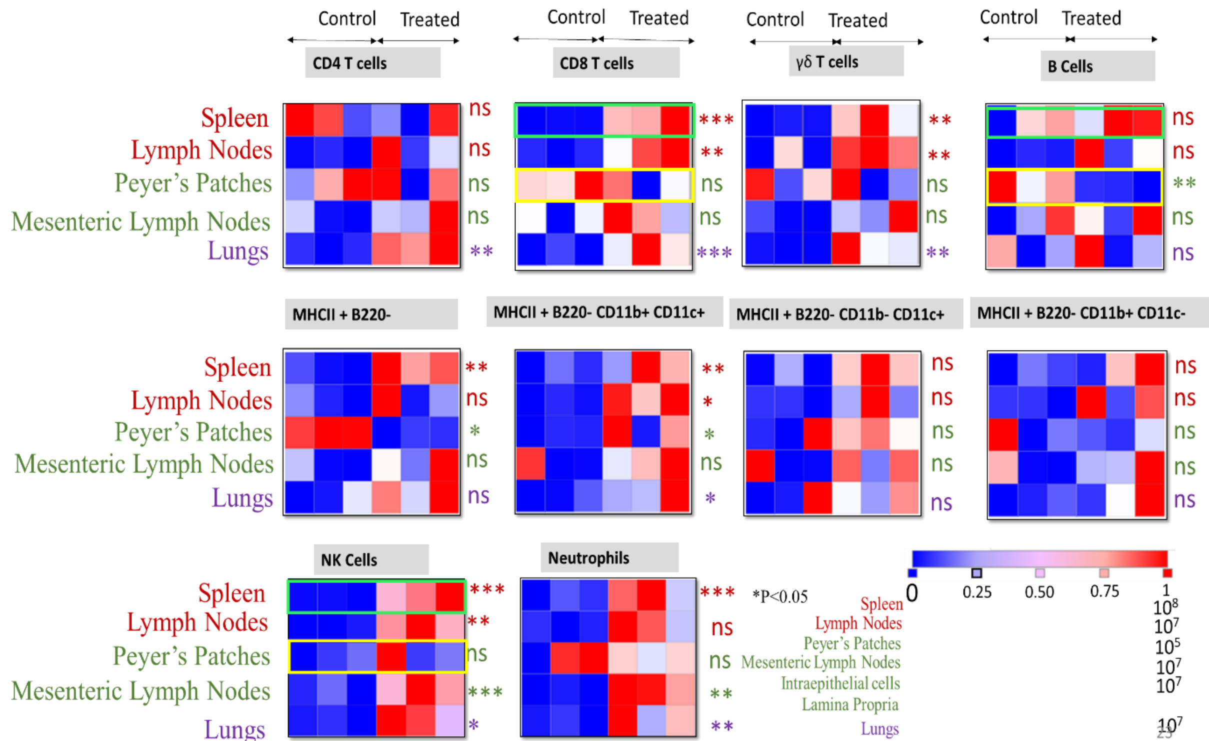
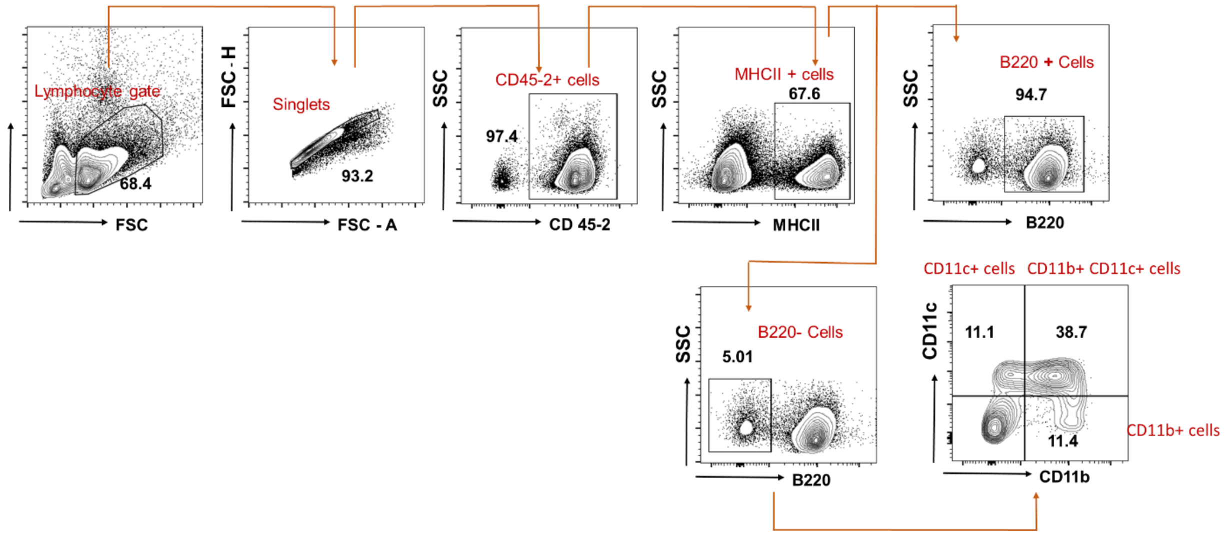


Figure 10:- **IL-2C Treatment differentially affects frequency and total cell population of B cell numbers in Peyer's Patches.**
Heat map depicting effect of IL-2 treatment on total cell numbers and frequency of all immune cell population as mentioned in the map. The heat map is only representative of three experiments with 2 mice. per experimental day, with a total of 3 mice per condition. Standard deviation and significant differences determined with unpaired, two-tailed, students *t*-tests ($\alpha = 0.05$, and * $P< 0.05$, ** $P< 0.005$, *** $P< 0.001$, **** $P< 0.0001$). NS represents non- significant.

A)



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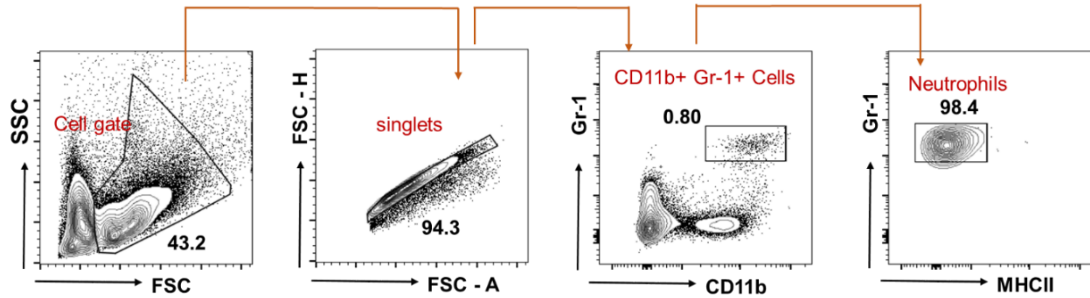

Figure 11:- IL-2C treatment differentially affects frequency and total cell population of B cell numbers in Peyer's Patches.

(A) Gating strategy to segregate B cells and Antigen Presenting cells using CD45-2, MHCII, B220, CD11b, CD11c (B) Gating strategy to segregate neutrophils using CD11b, Gr-1 and MHCII.

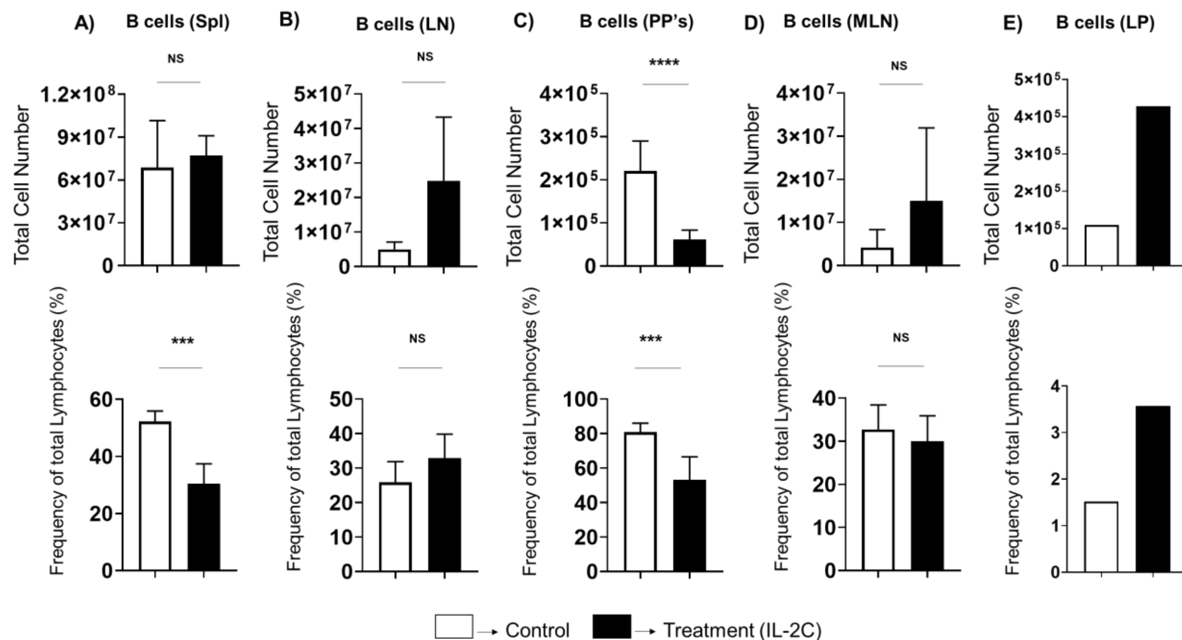

Figure 12:- **IL-2C treatment differentially affects frequency and total cell population of B cell numbers in Peyer's Patches.**

