ACTIVATION/INACTIVATION OF CD4+ T CELLS IN CONTEXT OF THE QUORUM HYPOTHESIS

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ABSTRACT

Understanding how immunological tolerance is established has been the subject of intense investigation and several models have been put forward to define the circumstances under which antigens activate or inactivate lymphocytes. According to the commonly held Danger Model, a single CD4+ T cell can be activated by an antigen presenting cell (APC) under appropriately "dangerous" conditions that instruct the APC to upregulate its expression of co-stimulatory molecules necessary for CD4+ T cell activation. In the absence of danger signals, CD4+ T cell inactivation ensues. Conversely, the Quorum Hypothesis postulates that the antigen-dependent, B cell-mediated cooperation between a minimum number of antigen-specific CD4+ T cells leads to their activation, whereas too few antigen-specific CD4+ T cells would be inactivated by antigen. Using enzyme-linked immunospot assay and flow cytometry, we investigated whether the activation of murine T cell receptor transgenic CD4+ T cells *in vitro* was quorum-dependent. The number of cultured CD4+ T cells was critical to their ability to generate IL-2 and/or interferon gamma (IFN-γ) producing cells. In general, relatively low numbers of CD4+ T cells did not generate cytokine-producing cells, medium numbers generated IL-2-producing cells, while higher numbers generated IL-2 and IFN-γ-producing cells. This quorum effect in the generation of cytokine-producing cells was not mediated by a difference in the proliferation of CD4+ T cells cultured under different conditions. Importantly, CD4+ T cells generated cytokine-producing cells without the deliberate use of Danger molecules, leading to the suggestion that Danger signals were not critical for CD4+ T cell activation. Moreover, our observations suggest that quorum sensing is mediated by a linked mechanism. CD4+ T cells specific for a non-crossreacting antigen optimally facilitated the activation of CD4+ T cells specific for the target antigen only if the two antigens were presented by the same APC. Lastly, our preliminary observations support a difference in the role of dendritic cells and B cells in mediating quorum sensing. In conclusion, our observations are consistent with the predictions of the Quorum Hypothesis. Further investigation is required to determine if CD4+ T cells are inactivated by antigen if cultured at limiting densities.

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"*If I have seen further, it is by standing on the shoulders of giants*"

- Sir Isaac Newton

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Peter and Popi, Popi and Peter. I could not have done this without you.

Lastly, I would like to thank my parents for their unconditional support throughout my career. They laid down the foundation that shaped the man I am today.

DEDICATION

I dedicate this thesis to my younger brother, Faisal Al-Yassin. May he find joy in whatever he pursues.

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LIST OF ABBREVIATIONS

2S2S	two-step, two-signal
2WO	two-week-old
APC	Antigen presenting cell
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BCR	B cell receptor
BSA	Bovine serum albumin
CD	Cluster of differentiation
CTL	Cytotoxic T lymphocyte
CTV	CellTrace Violet
DC	Dendritic cell
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
FDC	Follicular dendritic cell
FMO	Fluoresence minus one
FRC	Fibroblastic reticular cell
HEL	Hen egg lysozyme
HEV	High endothelial venule
HSA	Human serum albumin
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LN	Lymph node
LPS	Lipopolysaccharide
MACS	Magnetic-activated cell sorting
NBT	p-nitroblue tetrazolium chloride
NK	Natural killer
OVA	Ovalbumin
PBS	Phosphate buffered saline
PBST	PBS Tween 20
PSA	Pig serum albumin
RAG	Recombination activating gene
SAP	Streptavidin-conjugated alkaline phosphatase
SSA	Sheep serum albumin
TCR	T cell receptor
Treg	Regulatory T cell
tTreg	Thymic Treg

CHAPTER 1 - INTRODUCTION

1.1 Preamble

It was recognized early on that the immune system had evolved the ability to mount a response against virtually all antigens, even the unknown. In other words, the immune system had evolved to "anticipate", a characteristic some refer to as "promethean evolution" (1). Implied in this universality is the ability to mount a response against self, which was first observed in the form of autoimmune hemolytic anemia (AIHA) (2). We now know that the antigen-specific receptors, namely antibodies and T cell receptors (TCRs), are generated stochastically (3, 4), inevitably leading to the generation of lymphocytes bearing receptors specific for self-antigens (5, 6). Nevertheless, pathological autoimmunity is rarely observed, suggesting the existence of mechanisms by which the immune system discriminates between self and nonself.

Self-nonself (SNS) discrimination is a critical property of the immune system and has rightfully been the subject of intense investigation over the past six decades, and many models have been put forward to account for how SNS discrimination is realized. An example of such models is the Quorum Hypothesis (7). Acknowledging that no single framework is perfect, nor capable of accounting for all the diverse experimental observations, this introduction seeks to help the reader "stand on the shoulders of giants" by revisiting relevant classical and contemporary studies to gain insight into how the Quorum Hypothesis came about, and why I sought to examine it as a potential framework for understanding SNS discrimination.

1.2 A brief history of self-nonself discrimination leading up to the single-lymphocyte/multiple-lymphocyte model for the inactivation/activation of lymphocytes

Ehrlich and Morgenroth reported in a series of publications that goats immunized with allogenic red blood cells (RBCs) made antibodies that reacted with the donor's RBCs but not to the recipient's RBCs (8). Ehrlich envisaged that the immune system possessed an intrinsic ability to mount a response to the host's antigens, a possibility he referred to as *horror autotoxicus (9)*, but postulated that there were mechanisms in place to prevent such a response.