No significant effect of IL-2 treatment on total cell numbers and frequency of (A) B cells in the Spleen or (B) LN In contrast to spleen and lymph nodes 2-fold decrease of B cells observed in the C) PP's. Decrease in B cell numbers can define the decrease in absolute cell numbers in PP's. (E) No significant increase of B cells is observed in MLN. (Summary of 5-10 experiments with 2 mice per group). All error bars represent the standard deviation and significant differences determined with unpaired, two-tailed, students *t*-tests ($\alpha = 0.05$, and * $P< 0.05$, ** $P< 0.005$, *** $P< 0.001$, **** $P< 0.0001$). NS represents non- significant. Another gut organ – Lamina Propria also show an increase in total number of B cells and frequency by 2-3-fold. But the graphs are from only one experiment with a mouse per group. The graphs only give a hint towards the increase number of cells, but not infer any of the changes. More experiments are needed to observe a significant difference, if any.

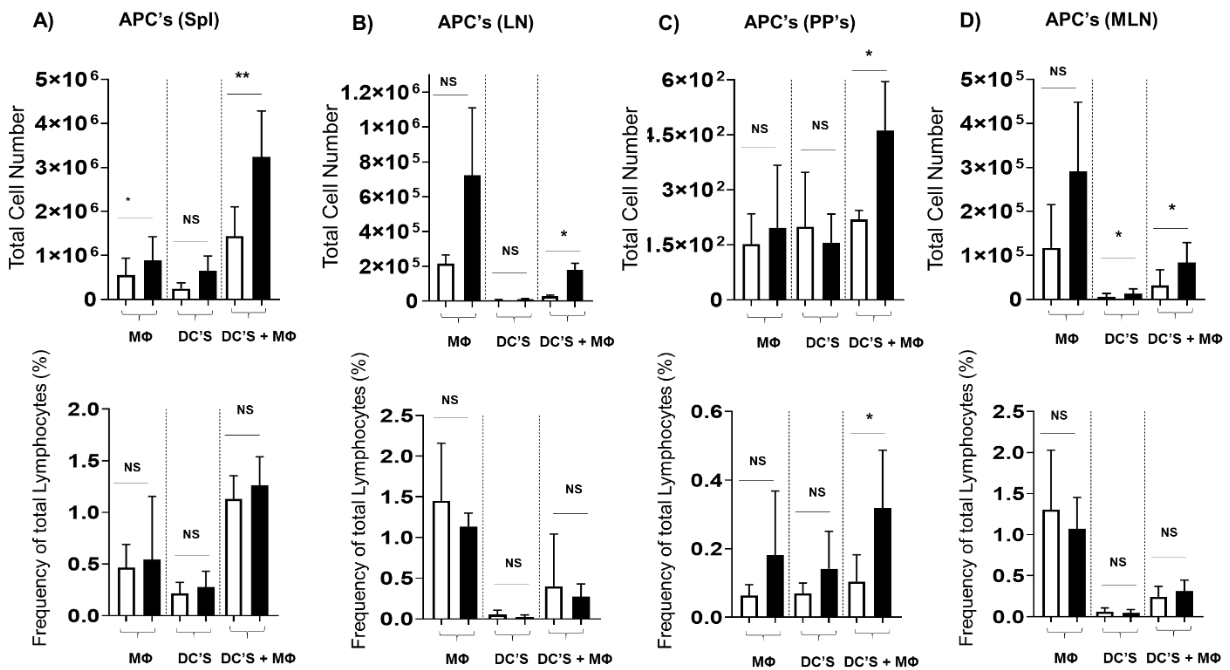

Figure 13:- **IL-2C treatment do not cause significant change in frequency and total cell population of MΦ and DC's in any of the organs**

No significant effect of IL-2 treatment observed on total cell numbers and frequency of DC's and MΦ of (A) Spleen – minimal increase observed in MΦ in the spleen but not in DC's (B) LN C) PP's (D) MLN (Summary of 5-10 experiments with 2 mice per group). All error bars represent the standard deviation and significant differences determined with unpaired, two-tailed, students *t*-tests ($\alpha = 0.05$, and * $P< 0.05$, ** $P< 0.005$, *** $P< 0.001$, **** $P< 0.0001$). NS represents non-significant.

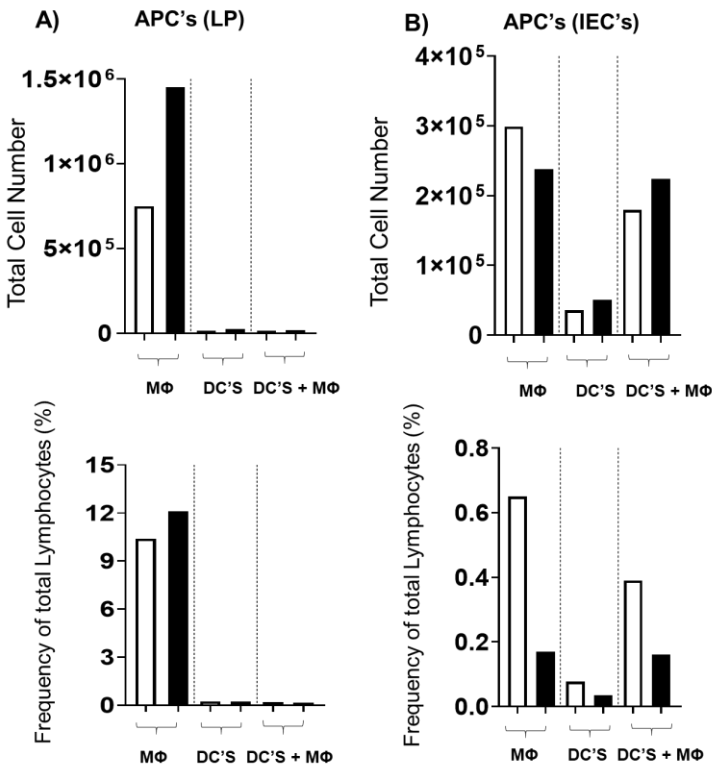

Figure 14:- IL-2C treatment do not seem to cause significant change in frequency and total cell population of MΦ and DC's in any of the organs

No significant effect of IL-2 treatment on total cell numbers and frequency of (A) Lamina Propria – minimal change observed in MΦ in the Lamina Propria(B) IEC's. But the graphs are from only one experiment with a mouse per group. The graphs only give a hint towards the increase number of cells, but not infer any of the changes. More experiments are needed to observe a significant difference, if any.

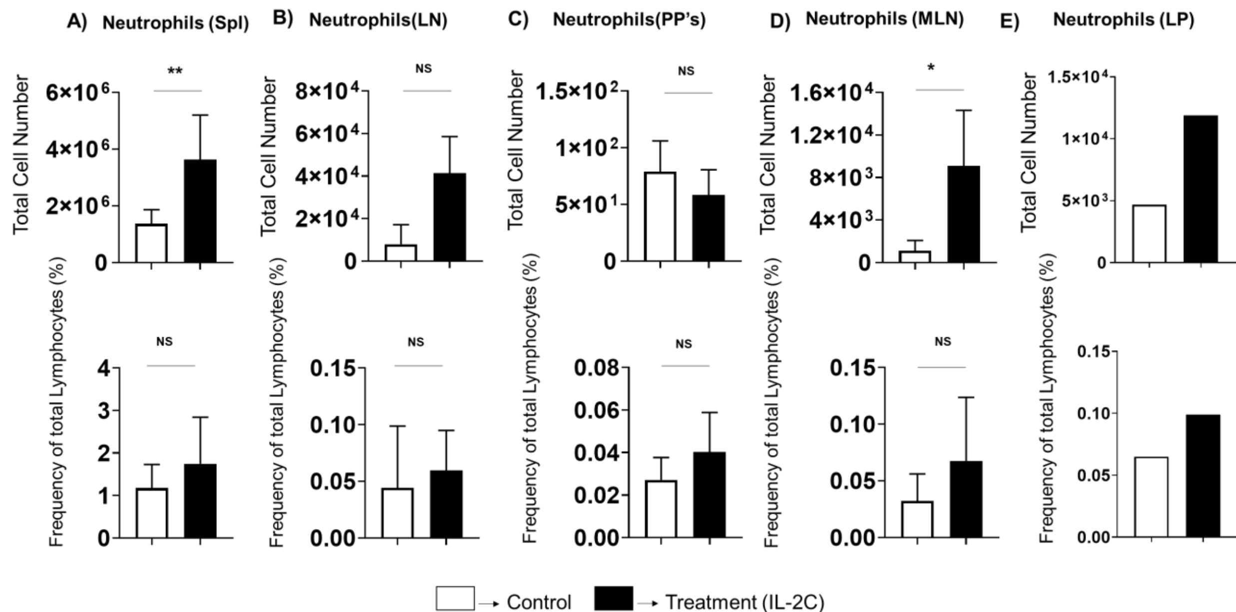

Figure 15:- **IL-2C treatment affects total cell population of neutrophils in spleen and MLN, but not any of the other gut organs.**

Significant effect of IL-2 treatment observed on total cell numbers but not frequency of (A) Spleen (D) MLN T cells. In contrast to spleen no other gut organ except MLN showed any significant difference All error bars represent the standard deviation and significant differences determined with unpaired, two-tailed, students *t*-tests ($\alpha = 0.05$, and * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$, **** $P < 0.0001$). NS represents non- significant. Another gut organ – Lamina Propria also show an increase in total number of neutrophils. But the graphs are from only one experiment with a mouse per group. The graphs only give a hint towards the increase number of cells, but not infer any of the changes. More experiments are needed to observe a significant difference, if any.

Result 3: - IL-2C treatment differentially affects frequency and total cell population of B cell numbers in PP's

In order to address the decrease in cell population in PP's further examination was conducted on changes in another cell population in PP's. Research indicates that PP's consists of approximately 60-70 % B cells and 10-15 % Antigen presenting cells (APC's) [146]. Additionally, MΦ and DC's are shown to be involved in CD, making it important to also explore the effect of IL-2 complex on these cells [94, 104, 163, 164]. We also looked for neutrophils – innate immune cells as they are found to be involved in CD [109, 115]. In summation, we further probed populations of B cells, APC's and neutrophils through flow cytometry in all the organs in order to determine how IL-2 signals impact their frequency and numbers (Fig 10).

The gating strategy employed in this experiment in order to dissect the B cells is illustrated in (Fig 11A). The first step involved in gating is using FSC- SSC to gate dead cells out along with doublet exclusion analysis, as described before (Fig 10A). Further to this, cells are distinguished using MHC class II antibody- as it is present on most of the B cells and professional APC's. We will differentiate B cells using the pan- B cell marker - B220. Further, drawing from the fact that APC's are $MHCII^{+}$ but $B220^{-}$, we will continue to differentiate the subset of this cell population utilizing two conventional markers for MΦ and DC's – CD11b and CD11c, respectively. In this study, we limit our analysis of APC subsets to the $MHCII^{+}$ $B220^{-}$ $CD11c^{+}$ $CD11b^{-}$ subset called as DC's and $MHCII^{+}$ $B220^{-}$ $CD11b^{+}$ $CD11c^{-}$ subsets as MΦ Other combinations ,such as $MHCII^{+}$ $B220^{-}$ $CD11b^{+}$ $CD11c^{+}$ - are also seen, but because of scientific disagreement over classification of

markers for DC's and MΦ in the gut, we have not analyzed APC further. Considering this dilemma, we chose to follow the conventional strategy as described above [145].

Effect of IL-2C on B Cells

We found that the frequency of B cells in PP's decreased by 30% and total cell population went down by 2-fold (Fig 12C). Thus, it is clear that the decrease in absolute numbers of cells in PP's is due in large part to a significant decrease in B cells. B cells were also investigated in other organs. Spleen and Lymph node demonstrated no significant change (Fig 12A, 12B). In comparison with the other organs of the gut except PP's, no change in B cells frequency or total cell number was observed in any of the other organs. Thus, change in B cells is exclusive to PP's. This represent a novel and exciting finding that supports our hypothesis of different actions of IL-2 administrations at different sites.

Based on one experiment B cells in Lamina Propria seems to be increasing by 4-fold (Fig 12E). This observation is again in contrast with the effect observed on spleen B cells after the treatment.

Effect of IL-2C on Antigen Presenting cells

The IL-2C treatment did not result in quantitative changes in DC's or MΦ in any organs except the spleen (in MΦ). A slight increase in total cell numbers was observed in the $MHCII^+$ $B220^-$ $CD11b^+$ $CD11c^+$ cells in the spleen, lymph nodes (Fig 13A, 13B). Parallelly, a slight increase was

also observed in PP's and also the MLN too (Fig 13C, 13D). However, LP demonstrated increase in macrophage number by 2-fold (Fig 14A). Since this is an observation from just one experiment, we cannot establish a concrete difference in the expression of these cells before and after the treatment.

Effect of IL-2C on Neutrophils

Neutrophils are separated from other leukocytes using the phenotypic markers Gr-1and CD11b. In mouse models, MDSCs (myeloid-derived suppressor cells) are found myeloid cells expressing high levels of CD11b (a classical myeloid lineage marker) and Gr-1 (granulocytic marker). High expression of these markers is used to distinguish neutrophils. CD11b(high), Gr-(high) and MHCII (-ve) cells are called as neutrophils in the gating strategy (Fig 11B). Robust and significant increase of total cell numbers and frequency was found in neutrophils in spleen (Fig 14A) Among the gut organs only MLN (Fig 15D) and LP (Fig 15E) only one experiment) showed increase in neutrophils. PP's did not show any difference either in the frequency nor in the total cell numbers. (Fig 15C).

Taken together, Antigen presenting B cells showed complete contrast in the total cell numbers and frequency before and after the S4B6 IL-2C treatment. There is no significant difference between the number of DC's or MΦ, but these cell types need further division based on more different markers mentioned in the literature. Hence, effect of IL-2C may be similar in some cases

between spleen and gut organs, but mostly administration of IL-2C have differential effects between the SLO and mucosal organs.

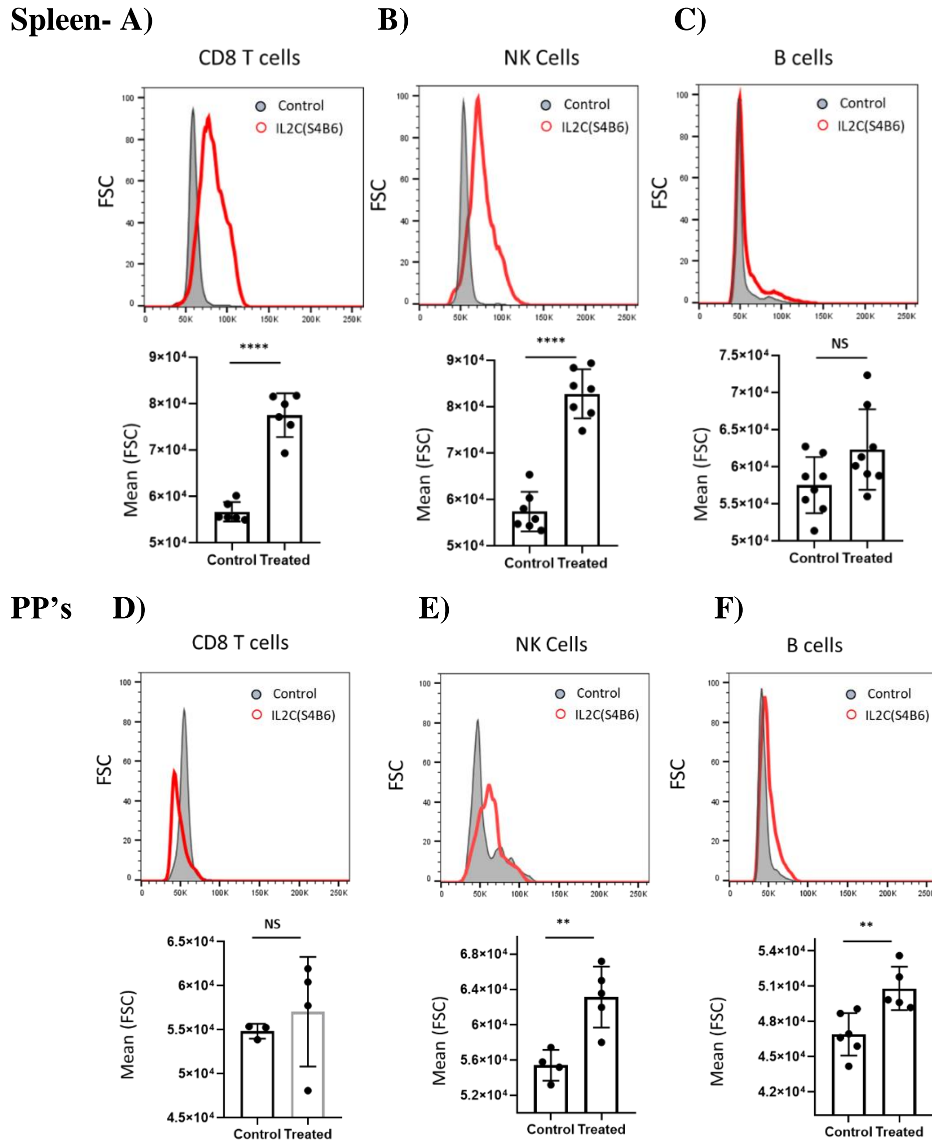

Figure 16:- IL-2C treatment do not cause increase in mean of FSC of CD8 T cells but cause increase in B cells in PP's.