The early 1900's was characterized by an intense debate between those espousing instructional theories and those espousing selectional theories on how antigen-specific antibodies were produced. Central to the formulation of these theories was providing a potential mechanism for how SNS could be achieved. In 1949, Burnet and Fenner, in their book *The Production of Antibodies*, proposed that "if, in embryonic life expendable cells from a genetically distinct race are implanted and established, no antibody response should develop against the foreign cell antigen when the animal takes an independent existence." (10). They envisaged tolerance to self-antigens to develop by virtue of the presence of self-antigens early in ontogeny, during which exposure of the developing immune system to antigens renders the immune system unresponsive to such antigens. Foreign antigens, on the other hand, impinge upon the immune system later in life, after the immune system had developed. Such an impingement results in the mounting of an immune response. This "Historical Postulate" was to an extent inspired by the earlier reports of Owen (11) and Traub (12) that early exposure to antigen resulted in a state of antigen-specific tolerance. Briefly, Owen reported that fraternal twin calves were exposed to each other's antigens by virtue of their sharing a placenta. These calves fail to produce antibodies to the twin's blood cells when immunized as adults. Traub reported that exposure of mice in utero to lymphocytic choriomeningitis virus (LCMV) was more likely to result in a chronic infection. The Historical Postulate was later corroborated by Medawar and colleagues (13, 14) and by Hašek and colleagues (15-17). I have previously discussed the significance of the findings of Medawar and colleagues elsewhere (18). In essence, Billingham, Brent, and Medawar reported that injecting murine fetuses with allogeneic tissue rendered these mice, as adults, more receptive to skin grafts from the same donors of the "tolerizing" cells. Interestingly, the older the fetuses at the time of injection, the lower the probability of tolerating the skin graft. Furthermore, mice injected with allogeneic cells on the first day of life, rather than during the fetal stage, rejected the skin grafts when receiving transplants as adults. These findings were interpreted as being consistent with the predictions of the Historical Postulate. Indeed, Burnet and Medawar were awarded the Nobel Prize in Physiology or Medicine for their "discovery of acquired immunological tolerance" in 1960 (19). Similarly, Hašek and colleagues reported that parabiosis of chick embryos rendered the chicks tolerant of each other's RBCs at both the antibody level and the level of cell-mediated immunity.

The Historical Postulate was challenged by Lederberg in his 1959 paper (20) where he made two important remarks: firstly, he postulated that lymphocytes, the critical cells of the adaptive immune system, were continuously generated throughout life. He made this proposal in view of evidence that the continuous presence of antigen was required to maintain tolerance to the antigen. A third remark could be made that autoimmune diseases can arise later in life, after the immune system has supposedly fully developed. These three remarks meant that the mechanism by which antigen-specific tolerance is induced should operate throughout the life, beyond merely early in development. Therefore, he proposed that when lymphocytes are first generated, they are in a sensitive/paralyzable state, whereby their interaction with antigens results in their physical or functional ablation. With time, sensitive lymphocytes that do not interact with antigen progress to a reactive/inducible state, whereby they can be activated by antigen. Lederberg's proposal is consistent with the Historical Postulate. Self-antigens are present early in development and continue to be present throughout life. Therefore, when self-specific lymphocytes are first generated, they would interact with self-antigens while in a sensitive state, resulting in their physical or functional deletion. The continued presence of self-antigens ensures the maintenance of the state of self-tolerance. On the other hand, lymphocytes specific for foreign antigens accumulate in the body and mature into a reactive state, until such foreign antigens impinge upon the immune system, leading to the activation of these lymphocytes. We shall see below that Lederberg's model seems to account for the mechanism by which central tolerance is established.

1.3 The single-lymphocyte/multiple-lymphocyte model for the inactivation/activation of lymphocytes

When Lederberg formulated his model, reactive lymphocytes were envisaged to be activated by the mere binding of antigen to the antigen-specific receptor on the surface of these lymphocytes, and so was the inactivation of sensitive lymphocytes. However, several reports emerged that were inconsistent with this antigen binding model and suggested that mature lymphocytes, deemed by Lederberg to be only inducible, seemed to be both inducible and paralyzable, depending on the circumstances under which they encountered the antigen. Bretscher and Cohn, in their famous 1970 paper "A theory of self-nonself discrimination" (21), provided an accounting of these reports, which can be summarized into four sets of observations. I shall briefly summarize these four sets

and defer a deeper discussion into one of them to a later section in this chapter. Firstly, immunization of immunocompetent animals with very low or very high doses of antigen precluded the subsequent generation of antibodies upon immunization with an immunogenic dose of same antigen (22). Secondly, the induction of an antibody response to haptens, especially macromolecular ones like poly-L-lysine (PLL), required the conjugation of these haptens to an immunogenic carrier (23). Importantly, the secondary response to the hapten required priming the animal to both the hapten and the carrier (24, 25). In other words, an animal primed with the hapten h coupled to the immunogenic carrier P (i.e., h-P) did not generate a secondary response when immunized with h-Q and P, where Q is an immunogenic carrier that does not crossreact with P. Thirdly, the optimal induction of an antibody response seemed to require, or least was greatly favoured, when the antigen to be was administered in aggregated form. The administration of de-aggregated or fragmented antigen induced unresponsiveness at the antibody level in some cases (reviewed in (26)). Lastly, the state of tolerance established in an animal to a foreign antigen could be "broken" by the administration of a crossreacting antigen (27). I shall discuss the latter observation in greater detail in later sections.

The above observations led to the suggestion that mature lymphocytes could interact with antigen in two different ways, one leading to their activation, and the other to their inactivation. These observations were not readily accounted for by Lederberg's model, according to which lymphocytes had one fate after antigen encounter, depending on which state they are in. Bretscher and Cohn sought to put forward a model that is consistent with the Historical Postulate and accounts for the observations listed above. According to their model, a lymphocyte is activated if it receives two signals, signal 1 and signal 2. Signal 1 is generated when the antigen binds to the antigen-specific receptor on the lymphocyte's surface. Signal 2 is generated and delivered to the lymphocyte after binding of a helper lymphocyte to another site of the same antigen, different from the site to which the precursor cell binds. In other words, the antigen was envisaged to act as a bridge between the target lymphocyte and the helper lymphocyte. Importantly, if a lymphocyte receives signal 1 without signal 2, it becomes inactivated (21).

Understanding the nature of signal 2 is critical to understanding the salient features of Bretscher and Cohn's model. Signal 2 was postulated to consist of membrane-membrane interactions or short-range cytokines, or both. Furthermore, evidence emerged supporting non-redundant roles for thymocytes and bone marrow cells in antibody production (28, 29). Therefore, the helper lymphocytes were postulated to be thymocytes (T cells) that were activated to express effector helper function. These T cells were also envisaged to be inducible and paralyzable, depending on whether they receive signal 1 and 2 or signal 1 alone, respectively, with the source of signal 2 being another activated effector helper T cells. With this in mind, Bretscher and Cohn's model essentially postulated that single lymphocytes were inactivated by antigen, while lymphocyte activation required the cooperation of multiple lymphocytes. Since the source of signal 2 was activated helper cells whose activation required activated helper cells, an important question arose: where did the first activated helper cell come from? This became known as the Priming Problem. I will address a potential solution to the priming problem in a subsequent chapter.