Effect of IL-2 treatment on the mean of FSC of CD8 T cells, NK cells and B cells in (A) Spleen CD8 T cells (4 times increase) (B) Spleen NK cells (3 times increase) (C) Spleen B cells – No significant increase (D) PP's CD8 T cells – No change in the size (E) PP's NK cells – Minimal increase in the size (F) PP's B cells – increased expansion of the mean of FSC the treatment The increase size can either be due to expansion of the size of the cells or proliferation.(Summary of at least 4-9 experiments with 1 mice per group). All error bars represent the standard deviation and significant differences determined with unpaired, two-tailed, students *t*-tests ($\alpha = 0.05$, and * $P< 0.05$, ** $P< 0.005$, *** $P< 0.001$, **** $P< 0.0001$

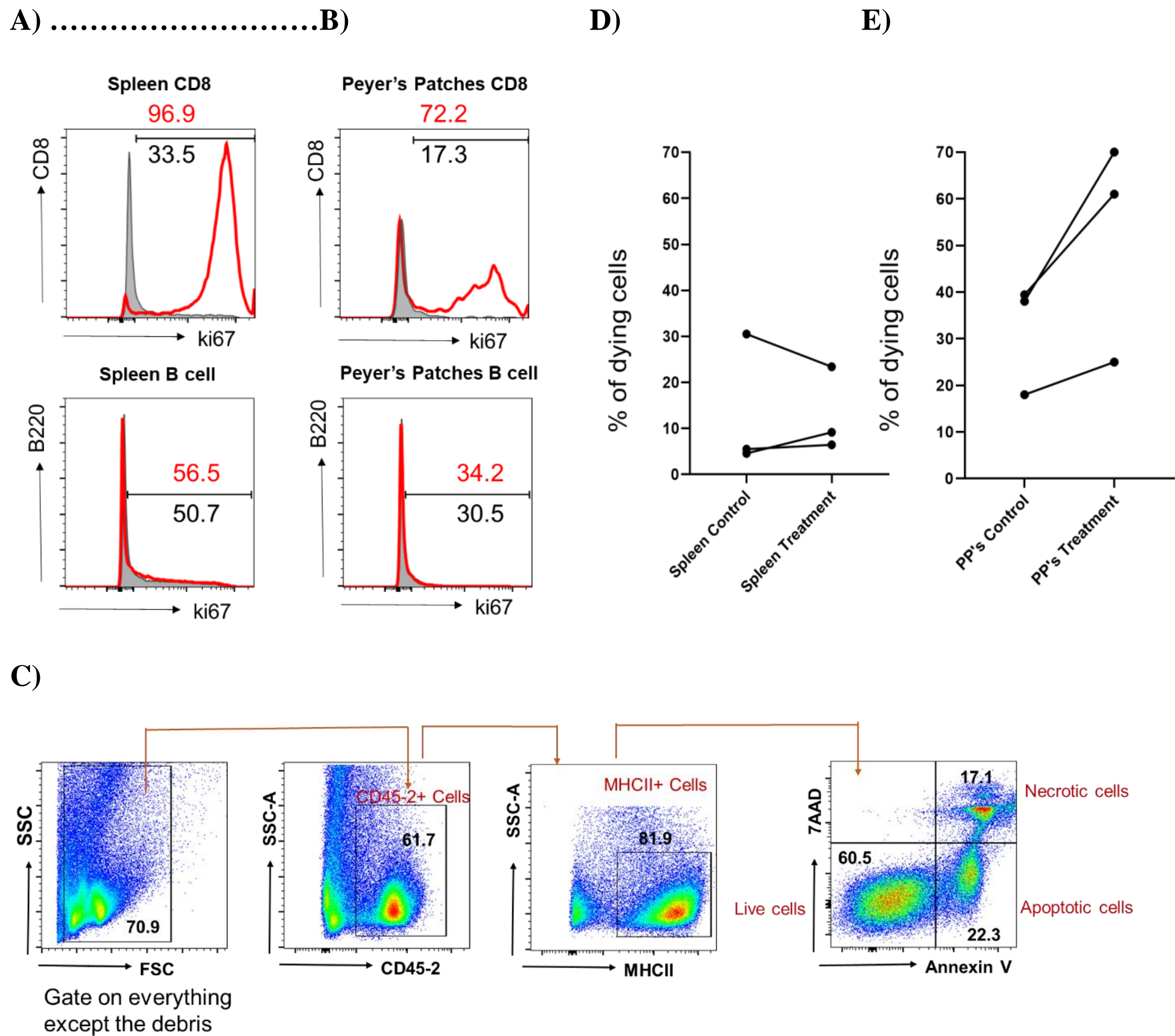

Figure 17:- IL-2C treatment cause death of B cells in PP's.
Effect of IL-2 treatment on proliferation of CD8 T cells and B cells measured by ki-67 staining in (A) Spleen- 2 times increase in proliferation of CD8 T cells but no change observed in B cells (B)PP's - 4 times increase in proliferation in CD8 T cells but no change in B cells. (C) Gating strategy to stain for live and dead cells using 7 AAD and annexin V staining. Measurement of dead B cells by performing 7AAD and annexin V staining in (D) Spleen. The percentage of dead cells are either same or less in treated mice which means that IL-2 is not causing any death of B cells after treatment in spleen (E) PP's: 2 times increased death of B cells after the treatment. (Summary of at least 4 experiments with a mouse per group).

Result 4: - IL-2C treatment cause death of B cells in PP's.

To further probe the finding that B cells are decreasing in total cell numbers in PP's we looked if cell size of B cells changes after IL-2C treatment. One easier way to confirm any alteration in the cell size is through comparing forward side scatter of the cells. Increase in size of cells of the forward side scatter of is suggestive of a possible activity of cell division or their activation.

We found that in the control organ spleen, expansion of CD8 T and NK cell size is observed (Fig 16A, 16B). This phenomenon is suggestive of the fact that they are either dividing or activating. These results also corroborate with the results in existing literature, thereby confirming the division of aforementioned cells after IL-2 treatment. Apart from CD8 T cells and NK cells, no alteration in the size of B cells is observed in spleen (Fig 16C).

In contrast to spleen, no significant difference was observed in the cell size of the $CD8^+$ T cells in PP's (Fig 16D). Although the size of B cells was noticed to have slightly increased, suggestive of either activation or proliferation (Fig 16F). Further analysis was needed to ascertain if this slight change was caused by cell expansion. So, we employed a strategy of staining the cells with staining marker - Ki67, a nuclear protein expressed in dividing cells. The results demonstrated 3 times proliferation of $CD8^+$ T cells in spleen ((Fig 17A) 33.5 % to 96.9 %). $CD8^+$ T cells in PP's also proliferated in the presence of IL-2 complex. But no significant change in size was observed in B cell (Fig 17B). The percentage increase in spleen B cells is from 50.7 to 56.5 (Fig 17A), whereas in PP's it is from 30.5 to 34.2 (Fig 17B) We found that proliferation of B cells is found neither in

spleen nor in Peyer's Patches after the treatment. In summation the observations suggest that change in size of B cells is not caused by proliferation.

Thus, as B cells are not proliferating, increasing in size and their decline in cell numbers raise the possibility that that B cells are getting activated and dying The validity of this assumption was confirmed by further analysis of apoptosis by staining for dead and dying cells using 7AAD and Annexin V - cell viability stains. Cells with compromised membranes will stain with 7-AAD, whereas live cells with intact cell membranes will remain dark. 7AAD intercalates with double-stranded DNA, with a high affinity for GC-rich regions, whereas annexin V distinguishes apoptotic cells by binding with phosphatidylserine (a marker of apoptosis when it is on the outer leaflet of the plasma membrane). The percentage of dead B cells in PP's were observed to increase almost 2 times after post-treatment (Fig 17E). However, in the control spleen no similar changes were observed. The frequency of dead B cells is almost the same as before and after the IL-2C treatment in spleen (Fig 17D). The gating strategy employed to look for dead cells is shown in (Fig 17C)

Significant death of B cells in PP's, but not in the spleen, after the regimen of injecting IL-2 for three days, implies that IL-2 is killing B cells in PP's, which contrasts with the established activity of IL-2 – i.e. no effect on B cells in the spleen.