Bretscher and Cohn envisaged their single-lymphocyte/multiple-lymphocyte model to account for SNS discrimination as follows: During development, lymphocytes are generated only one or a few at a time from primary lymphoid organs. If some of these lymphocytes happen to be specific for a self-antigen, they will receive signal 1, as self-antigens are usually present early in development. However, when the self-antigen is encountered, there will be too few antigen-specific lymphocytes for sufficient cooperation to occur, so these lymphocytes will be inactivated or deleted. Since self-antigens continue to be present throughout life, the immune system maintains low numbers of self-specific lymphocytes, thereby maintaining a state of self-tolerance. On the other hand, lymphocytes specific for foreign antigens accumulate in the body such that when the foreign antigen impinges upon the immune system, the antigen-specific lymphocytes will sufficiently cooperate to become effector cells (21).

1.4 Early studies on the nature of signal 2 and their influence on future models of self-nonself discrimination

Lafferty and Cunningham proposed that T cell activation required a second signal in addition to antigen recognition (30). They referred to this second signal as the co-stimulatory signal (31), and based their proposal on their observation that the ability of cells, from an MHC disparate donor, to activate T cells did not correlate with their MHC expression, but rather with the expression of what they postulated was a co-stimulatory factor on the donor cells. A series of studies in the 1980's (reviewed in (32)) demonstrated that the TCR signal (i.e. signal 1) and the co-stimulatory signal were indeed different. Furthermore, Quill and Schwartz reported that stimulation of T cells with peptide/MHC complexes inserted into planar membranes resulted in long-term unresponsiveness to future stimuli (33), consistent with Bretscher and Cohn's proposal that signal 1 alone leads to inactivation. Concurrently, Jenkins and Schwartz reported that antigen presenting cells (APCs) that were chemically fixed in a manner that did not impair their antigen-presentation resulted in clonal deletion of antigens-specific T cells, suggesting a role for APCs as providers of co-stimulatory signals for T cells (34). Several studies, reviewed in (35), identified the important role of B7-CD28 signaling in T cell activation, with B7 being expressed on APCs and CD28 on T cells, further highlighting the role of APCs in T cell activation. It seems to me that these pivotal studies led to the establishment of co-stimulation as the molecular form of signal 2. Therefore, it became accepted that a single T cell could be activated by an APC, as long as the APC expressed sufficient co-stimulatory molecules, such as B7, which is inconsistent with Bretscher and Cohn's model. As a result, I believe that subsequent endeavors to provide an accounting of how SNS is achieved shifted from antigen-mediated lymphocyte cooperation towards determination of how the level of expression of co-stimulatory molecules is controlled.

Substantial efforts over the two decades following Bretscher and Cohn's proposal led to significant findings. B cells were identified as the antibody-producing cells (36) that originate in mammals from the bone marrow (from the liver during fetal development) (37, 38) and two functionally distinct subsets of T cells were defined (39), CD4+ T cells, the helper cells, and CD8+ T cells, the precursor of cytotoxic T cells, both of which develop in the thymus (40, 41). Studies by Metcalf and Klinman (42) and by Goodnow and colleagues (43) demonstrated that exposure of B cells to

antigen in the absence of helper T cells led to the inactivation of B cells. Similarly, Keene and Forman demonstrated that CD8+ T cell activation required help from CD4+ T cells (44); in the absence of helper T cells, CD8+ T cells were inactivated (45). The role of activated T helper cells in activating B cells and CD8+ T cells, and in preventing their inactivation by antigen, highlighted the significance of the different circumstances leading to the activation and inactivation of CD4+ T cells in SNS discrimination.

1.5 Central tolerance

In writing this section, I'm incredibly grateful to the tremendous effort of Dr. Anderson, Dr. Baldwin, and Dr. Bretscher, who co-wrote a chapter on Immunologic Tolerance for the 2021 edition of Fundamental Immunology, the original having been edited by WE Paul.

To set the stage for discussing models of peripheral SNS discrimination, I think it important to discuss central tolerance. In essence, central tolerance is a state established by the deletion, in primary lymphoid organs, of the majority of self-specific lymphocytes. Such deletion, referred to as negative selection, occurs during the development of B cells and T cells, when they exist in a paralyzable state such that their exposure to antigens expressed in the primary lymphoid organs leads to their deletion. In other words, central tolerance seems to be established by a mechanism similar to what Lederberg postulated (20). The first evidence of negative selection in T cells came from the use of a proviral superantigen isolated from mouse mammary tumor virus. This superantigen was able to crosslink TCRs expressing a particular Vβ family with a particular MHC, thereby causing the stimulation of T cells expressing these TCRs. It was found that administration of the superantigen resulted in the absence of detectable single positive T cells expressing the particular Vβ family (46). Similar observations were made using mice transgenic for a TCR that recognized a male antigen expressed on the Y chromosome (H-Y antigen) in context of MHC I. These studies reported greatly reduced numbers of single positive CD8+ T cells expressing the transgene in the thymus and the periphery of male mice (47, 48). Negative selection of MHC II restricted T cells has also been demonstrated in vitro and in vivo (49). Later studies found this negative selection to occur by apoptosis (50, 51).

Since negative selection is mediated by antigens expressed in the primary lymphoid organs, it was initially thought that thymic negative selection was of circumscribed physiologic significance, as it would be restricted to antigens expressed in the thymus. However, later studies demonstrated the presence of many more antigens in the thymus than originally anticipated, including antigens previously thought to be tissue specific and so peripheral (52-55). This "promiscuous" expression of antigens was correlated to the expression of the Autoimmune Regulator (*AIRE*) gene in the thymus (56, 57) and bolsters the state of central tolerance established by thymic negative selection. As mentioned later, expression of self-antigens under the control of AIRE can also lead to generation of thymic regulatory T cells (tTregs).

Similarly, B cell negative selection has also been well documented using mice transgenic for a B cell receptor (BCR) specific for a self-antigen (58-61). The self-specific B cell's path towards deletion was found to occur in two stages: a transient reversible stage, followed by cell death (62).

It is worthwhile noting that deletion is not the only outcome of interaction with self-antigens in primary lymphoid organs. Both B cells (63-65) and T cells (66, 67) have been shown to undergo receptor editing if the lymphocyte expressed a self-reactive receptor, although the evidence is less clear for the latter (68). Furthermore, some MHC II restricted, self-reactive T cells can differentiate into Tregs, further contributing to immunological tolerance (69). The circumstances leading to receptor editing and tTreg differentiation is beyond the scope of this section.

Despite the significant role of central tolerance, some mature self-reactive lymphocytes emigrate to the periphery, thereby creating a potential for autoimmunity. Furthermore, not all self-antigens are present in primary lymphoid organs at sufficient levels to ensure the robust deletion of lymphocytes bearing receptors specific for such antigens. Therefore, mechanisms for peripheral tolerance are needed to prevent autoimmunity.

1.6 Models of peripheral tolerance

1.6.1 The PAMP and Danger models

Charles Janeway, in his address at the 3rd Cold Spring Harbor Symposium in 1989, argued that the immune system did not evolve to discriminate between self and nonself, but rather between infectious and non-infectious entities (70). He distinguished the relatively young vertebrate immune system from its "primitive" predecessor by two properties: clonality and diversity. In essence, the vertebral immune system relies on a diverse repertoire of somatically generated antigen-specific receptors that are expressed on monospecific lymphocytes that divide to form clones upon stimulation by antigen, whereas the primitive immune system utilized germline encoded receptors. Janeway coined the term Pattern Recognition Receptors (PRRs) to refer to such germline encoded receptors that recognize conserved pathogen associated molecular patterns (PAMPs) on infectious entities, such as lipopolysaccharide (LPS), and argued that the vertebrate immune system had evolved to utilize these PRRs to control the expression of costimulatory molecules on APCs. He supported his argument with three lines of thought:

1) It was known that LPS stimulated B cells in a polyclonal manner and activated macrophages to express pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α, suggesting the presence of an LPS-receptor expressed in B cells and macrophages
2) Various germline encoded receptors were known to be involved in the activation of the innate system, such as the activation of the alternative complement pathway and natural killer (NK) cells
3) The induction of an optimal immune response required the use of adjuvants, which he famously referred to as the "immunologist's dirty little secret". These adjuvants contained microbial components that Janeway postulated to interact with the PRRs, thereby mediating the generation of a robust immune response.

Therefore, according to this PAMP model, infectious entities possess PAMPs that act on PRRs on APCs, leading to the upregulation of co-stimulatory molecules, allowing the delivery of signal 2

to T cells. Non-infectious entities, such as self-antigens, do not possess such PAMPs; therefore, APCs will present self-antigens without sufficient co-stimulation to deliver signal 2, leading to inactivation of self-reactive T cells. Janeway and colleagues provided observations in support of this PAMP model (71). They reported that stimulation of a polyclonal population of T cells with anti-CD3 in the presence of B cells pretreated with LPS or poly-I:C, or with macrophages pretreated with zymosan, caused the robust proliferation of the T cells, while stimulation of T cells with anti-CD3 alone or with anti-CD3 in the presence of untreated APCs failed to do so.

It was known that signaling through the Toll protein in Drosophila was similar to that of the IL-1 receptor (IL-1R) signaling, with the former leading to the induction of a transcription factor, Dorsal, that was homologous to the transcription factor NF-κb (72-74), important in the activation of the innate immune system and pro-inflammatory pathways (75). Furthermore, the cytoplasmic domain of Toll was homologous to that of IL-1R (76). Importantly, Toll was shown to be critical in the Drosophila's antifungal response (77). These findings hinted at the possibility of Toll being involved in the Drosophila's defense system, which lacks an adaptive immune system, by functioning as a PRR. Interested in finding such PRRs and elucidating their potential role in the vertebral immune system, Ruslan Medzhitov, at the time a post-doctoral fellow in Janeway's lab, screened a cDNA library of human splenocytes for genes homologous to the Drosophila Toll protein (dToll) and identified a gene coding for a protein they termed hToll that was highly expressed in a variety of cells of the immune system, both innate and adaptive (78). Since no ligand was known for hToll, they constructed a gene that coded for a constitutively active form of hToll and transfected it into human cells, which led to the upregulation of proinflammatory cytokines, as well as B7. These findings supported the PAMP model and proved the existence of receptors whose activation led to the upregulation of costimulatory molecules. Several studies afterwards identified a family of such Toll-like receptors (TLRs) in both murine and human cells, reviewed in (79).

Polly Matzinger, in her 1994 essay (80), made a reference to the immune system's ability to mount a response to sterile mammalian tissues that were virtually devoid of PAMPs, an observation that

is inconsistent with the PAMP model. Therefore, she put forward the Danger Model, which postulates that a stressful or a dangerous environment can be sensed by APCs, causing the upregulation of the necessary co-stimulatory molecules required for CD4+ T cell activation. The danger signals comprise a broad group of molecules, such as PAMPs, pro-inflammatory cytokines, mammalian nucleic acids, mammalian heat shock proteins, alarmins, and other molecules released during tissue necrosis (as opposed to apoptosis) (81, 82). In addition to expanding the diversity of signals contributing to the induction of co-stimulatory molecules on the surface of APCs, Matzinger recognized the potential for autoimmunity inherent to the PAMP model. In essence, an APC presenting self-antigens can become activated by PAMPs, leading to the activation of autoreactive CD4+ T cells. Therefore, she introduced a set of laws, which she terms "The Laws of Lymphotics". to minimize the risk of autoimmunity (80). Of these laws, two stood out to me; the first was that B cells should not be able to provide signal 2 to naïve T cells but do so for memory T cells; the second was that effector cells were short lived. I shall explain the significance of these two rules below.

Matzinger provided two theoretical points on why the B cells should not be able to provide co-stimulation to naïve T cells. The first pertained to Jerne's proposal that the immune system should not be tolerant of every antibody idiotype, otherwise the hole in the repertoire would be too large and so would undermine universality. Conversely, the immune system should not be able to mount a response against all idiotypes, otherwise generalized autoimmunity ensues (83). The second was due to the B cell's ability to undergo somatic hypermutation, which can alter the specificity of an activated, foreign-antigen-specific B cell such that it can interact with a self-antigen. This mutated B cell would, in Matzinger's view, concentrate the self-antigen on its surface to a level higher than the tolerance cut off for T cells. In other words, the B cell would concentrate the antigen on its surface to a level such that antigen-specific T cells that were otherwise tolerant of the lower antigen level on the surface of other APCs would now become activated.