A)

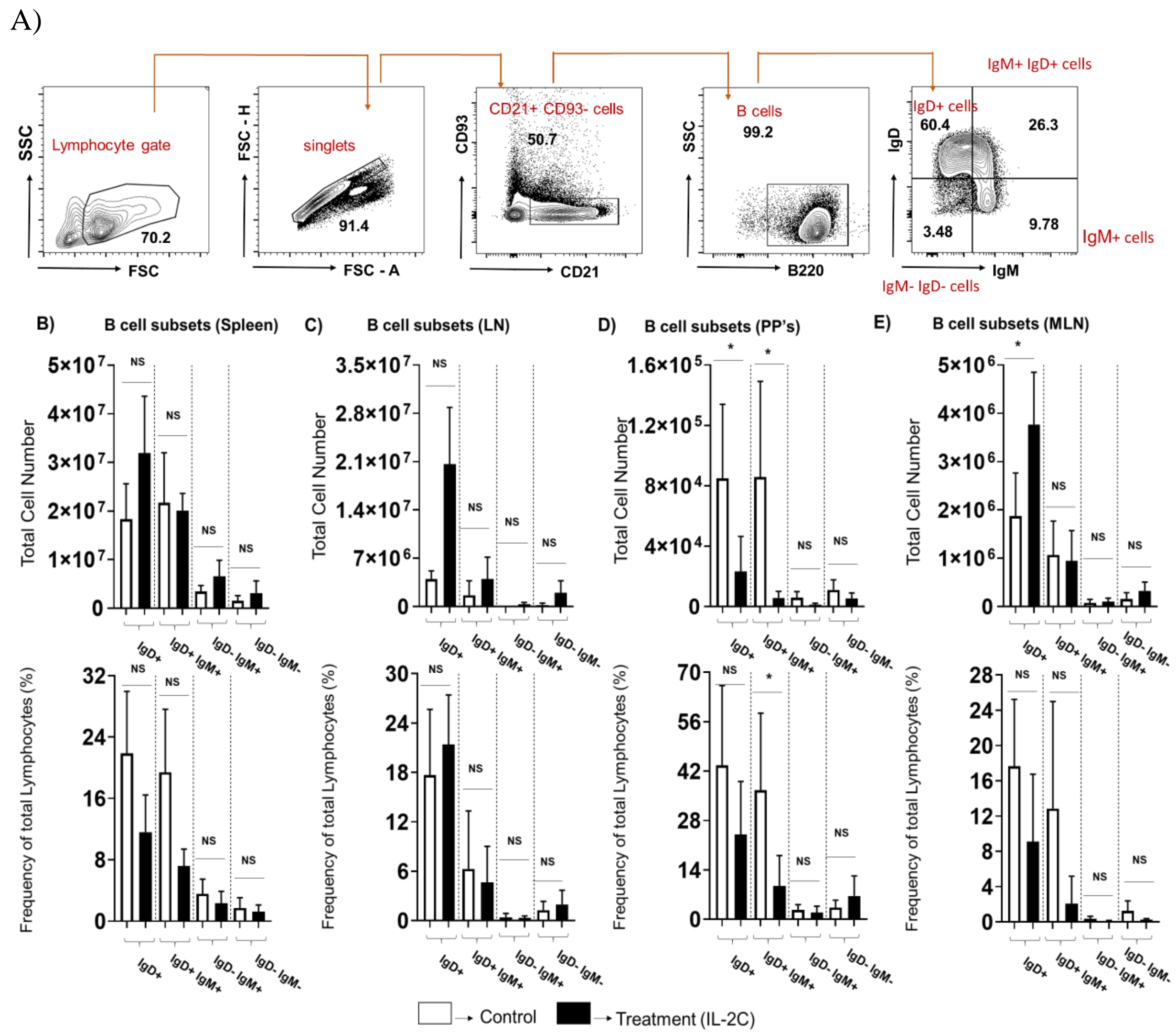

Figure 18:- IL-2C cause targeted killing of naïve B cells in Peyer's Patches

Gating strategy to segregate naïve and effector B cells using IgM, IgD, CD21, CD93 and B220 antibody. Effect of IL-2C treatment on subtypes of B cells in (B) Spleen – Both naïve and effector B cells are not affected (B) Lymph Node – No significant difference in any of the subtypes after the treatment. (C) Peyer's Patches- the naïve subtypes of B cells – IgD+ and IgM+ IgD+ are affected and are decreasing in number and frequency. Also, the effector cell population IgM- IgD- do not show any significant difference. (D) MLN – IgD+ population is showing a minimal increase after the treatment. (Summary of at least 3-4 experiments with a mouse per group). All error bars represent the standard deviation and significant differences determined with unpaired, two-tailed, students *t*-tests (α = 0.05, and * $P< 0.05$, ** $P< 0.005$, *** $P< 0.001$, **** $P< 0.0001$).

Result 5: - IL-2C cause targeted killing of naïve B cells in Peyer's Patches.

We next stained for naïve and mature subsets of B cells to see if specific cohort of B cells are dying in PP's after the IL-2C treatment. The gating strategy employed (Fig 14A) to look for different subsets involves using IgM, IgD - naïve mature B cells (IgM^+ IgD^+) [165], plasma cells (IgD^-) or memory cells, $CD21^+$ - strongly expressed on mature B cells (not clear if expressed on Lamina Propria B cells [166, 167], $CD93^-$ - a marker of recent B-cell generation and B220.

This study has demonstrated low numbers of naive IgM^+ IgD^+ and IgD^+ B cells in Peyer's Patches (Fig 18D) after the IL-2 treatment, but no significant differences were observed in any of the IgM^- IgD^- population. In contrast to spleen, no significant difference was observed in any of the sub types (Fig 18B). IgM^- IgD^- subtype could be consisting of most of the plasma and effector memory cells. No significant changes in this subtype in PP's and decrease in naïve B cells, pointed that B cell effectors or memory cells are not affected by IL-2C signals whereas mature naïve B cells are targeted resulting in their death in PP's.

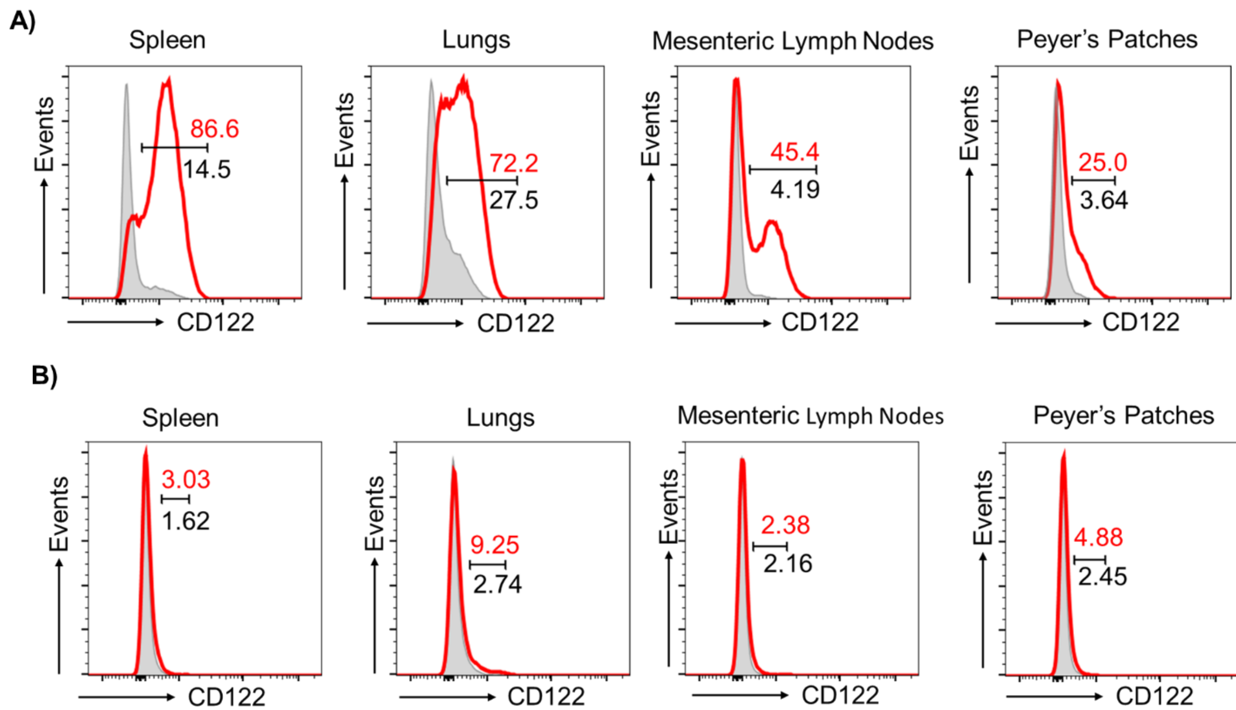

Figure 19:- IL-2C treatment might be affecting B cells indirectly.
Effect of IL-2 treatment on CD122 receptor of (A) T cells - IL-2 CD122 expression was upregulated by many folds in spleen, relatively less in MLN followed by PP's. (B) B cells- A very subtle upregulation of CD122 is observed in B cells. It shows that IL-2 complex does not upregulate CD122 as strong as it does with T cells.

Result 6: - IL-2C might be affecting B cells indirectly.

Overall after the study, it is evident that IL-2C treatment is decreasing B cell numbers in PP's and the cause of this decrease could be the apoptosis of naïve B cells. Since B cells were dying, the next step was to understand the cause of death of these cells. Prior studies have documented that expression of receptor CD122 is highly enhanced in CD8 T cells after IL-2C treatment and is a direct effect of IL-2 signaling [168]. Therefore, to understand if IL-2C is impacting the CD122 receptor expression on B cells similarly as it impacts T cells, we looked for CD122 receptor expression on B cells and T cells in spleen and PP's. If IL-2C is impacting CD122 receptor expression similarly as that in $CD8^+$ T cells in spleen, then it could be hypothesized that IL-2 may have a direct effect on B cells. The gating strategy implemented during this experimentation involves the use of thymocyte marker CD90-2, pan B cell marker B220, CD45-2 and CD122.

Our experiments were evident of increased expression of CD122 in spleen T cells by almost 7 times (Fig 19A) following IL-2C treatment. In contrast to $CD8^+$ T cells, no changes were observed in CD122 expression of B cells after the treatment (Fig 19B). This could imply that IL-2 has an indirect effect on B cells, which needs to be proven with further experiments. Hence all the differences shown between the GALT and the spleen proves that IL-2 is differentially regulated in various immune compartments.

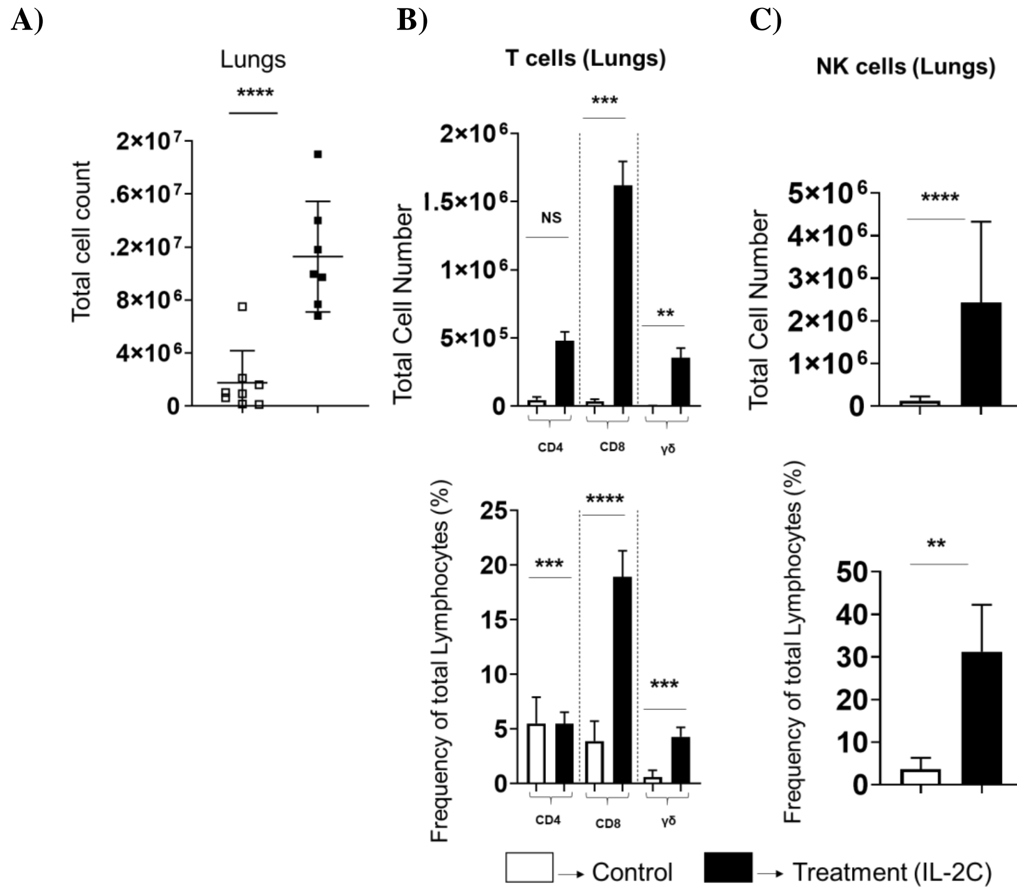

Figure 20:- **IL-2C selectively increases CD8$^+$ T cell and NK cell populations in lungs.**
A) Increase in absolute cell numbers of immune cell populations in the lungs after IL-2C treatment. Effect of IL-2 treatment on total cell numbers and frequency of (B) Lungs T cells (3 fold) (C) Lungs NK cells (2 fold) (Summary of at least 5- 9 experiments with at 2 mice per group). All error bars represent the standard deviation and significant differences determined with unpaired, two-tailed, students *t*-tests ($\alpha = 0.05$, and * $P< 0.05$, ** $P< 0.005$, *** $P< 0.001$, **** $P< 0.0001$). NS represents non- significant.

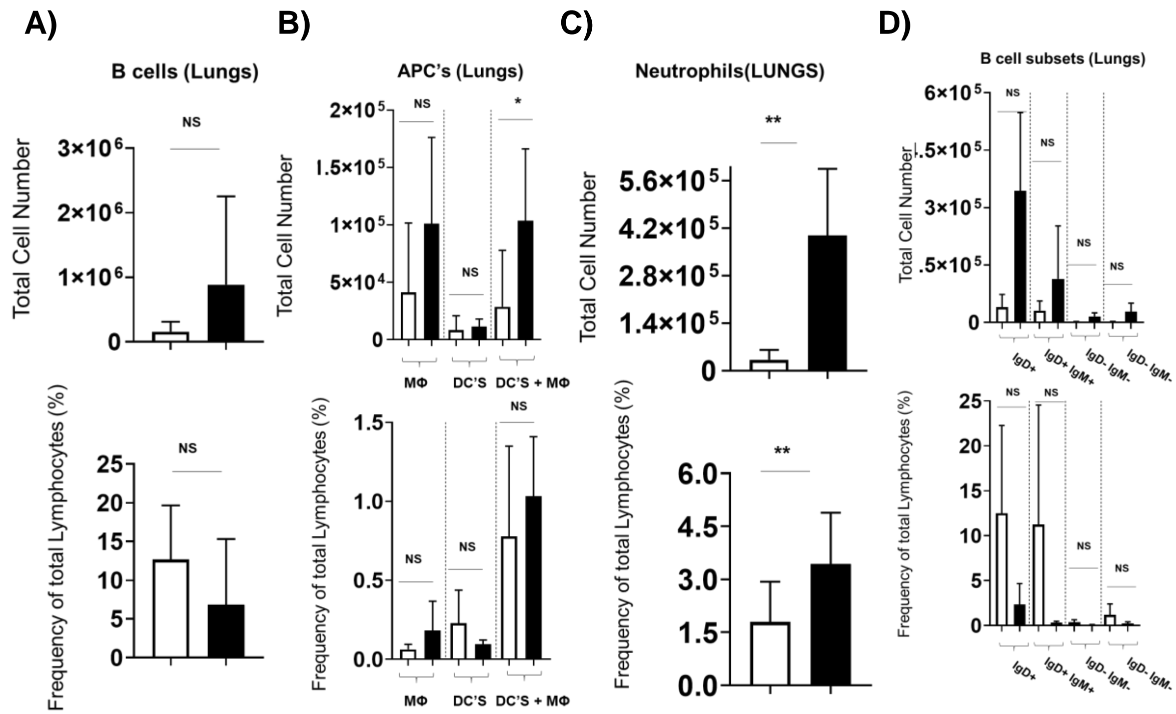

Figure 21:- Unlike gut organs, IL-2C treatment does not cause significant changes in B cells but results in expansion of Neutrophils.
Effect of IL-2 treatment on total cell numbers and frequency of (A) Lungs B cells (No significant change) (F) Lungs APC's – No significant change (C) Lungs - Neutrophils (2 fold) (D) No significant change in naïve and effector B cells in lungs (Summary of at least 5-9 experiments with a mouse per group). All error bars represent the standard deviation and significant differences determined with unpaired, two-tailed, students *t*-tests ($\alpha = 0.05$, and * $P< 0.05$, ** $P< 0.005$, *** $P< 0.001$, **** $P< 0.0001$). NS represents non- significant.

HYPOTHESIS II

We hypothesize that treatment with IL-2C elicits similar quantitative changes in lymphocytes in the lungs versus the GALT.

<u>Result 7: - Insignificant change in B cell populations in lungs after treatment with IL-2C.</u>

Lungs are most-studied mucosal sites in the body. The lung encounters a myriad of potentially foreign, particles on a daily basis. As such, immune cells in this site, like GALT must gauge whether to respond or not. Therefore, to compare changes observed in different mucosal sites, we selected lungs versus other gut organs - PP's, MLN, LP and IEC's. GALT immune system comprises of four different immune sites working together to maintain the homeostasis of the immune system, whereas such different immune compartments are not marked in the immune system of the lungs. Therefore, lungs will be discussed with respect to each organ to paint a complete picture of similarities or dissimilarities between the two.

We hypothesized that lungs will exhibit similar changes as the gut after the treatment, which means either no change in $CD8^{+}$ T cell numbers or a decrease in B cells. Lungs are a complex mucosal site fighting several airborne antigens, much like the gut which fights several foods- borne and other antigens.