I find Matzinger's views on B cells rather problematic. I shall first address the two theoretical points provided above and then provide more evidence supporting a role for B cells in T cell

activation in subsequent sections. Firstly, while Matzinger's proposal explained how rampant autoimmunity brought about by the immune response to antibody ideotypes could be prevented, it did not address the other side of the coin, namely tolerance, especially when she reported that B cells induced tolerance in virgin T cells (84). Secondly, Dr. Bretscher, Dr. Anderson and I recently published an article making the case that number of cognate peptide/MHC complexes on the surface of APCs required for their inactivation by antigen must not be greater than the number required for their activation (85), which contradicts Matzinger's proposal.

Matzinger, Lassila and Vainio coined the term "professional APCs" to describe APCs capable of activating naïve T cells and bestowed such a title upon dendritic cells (DCs) (86). However, Matzinger recognized the autoimmunity potential associated with DCs being antigen-nonspecific APCs. Specifically, a DC, presenting self-antigens, could take up a PAMP-laden pathogen, upregulate its expression of co-stimulatory molecules, and activate T cells specific for the self-antigens and the pathogen alike. Therefore, Matzinger put forward two mechanisms that aim to limit the likelihood and/or the damage caused by autoimmunity. Firstly, she proposed that the T cells would be tolerant to the self-antigens typically found on the surface of DCs by virtue of the ability of DCs to cause negative selection in the thymus (87). Furthermore, in the absence of "danger", peripheral tissue would induce T cell tolerance. Therefore, an immune response induced to antigens expressed on the surface of activated DCs would likely be focused on foreign antigens rather than self-antigens typically found on the surface of these DCs, thereby minimizing the risk of autoimmunity. Matzinger has briefly summarized this point in a recent discussion I had with her (18, 88). Secondly, if a self-reactive T cell were to give rise to effector cells, the Laws of Lymphotics postulate that the effector cells generated would be short lived, thereby minimizing the damage from the autoimmunity (80).

While the Danger Model is currently the most widely accepted framework for how SNS discrimination is achieved, I believe it fails to account for several observations in classical and modern literature. It might seem unfair to expect any particular framework to account for seemingly arbitrary observations; however, the fact that many of the observations I shall list in

subsequent sections were made in intact animals with minimal manipulation renders such observations, in my opinion, rather critical to our understanding of how SNS is achieved. Prior to discussing these observations, I shall first describe the salient features of the Quorum Hypothesis that I argue provides a more comprehensive framework for understanding how SNS discrimination is realized and accounts for the critical observations I shall discuss.

1.6.2 The Quorum Hypothesis

Bretscher and Cohn's two-signal model was formulated before the roles of B cells and T cells were formally elucidated and before the mechanism of how the TCR interacts with antigen was described. Therefore, Bretscher revisited the original model to emphasize the role of B cells in mediating CD4+ T cell cooperation required for the generation of signal 2 required for CD4+ T cell activation, as envisaged in the original model. Furthermore, this two-step, two-signal (2S2S) (89) model provided a possible solution to the Priming Problem encountered in the original two signal model, as I shall explain below.

As described above, CD4+ T cells play an important role in the activation of B cells and CD8+ T cells. Therefore, I shall focus on how CD4+ T cells are envisaged to be activated in context of the 2S2S model. Briefly, CD4+ T cells are activated in two steps, receiving two signals in each. In the first step, signal 1 is generated and delivered to the CD4+ T cells upon interaction of their TCRs with the cognate peptide/MHC II complexes present on the surface of DCs or macrophages. Signal 2 is generated and delivered to CD4+ T cell upon binding of co-stimulatory molecules expressed on the surface of DCs to their respective receptors on the surface of CD4+ T cells. Step 1-primed CD4+ T cells proliferate but do not generate fully activated effector CD4+ T cells. These step 1-primed CD4+ T cells then proceed to step 2, where they receive signal 1 when their TCRs interact with the cognate peptide/MHC II complex present on the surface of antigen-specific B cells. The nature of signal 2 in step 2 is more sophisticated than signal 2 in step 1 and requires CD4+ T cell cooperation, mediated by antigen-specific B cells, to generate and deliver signal 2 to step 1-primed CD4+ T cells. Furthermore, there must be a sufficient number of step 1-primed CD4+ T cells in step 2 to achieve the required level of cooperation to generate signal 2 (89-93). Failure to complete

step 2 would result in a failure to generate signal 2, and deletion of the step 1-primed CD4+ T cells ensues.

The role of B cells in mediating CD4+ T cell cooperation is an essential feature of the 2S2S model. Unlike DCs and macrophages, B cells only efficiently present antigens for which they are specific (94). Therefore, such a requirement for B cells would ensure that only CD4+ T cells specific for the antigen of interest would cooperate and would not interfere with the activation/inactivation of CD4+ T cells specific for other non-crossreacting antigens. Consider two antigens, Q and R, that do not cross-react. The state of immunity/tolerance, as well as the phenotype of the response, to Q should not interfere with those to R, even if Q and R simultaneously impinged upon the immune system. While it is well documented that some overwhelming parasitic infection can skew immune responses towards Th2 phenotype in an antigen-non-specific manner, these cases are considered pathologic rather than the general rule. To achieve such independence, the critical APC mediating CD4+ T cell cooperation must be antigen-specific. DCs will take up both R and Q, if they are simultaneously present, and will present R- and Q-derived epitopes to antigen-specific CD4+ T cells. If DCs were the critical APCs, R- and Q-specific CD4+ T cells would interfere with each other, for example, by secretion of cytokines or by activating/suppressing the DC. However, this will not be the case for B cells. Since Q and R do not cross-react, their epitopes will unlikely be presented on the same B cell. Therefore, the decision made by R-specific CD4+ T cells interacting with R-specific B cells will be independent from the decision made by Q-specific CD4+ T cells interacting with Q-specific B cells (7, 90, 95).