<u>Lungs comparison with PP's</u>

Surprisingly, lungs behaved like spleen in terms of significant increase in absolute cell numbers and not like PP's (Fig 20A). 2-fold to 4-fold increase in frequency of CD8 T cells and NK cells, and 5-fold increase in total cell numbers of CD8 T cells and 2-fold increase of NK cells was seen in lungs after IL-2C (Fig 20B, 20C) treatment. Also, there was a notable expansion in the size of

the above-mentioned cells verifying their proliferation after a regime of IL-2 treatment. These results suggest that IL-2C complex treatment causes expansion of cell numbers in the lungs like spleen. Additionally, total cell numbers did not change for B cells in the lungs (Fig 21A).

Lungs comparison with MLN

IL-2C induced changes in the lungs is in sharp contrast with MLN with respect to T cells. MLN showed no significant differences in total cell count neither for T cells nor for B cells. Although lungs behaved like MLN in relation to neutrophils (Fig 21C) and NK cells demonstrating increase in cell numbers.

Therefore, considering all the results explained above it is clear that it is difficult to compare changes in lungs with respect to gut as a whole, but it can be done with respect to one GALT organ at a time, and more experiments will be needed to make a whole comparison. In the data described above two gut organs are behaving are completely opposite.

Thus, it is evident that IL-2 does not behave the same way in all the mucosal immune compartments, especially in the gut. It behaves differently in different mucosal organs, albeit very differently with respect to lungs.

CHAPTER 5: - CONCLUSIONS/DISCUSSIONS

We show that IL-2 does not similarly affect immune cell populations at different sites in the body, but differentially impacts leukocytes in systemic lymphoid tissues such as spleen and lymph nodes versus the GALT. Furthermore, our studies identify a novel effect of systemic IL-2 administration in increasing acute death of B cells in PP's, which is surprising, and interesting. Additionally, we showed that the dying B cells are only naïve B cells and the involvement of IL-2 in there could be indirect. Finally, we show that IL-2 impacts immune cells differentially at different mucosal sites (i.e. Lungs versus the gut).

Boyman and sprent demonstrated a very large increase in the total numbers of $CD8^+$ T cells and NK cells in the spleen and lymph nodes after treating mice with injections of IL-2 and IL-2 mAb (S4B6), with marked enlargement of these organs. They also found minimal effects on other cells, including $CD4^+$ T cells and $B220^+$ B cells. Our results clearly indicated no such expansion of CD8 T cells and NK cells in the GALT in mice treated with IL-2 and IL-2 mAb (S4B6). Gut tissues like PP's (20% of T cells), MLN (80% T cells), LP (60-70% CD4 T cells [169]) and intra epithelial layer (mostly CD8 T cells or γδ T cells [170]) constitutes T cells in fairly good frequency. It was surprising to see that instead of having a predominance of T cells in these organs, the impact on gut by the IL-2 treatment is very different as observed in spleen and lymph nodes. No significant change was observed in PP's and MLN. Additionally, in contrast to T cells, we found that the treatment impacted B cells in Peyer's Patches.

PP's involved in the pathogenesis of CD's: -We found that the number of B cells decline in PP after an IL-2C treatment. This finding instigates a question of this mechanism's relevance to CD which prompted us to investigate relevant literature. Previous studies have indicated the importance of PP's in CD because they are abundantly present in the terminal ileum, an area predisposed to CD [171]. In 1960 and 1996, two separate groups reported that the earliest microscopic changes in CD were ulceration of lymphoid follicles and PP's in the terminal ileum [172, 173]. Further endoscopic observations of the PP's in a CD patients [174, 175] showed altered irregular surface which harbors microgranulomas and lymphoid hyperplasia when compared to controls. Similar observations were also noticed in patients suffering from UC [176]. As PP's are an important site to sample luminal antigens, their disruption can cause an increase in the intestinal permeability to unwanted luminal antigens. If such a state persisted for a long time, it can disrupt the intestinal layer and grant entry to unwanted organism causing inflammation. Taken together, these results suggest that PP's are an important organ and the alterations in PP's in CD demands further investigation.

Deletion of B cells could cause IBD like symptoms:- Moreover, Peyer's Patches consists of 70-80% of B cells [146] and through this study we found that these some of these cells die after IL-2C treatment. Different biological therapies in various disease settings produce unwanted adverse effects to treatments. In view of this is one such therapy using Rituximab, a B lymphocyte depleting agent. Rituximab is widely used in the initial treatment of CD20-positive hematologic malignancies and various autoimmune disorders. It was reported to exacerbate UC in a patient

with refractory disease [177] . A case of a 45-year-old patient was reported in 2008, who developed an UC after undergoing treatment with this antibody and was not predisposed to IBD or received any other therapy before this [178]. Recently, a patient developed 2 episodes of fulminant colitis after 2 infusions with Rituximab for the treatment of disseminated B-cell marginal lymphoma [179]. The studies above suggest that the decline in B cells may foster the development of colitis in patients undergoing the therapy, although, the causation of acute severe colitis after rituximab therapy is not known. Considering this, more work is needed to understand the mechanism of how reduction in B cells can foster an environment that might lead to colitis, if at all B cells are at fault.

Mice studies showing involvement of B cells in IBD: - In addition to the clinical cases mentioned above, some in-vivo murine studies have attempted to describe the role of B cells in colitis. Mizoguchi *et al* have found severe colitis in a double mutant mice that lack TCR-$\alpha^{-/-}$ (lack T cell receptor) × immunoglobulin (Ig) $\mu^{-/-}$ (lack B cells) [180]. The findings of their study suggest that B cells are not required for the initiation of colitis but can suppress colitis through mucosal clearance of apoptotic cells. Further research by Mizoguchi *et al*. show that B cell subset suppresses the progression of intestinal inflammation by producing interleukin-10 [181], a cytokine which maintains immune equilibrium and prevents the development of autoimmune disease [182, 183]. Patricia *et al* [184] showed alterations in the cytokine environment (i.e diminished up-regulation of IL-4, IL-10, and TGF-β) in the gut of a B cell deficient mice (μMT) when compared to a wild-type mice after treatment with low doses of antigen given orally. These cytokines are associated with the induction of cytokine-mediated active suppression. Along with

this, increased Ag was also detected in the lymph of the µMT mice. The paper does not unravel the actual mechanisms underlying these observations. Additionally, Wang and Chu [185] confirm that B cells present in the gut are implicated in the resolution of colitis, using a sulfate sodium (DSS)-induced colitis model. More severe disease developed in the absence of B cells, and that the adoptive transfer of total WT B cells to B cells-deficient mice attenuated the disease while the mechanisms induced are unclear. It has also been suggested that the transfer of IL-10(-/-) B cells attenuated colitis, indicating that B cells inhibit colitis through an interleukin-10 (IL-10)-independent pathway. Moreover, they found $CD4^+$ T cells to be the major contributor to the IL-10 pool in the gut. Furthermore, Wang and Chu also showed that antibody depletion of Tregs resulted in an exacerbated colitis B cells- deficient model mice [186]. Interestingly, it has been found that B cells induce proliferation of Tregs which in turn promotes B-cell differentiation into IgA-producing plasma cells. These results demonstrate that B cells and Tregs interact and may cooperate to prevent excessive immune responses which can ultimately lead to colitis.

In view of the above observations and our observation of IL-2 induced B cell apoptosis we hypothesized that the decrease in B cells after an IL-2C treatment could be responsible for generating an inflammatory environment in the PP's. Such an environment created (as a result of an IL-2C treatment) in the PP's may be more susceptible to infections as the equilibrium of tolerance can get disturbed. This could result either in increased intestinal permeability in the gut, causing more antigens to cross the epithelial barrier, and/or due to the unavailability or low luminal IgA which is normally produced by B cells. IgA is a major immunoglobulin produced in the GALT

and is responsible for generating a immune response against the harmful microbiota residing in the gut. Thus, disruption of equilibrium in susceptible individuals caused due to deletion of B cells may result in severe colitis.

Treating mice with IL-2 at different time points: - Currently we have treated the mice with IL-2C for three days and assessed changes in immune cell subsets on the fourth day (i.e 1 day after 3rd day treatment). And this time we have undertaken the task of understanding the change in immune cell subsets in various regions of the GALT. However, changes observed after three days of injection, are like a snapshot in time. We do not understand what occurs prior and after the third day timepoint. Therefore, we feel it is important to perform many experiments that are possible in context of exploring the effect of IL-2 at different time-points including day 1 or 2 or post day 4. This will help us in understanding if, the changes that we see after IL-2 treatment are acute or chronic. The observations from the suggested future study will throw light whether one day treatment of IL-2 is enough to observe the acute changes or the difference obtained in the immune cell population is chronic and persist for a long time.

If the changes observed are chronic it can be hypothesized that deficiency of B cells in the PP's can be of real consequence to CD's. It can also be hypothesized that IL-2 can be involved in the pathogenesis of CD because of its potential ability to kill B cells and expose the gut to several unwanted pathogens. If changes in PP's continue to remain the same even after dis-continued exposure of IL-2C, then the skewed environment that is letting loads of antigens to cross the barrier

through PP's persist for an elongated interval and can ultimately result in IBD. In the gut, exposure to low amount of particular antigen is not harmful. But regular exposure can initiate a surmountable response of inflammation homing the gut. There this state of continuous inflammation can be one of the in the etiology in some cases of IBD.