As for autoimmunity, the 2S2S model imposes two checkpoints to decrease the likelihood of activating autoreactive lymphocytes. Firstly, antigen-specific B cells must exist to mediate the cooperation between CD4+ T cells. B cells specific for self-antigens are eliminated in the bone marrow by negative selection and in the periphery when they interact with self-antigens without receiving help from CD4+ T cells. Therefore, the immune system maintains a low number of self-specific B cells, which decreases the likelihood of self-specific CD4+ T cells completing step 2. Secondly, a sufficient number of antigen-specific CD4+ T cells must be present in step 2 for

sufficient cooperation to occur to generate signal 2. While negative selection in the thymus removes a large number of self-specific CD4+ T cells, the immune system maintains too low a number of CD4+ T cells specific to peripheral self-antigens by a mechanism similar to that described in section 1.3, above. Furthermore, the 2S2S model provided a solution to the Priming Problem, described above, that arose when the original two signal model was formulated. Briefly, a target lymphocyte was envisaged to be activated when it received signal 2 from an activated helper lymphocyte whose activation required help from another helper lymphocyte. The Priming Problem refers to the difficulty in determining how the first helper lymphocyte was activated, given that its own activation would have required an activated helper lymphocyte. The solution proposed by Bretscher solves the Priming Problem by endowing the naïve CD4+ T cells with a basal helper activity such that if a sufficient number of such naïve helper lymphocytes cooperate by interacting with the same antigen-specific B cell, enough helper activity would be expressed to generate the signal 2 required for the activation of the CD4+ T cells (89, 96).

Additionally, the 2S2S model provided a potential solution to the "Scarcity Problem". While the immune system maintains the ability to respond to a diverse repertoire of antigens, only a few lymphocytes specific for a particular antigen exist in an immunologically naïve animal. Therefore, in context of the original Bretscher and Cohn model, it was difficult to envisage how such scarce antigen-specific lymphocytes could cooperate to initiate an antigen-specific immune response. The proliferation of CD4+ T cells in step 1 provides a partial solution to this Scarcity Problem. B cell proliferation, lymphocyte recirculation and lymph node organization complement this solution (97-100). The cooperation between scarce antigen-specific CD4+ T cells will be revisited in chapter 9 below.

Having explained the essential elements of the 2S2S mode, I think it helpful to present a minimal model of how CD4+ T cells are envisaged to cooperate. As mentioned above, naïve CD4+ T cells are believed to possess a low level of basal helper activity. Therefore, CD4+ T cell cooperation involves the integration of their basal helper activity as they interact with the same antigen-specific B cell. Such integration could occur at the level of the B cell, CD4+ T cell or both. Once the B cell

or the CD4+ T cell accumulates sufficient helper signals, its progeny become effector lymphocytes that are more efficient at activating CD4+ T cells. Therefore, the greater the number of CD4+ T cells in step 2, the more basal helper signals would be integrated, and the more likely effector lymphocytes would be generated. An important prediction of this model is that the presence of activated self-specific B cells would greatly facilitate the activation self-specific CD4+ T cells. Indeed, evidence exists that is consistent with such a prediction as I shall describe below.

In our recent communications (7), Dr. Bretscher and I opted to refer to the 2S2S model as the Quorum Hypothesis. We believe that such a parsimonious title is more relatable and facilitates the abstract imagination of how CD4+ T cell activation and cooperation may be realized. I shall, therefore, use "Quorum Hypothesis" to refer to the 2S2S model.

1.7 B cells and DCs: a comparison

The requirement for an antigen-specific B cell in CD4+ T cell activation, as proposed by the Quorum Hypothesis, has made some immunologists uneasy, as evident from my personal interactions. This is not surprising given the emphasis on the roles DCs have received over the past two decades. I argue that DCs and B cells serve non-redundant roles in immunity and therefore, their roles must be analyzed in the appropriate contexts. In this section, I compare DCs and B cells and attempt to critically analyse their roles in context of the immune system as a whole. I first list four criteria that I believe minimally define an APC:

1) Possesses the ability to internalize antigens from the extracellular compartment and express peptides derived from these antigens on the relevant MHC.
2) Constitutively expresses MHC I and MHC II.
3) Possesses the ability to provide the necessary co-stimulation to T cells.
4) Be inducible under appropriate conditions to become more potent, for example through the upregulation of MHC and/or co-stimulatory molecules).

By these criteria, B cells and DCs are very similar (101). Both express a wide range of PRRs and respond to a large variety of PAMPs (102-106). B cell activation through BCR and CD40 stimulation results in upregulation of MHC II and of co-stimulatory molecules, resulting in them becoming very potent APCs (107). Similarly, immature DCs under steady state are not potent stimulators of T cell activation and have been implicated in tolerance induction (108). In contrast, activation and maturation of DCs, such as in the presence of PAMPs, renders them very potent stimulatory APCs, due to their upregulation of MHC II and co-stimulatory molecules (104, 109).

Besides their ability to present exogenous antigens and provide co-stimulation, both DCs and B cells can modulate immune responses through secretion of an array of cytokines. Importantly, the cytokines produced varied based on exogenous stimuli. For example, Banchereau and colleagues (110) reported that, upon exposure to influenza virus, human myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) secreted coordinated waves of chemokines *in vitro* that appear to correlate with the location of the DCs. Shortly after exposure, the DCs secreted chemokines that recruit neutrophils and NK cells. This occurs when DCs are thought to be present in the tissue, at the site of exposure. After 24-48 hours, when DCs are thought to have migrated to the lymph nodes (LNs), the DCs secreted chemokines (CCL19, CCL21 and CXCL13) that attract naïve B cells and T cells. In addition, DCs can secrete a variety of pro-inflammatory cytokines, such as IL-12 (important for generation of Th1 cells), IL-23 (important for generation of Th17 cells) and TNF-α. Despite being considered poor antigen presenters, pDCs are an important source of type I interferons during viral infections (111). In addition to their pro-inflammatory role, DCs can also assume a regulatory/suppressive role. $CD103^+$ DCs in the gut convert vitamin A to retinoic acid using the enzyme aldehyde dehydrogenase. This production of retinoic acid has been shown to be important for conversion of naïve CD4+ T cells to regulatory cells (Tregs) in the gut (108, 112). Moreover, DCs can be programed to express IL-10, which in turn can suppress pathologic immune responses and can promote conversion of naïve CD4+ T cells into Tregs (108, 112).

Similarly, B cells can assume pro-inflammatory and anti-inflammatory roles. Under inflammatory conditions, B cells constitute an important source of IL-2, IL-6, TNF-a and lymphotoxin (LT)

(113, 114). Moreover, B cells can be programmed in the presence of Th1 and Th2 cells to express Th1- or Th2-promoting cytokines (Be 1 and Be 2 B cells, respectively). Once programmed, Be 1 and Be 2 B cells can promote the conversion of naïve CD4+ T cells into Th1 or Th2 phenotype, respectively (115). Conversely, a relatively new phenotype of suppressive B cells has been identified, known as regulatory B cells (Bregs). While Bregs have not yet been fully characterized, it is believed that they exert their suppressive function through production of IL-10, TGF-β or IL-35 (116, 117).

Lastly, both B cells and DCs contribute to the establishment of central tolerance at the T cell level. Thymic B cells and DCs can either develop *in situ* from precursor cells in the thymus, or they can migrate to the thymus from the periphery, where they can induce negative selection in MHC-dependent manner (108, 118).

In addition to B cells being antigen-specific APCs while DCs are not, it is well known that DCs and B cells play non-redundant roles in the generation and modulation of immune responses. In the tissues, outside of LNs, DCs act as sentinels and play an important role in immune surveillance. Indeed, some cellular properties of DCs help with this process; for example, DCs have the ability to extend protrusions across epithelial barriers and into the gut lumen, a process that appears to be enhanced when gut epithelium senses microbial stimuli (119). When they encounter an antigen under circumstances that lead to their maturation (ex: in the presence of danger signals), DCs cease their phagocytic activity, enhance their surface expression of MHC II and co-stimulatory molecules, and migrate to the T cell zone in the paracortex of the draining LN (101, 119). Trafficking of cells inside the LN is very sophisticated, organized into an elaborate network of conduits laid down by follicular dendritic cells (FDCs) in the cortex and fibroblastic reticular cells (FRCs) in the paracortex. When T cells and B cells arrive to the LN via the high endothelial venules (HEVs), they navigate the cortex by moving along the conduits of the FRCs (B cells then switch from FRC conduits to FDC conduits as they upregulate their expression of CXCR5). Interestingly, these FRC conduits are heavily populated with DCs, and when DCs migrate to the LN from the tissue, they stay in these conduits (119). Therefore, it appears that LN organization places the DCs

in intimate contact with T cells; it is perhaps not surprising that a single DC was reported to contact approximately 5000 CD4+ T cells per hour (120). This suggests that DCs are well suited to be the initiating APCs for T cell activation. In addition, DCs in the paracortex can also present intact antigen to newly-arriving B cells and, to a lesser extent, can migrate to the B cell follicles and participate in B cell activation (119, 121).

B cells, on the other hand, play a different role. B cells reside mostly in the follicles, in the cortex of LNs, where they can receive antigens arriving through the lymph either directly or from other APCs. Using fluorescence microscopy and flow cytometry, Jenkins and colleagues reported that antigen-specific B cells in the draining LN were able to acquire antigen within 10 minutes, present antigen-derived peptides on MHC II within 4 hours, and proliferate within 24 hours following intradermal injection of the antigen (122). Antigen-specific B cells were also very efficient at taking up antigen in the blood before arriving to the draining LN. In addition to their efficiency in acquisition, processing and presentation of antigen, B cells play an important role in promoting the formation of follicles and FDCs in secondary lymphoid organs (123, 124). Importantly, during chronic immune responses, B cells were reported to be critical for the formation of tertiary lymphoid organs, perhaps due to the B cells' ability to produce lymphotoxin (LTα1β2) (123, 124). In addition, B cells can modulate immune responses through their ability to produce antigen-specific antibodies. The broadly-reactive 'natural' antibodies produced by B-1a B cells play an important role in opsonization of pathogens (125); the different classes of antibodies produced by B-2 B cells have different effects, such as opsonization, complement activation, and activation of mast cells and NK cells. Moreover, some antibodies have anti-inflammatory properties, specifically IgA and IgG4 antibodies.

An important question that remains to be answered is whether B cells are the critical APCs required to activate CD4+ T cells. Much controversy exists in the literature. Some systems demonstrate adequate, albeit slightly reduced, priming of CD4+ T cells in B cell-deficient mice, while others demonstrate an unequivocal requirement for B cells (107). I would like to raise three issues that I believe to be worthy of consideration when analysing these systems:

1) Mice with a genetic deficiency in B cells are partially lymphopenic. Lymphopenia might lower the threshold for CD4+ T cell activation and can contribute to autoimmunity (126, 127). Alternatively, as we have recently argued (85), lymphopenic expansion of CD4+ T cells might facilitate the sufficient cooperation, leading to activation of autoreactive CD4+ T cells.
2) Genetically B cell deficient mice have abnormal or absent development of follicles in LN. This might result in CD4+ T cells spending more time proliferating in the T cell zone and engage in longer encounters with DCs than wild type mice.
3) High doses of adjuvants are usually required. PAMPs in these adjuvants might stimulate DCs to levels that cannot be attained under natural circumstances, such as during infections, thereby creating the impression that DCs are sufficient to activate CD4+ T cells.

Indeed, animal models of autoimmunity employing mice with "semi-intact" B cell compartment, such as mice with B cell-specific MHC II deficiency (128), with B cells of very restricted repertoire (129), or even wild type mice (130) tell a different story. In these systems, B cells were required for clinical disease or the generation of autoreactive CD4+ T cells, even if adjuvants were used. This requirement was independent of the B cells' ability to secrete antibodies. One line of evidence supporting a critical role for B cells in activation of autoreactive T cells was provided by Janeway and colleagues (130) and I believe is worthy of highlighting. They reported that immunization of C3H mice with murine cytochrome C (MCC) in complete Freund's adjuvant (CFA) failed to generate MCC-specific B cell or T cell responses. This finding leads to two possible conclusions: A) the state of tolerance to MCC is very robust in C3H mice such that no MCC-specific lymphocytes exist, or B) DCs, despite being activated by the PAMPs in CFA, are not sufficient to activated MCC-specific lymphocytes. However, the similar immunization of naïve C3H mice with human cytochrome C (HCC), which crossreacts with MCC, generates antibodies that react with MCC and HCC, suggesting the presence of MCC-specific B cells in naïve C3H mice that could not be activated by the immunization with MCC in CFA. Janeway and colleagues then transferred B cells from HCC-immunized C3H mice to naïve C3H mice and challenged the recipients with MCC in CFA. Interestingly, the recipients generated MCC-specific T cell responses upon

immunization of MCC. Therefore, it appears that activated MCC-specific B cells were essential for the activation of MCC-specific T cells, consistent with the prediction of the Quorum Hypothesis stated in section 1.6.2, above. It is therefore not surprising that B cell depletion has been shown to be effective in treatment of some autoimmune diseases, even those where autoantibodies are not responsible for the immunopathogenesis (131, 132).