Additionally, the disruption of the intestine layer can be caused due to altered production of IgA^+ B cells, result in shifting of the equilibrium towards the unwanted pathogens. Thus, this could cause alteration in the microbiome of the gut in a healthy individual. Through our experiments we show that there is increase in absolute numbers of $CD8^+$ T cells and NK cells. Combined this increase of $CD8^+$ T cells with decrease in B cells can alter the environment in number of ways. Selective transport of polymeric IgA across epithelial cells lining the mucosal surface is an important phenomenon to keep the commensal organisms in check. If this property of B cells is altered, followed by increase in $CD8^+$ T cells effector cell populations, then the regulatory mechanism that control the local immune response to luminal antigens will get disturbed. Thus, alterations in this way can jeopardize the equilibrium of the whole gut immune system and will not spare the vital structures of the intestine from immune mediated destruction.

Increased secretion of IL-2 can create a skewed environment: - Our observations suggest that naïve B cells are dying in Peyer's Patches after IL-2C treatment. We also attempted to find out if death of B cells was a direct effect or an indirect effect due to IL-2 signals (CD122). CD122 gets upregulated after interacting with IL-2, which is clearly seen with CD8 T cells. But no such

upregulation of CD122 signal was observed in B cells. This supports that it can be an indirect effect of IL-2. There could be many reasons for the indirect effect observed. IL-2 triggers other cells populations, i.e. CD8 T cells and NK cells to expand and secrete cytokines that create pro - inflammatory condition, which can result in killing of B cells. This assumption has prompted a thought that, increased secretion of IL-2 without antigen exposure can result in a skewed environment, thus IL-2 being a dangerous cytokine, if not regulated optimally. Some light on this thought have shown by Strutt and colleagues [159]. They demonstrated that IL-2 administration can cause extremely potent lung inflammation involving NK cells, and that lung environment is particularly sensitive to IL-2-induced inflammation during viral infection. Thus, future studies can test the effector cytokines such as TNF, IL-1α, IL-1β, IFN-γ etc released by these cells at various intervals of time and can test to see if these cytokines are responsible for B cell death.

IL-2 secretion can activate death receptors: - Within the immune system, FasL is mostly expressed by cytotoxic $CD8^+$ T lymphocytes and NK cells that are activated in response to viral infection and malignancies. We can hypothesize that IL-2 signals can initiate such response from $CD8^+$ T cells and NK cells and thus can kill B cells by activating the death receptors on them. Also, B cells death receptors can get activated after interacting with helper T cells. In addition to the extrinsic ligands, apoptotic signals can also be initiated through the intrinsic factors associated with Bcl-2 protein family, mitochondrial permeability and apoptosome formation. Therefore, studies can look for the molecules involved in the extrinsic pathway [187] on B cells and intrinsic pathway to confirm the reason of B cell death.

Subsets of APC's present in gut and involved in CD's: -. In addition to the B cells, we also looked for APCs. We use the traditional surface markers to phenotype classical DC's ($CD11b^-$ $CD11c^+$) and MΦ ($CD11b^+$ $CD11c^-$). However, in the gut combination of other phenotypic markers are used to classify APC's that have been interchangeably used to define DC's and MΦ, such as F4/80, CX3CR1 CD103, CD14 and CD64 etc. For broader understanding, the reader is referred to the suggested reviews [188, 189]. Therefore, classification of these cells requires a multiparameter approach, which was beyond the scope of this study.

MΦ and DC's have been known to later markedly in IBD environment in the gut. In humans IBD has been marked with accumulation of CD14(high)CD11c(high) MΦ, rather than CD14(low) MΦ during the homeostatic state. Anti TNF treatment have also been shown to be accompanied by loss of these CD14(high) cells [190, 191]. In different mice models of inflammation, intermediates levels of CX3CR1 MΦ, and a unique subset of $CD169^+$ $CX3CR1^+$ have been mentioned in the cases of IBD [192, 193]. As per platt *et al* [194] MΦ in steady state condition are $F4/80^+TLR^-$ $CCR2^-$ CX3CR1(high) and do not produce TNF-alpha in response to stimulation. But during experimental colitis, $TLR2^+$ $CCR2^+$ CX3CR1(int) Ly6C(high) $Gr\text{-}1^+$, TNF-α-producing macrophage are present. They also showed that there is preferential CCR2-dependent recruitment of the proinflammatory population during colitis. CCR2 knockout mice show reduced susceptibility to colitis and lack the recruitment of $TLR2^+$ $CCR2^+$ $Gr\text{-}1^+$, TNF-alpha-producing MΦ.

Therefore, future studies can investigate these subsets and many others as various subsets have been mentioned to play an inflammatory role in cases of CD (colitis) in humans as well as mice, and their role are only starting to be understood. It could be a possibility that these subsets are affected by IL-2 treatment

<u>Cross Talks between APC's: -</u> APC's seem to be specific to local environment in the gut. DC, MΦ and B-cells exhibit cross-talks among themselves for supporting critical roles. Although each type of APC exhibits unique functions allowing them to participate in gut immunity, with their official functions of activating T-cell responses by showing an antigen, APC can also interact with one another to directly shape immune responses. Therefore, the disturbance in the interconnections between these cells could result in disequilibrium, promoting a chronic state of inflammation, detrimental in CD or IBD. Therefore, these specific subsets need to be looked in detail to understand if by any way, IL-2 is causing a change in any specific markers on the cells. It can cause cascade of changes in all the attached mechanisms causing destruction of tolerance.

<u>Causes of CD like MAP could result in flare-ups of IL-2: -</u> CD is considered to be a chronic inflammatory bowel disease. Many explanations have been proceeded to explain the cause for this disease however, its causation remains unclear. Genetic causes, environment changes, high fat diet [195] and exposure to an mycobacterium- MAP are various proposed possibilities [158]. It can be a possibility that the flare up and inducement in IL-2 can be based upon the above-mentioned

rationale, that these reasons can be the initiating triggers of release of IL-2. Therefore, the next step would be to test this into a diseased model where all these factors can be tested to see if they are actually resulting in flare ups. There is an entire possibility that increase in IL-2 due to these causes could create a disruption in B cells.

Effect of IL-2 on lungs: - Our second aim of the project was to compare the effects of IL-2C at distinct mucosal sites, and we chose lungs as the representative mucosal organ. The results clearly showed that the effect of IL-2C was different in lungs as compared in the gut, even though both are mucosal sites. It is unclear what underlies these results. Some possibilities are mentioned below.

Firstly, the difference could be because of the different cytokine mediated environment of the gut and the lungs [196-198]. The source of antigens found in the lungs are quite dissimilar as to the source of antigens present in the gut. Airborne antigens are found in the lung, whereas gut gets the continuous influx of food antigens and the commensal microorganisms. Thus, there are different kinds of signals generated and initiation of different pathways through the interaction of these antigens with different Toll like receptors and pattern associated molecular patterns. These signals can sensitize the IL-2 signals differently at both the mucosal sites. Therefore, difference in the mucosal environments can be one of the reasons for the difference observed in both the mucosal sites. Second, as mentioned above different types of APC's are present at these two different sites. Differential APC subsets might respond differently to these antigens leading to differential

susceptibility to apoptosis after IL-2 treatment in both the organs. Third, it has been established that the vascular leak syndrome caused due to IL-2 treatment in the lungs is because of the presence of IL-2 receptors on the endothelial cells [199]. It is possible that the type and the number of IL-2 receptors present on the endothelial lining of the gut tissues versus the lungs are different. These differences can cause IL-2 to react in opposite ways at both the sites, thus giving a completely different outcome. Therefore, studies need to be performed to tests these possibilities to understand which of these reasons are responsible for varied impact of IL-2 and its differential regulation at different organs.

Summary: - The use of S4B6 IL-2C is a popular regime in studying the effects of IL-2 in various disease models such as cancer and many autoimmune diseases [200-203]. But the studies concluding the effects of IL-2 in the gut are extremely limited. These studies fail to scrutinize the possibility of IL-2's adverse effects on other organs such as gut, lungs, etc. thereby, confining the focus upon organs such as the spleen and lymph nodes. Also, this limitation extends to the inclusion of looking the effects only to cell types such as CD8T cells and NK cells. However, our study illustrates the effects of IL-2 on B cells and it is not limited to the aforementioned cells. The differential effect of IL-2 on CD8 T cells, NK cells and B cells is quite clearly observed between the systemic lymphoid organs and Gut associated lymphoid tissue. The effect of IL-2 on these cells in the gut is varied than observed in the spleen. These effects could be involved in pathogenesis and etiology of some of the cases of CD. Therefore, there is abundant room for further research on the alterations caused in the immune cell subsets by IL-2 specially in the conditions of IBD.

The effects of IL-2C on immune cells should be understood holistically. This would bring in expanded awareness about events occurring in other sites and how it could affect the given treatment.

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