A stark difference between DCs and B cells is seen in CD8+ T cell activation. Evidence that B cells cross-present exogenous antigens not capable of replicating in the B cell is limited (133-136). However, clear of evidence exists that DCs are capable of such cross-presentation, as demonstrated by Francis Carbone and Michael Bevan (137) [initially demonstrated by Bevan in 1976 (138)]. Cross presentation is largely attributed to a special subset of CD8+ DCs in mice (139), and a similar subset has been found in humans, but they lack CD8 expression (140). The detrimental effect of B cell deficiency on CD8+ T cell responses in some systems might be due to compromised CD4+ T cell priming. Indeed, reconstitution of B cell deficiency with MHC I-deficient B cells appeared to ameliorate the deficiency in CD8+ T cell responses (141). Therefore, DCs appeared to be sufficient for the generation of effector and memory CD8+ T cells (142). However, there is some agreement that B cells are required for the generation of memory CD4+ T cells (143, 144).

In conclusion, DCs and B cells are both essential APCs that synergize to support the generation of robust immune responses. DCs, by virtue of their ability to survey their environment and their intimate contact with T cells, are well poised to initiate T cell activation. Antigen-specific B cells, due to their low frequency, are not the initiators of T cell activation. They are initially separated from T cells but are very responsive to antigen, as discussed above. After antigen encounter, they divide to overcome their low frequency and migrate towards the T cell zone. CD4+ T cells, being primed by DCs, upregulate their expression of CD40L and migrate towards the B cell zone and interact with antigen-specific B cells, where both B cells and CD4+ T cells can finish their activation. In my view, it is not unreasonable to entertain the possibility that CD4+ T cells can be partially activated upon their stimulation by DCs; however, I believe that such stimulation should

not result in activation that is of similar quality to that achieved after interaction with an antigen-specific B cell.

1.8 Observations paradoxical in context of the Danger Model but accounted for by the Quorum Hypothesis

As mentioned above, I favour the Quorum Hypothesis as a framework for understanding how the immune system distinguishes between self and foreign antigens. This position is largely driven by the ability of the Quorum Hypothesis to account for diverse observations that seem paradoxical in context of the Danger Model. We have recently published our account of these observations in context of the Quorum Hypothesis (7). I discuss below some of these pertinent observations.

It was known by the 1960's that the administration of large doses of some foreign proteins to animals as neonates rendered these animals unresponsive, at the antibody level, when challenged with an immunogenic dose as adults (145-147), most likely by depletion of antigen-specific lymphocytes. There is no agreement on the mechanism behind such deletion; however, I speculate that the systemic administration of large doses of a foreign antigen causes the dissemination of antigen-specific lymphocytes across many lymph nodes, thereby impeding their ability to cooperate. This would be further facilitated by the presence of a relatively few lymphocytes during the neonatal stage. William Weigle took advantage of this tolerance protocol and conducted further experiments that contributed to our understanding of how peripheral tolerance might be achieved (27, 148). Firstly, he demonstrated that rabbits given large doses of the protein bovine serum albumin (BSA) shortly after birth became unresponsive, at the antibody level, to an immunogenic dose of BSA when given during adulthood, at three months of age. Human serum albumin (HSA) crossreacts with BSA at the antibody level such that 15% of the anti-BSA antibodies raised in rabbits react with HSA. Weigle then demonstrated that the state of tolerance established in rabbits to BSA could be broken by the repeated administration of HSA, as assessed by the ability of these rabbits to produce antibodies that bind to both HSA and BSA (27). I believe these findings to be difficult to explain in context of the Danger Model, but are readily explicable in context of the Quorum Hypothesis. BSA-specific lymphocytes exist in BSA-tolerant rabbit that

could not be activated by immunization with BSA, but could be activated by immunization with HSA. I hypothesize that BSA-tolerant rabbits contain too few BSA-specific lymphocytes to sufficiently cooperate and mount a BSA-specific antibody response upon immunization with BSA. However, these BSA-tolerant rabbits contain a sufficient number of HSA-specific lymphocytes that cooperate and become activated, including the lymphocytes specific for the crossreacting epitopes, thereby resulting in the production of antibodies specific for BSA and HSA. Interestingly, upon receiving multiple doses of HSA, the BSA-tolerant rabbits eventually regained the ability to respond, at the antibody level, to BSA (148), a finding that can also be explained in context of the Quorum Hypothesis whereby lymphocyte cooperation results in the activation of lymphocytes specific for new epitopes that are linked to the epitopes against which there are ongoing responses, a phenomenon known as epitope spreading in context of autoimmunity. I believe the above analysis sheds light on the mechanism by which pathogens that crossreact with self-antigens induce autoimmunity.

Weigle then repeated the above experiment to test the ability of other serum albumins, namely pig and sheep serum albumins (PSA and SSA, respectively), to break the unresponsive state in BSA-tolerant rabbits. PSA is 32% crossreactive with BSA and SSA is 75% crossreactive. Weigle reported that while 85% of BSA-tolerant rabbits lost the unresponsiveness to BSA upon immunization with HSA, only 40% of them lost the unresponsiveness upon immunization with PSA and none of them lost tolerance upon immunization with SSA. In other words, the greater the crossreaction between BSA and the serum albumin used to attempt to break the unresponsive state, the lower the ability of the serum albumin to do so. This makes sense in context of the Quorum Hypothesis. The greater the degree of crossreaction between BSA and the serum albumin, the more lymphocytes specific for the serum albumin would be deleted when BSA tolerance is induced during the neonatal stage. The remaining lymphocytes would be too few for sufficient cooperation to take place. This would not be the case if the degree of crossreaction was low. HSA crossreacts the least with BSA, and therefore has the highest likelihood of breaking the unresponsive state to BSA.

Another line of evidence supporting the Quorum Hypothesis was provided by Anderson and colleagues (149). They transplanted recombination activating gene knockout (RAG-KO) female mice, which lack B cells and T cells, with foreign grafts, such as heart and islet tissues, that were grafted under the kidney capsule. These grafts were allowed to heal before the mice were then reconstituted with bone marrow from RAG-sufficient donors. Grafts bearing multiple different minor antigens were rejected post-reconstitution, while those bearing a single minor antigen were not, and induced unresponsiveness to the antigen instead. These findings could be readily explained in context of the Quorum Hypothesis: the more foreign a graft is, the more lymphocytes there will be specific for it. Therefore, lymphocyte cooperation would be more readily facilitated in the presence of more foreign grafts than less foreign ones.

To provide more direct evidence that CD4+ T cell activation requires CD4+ T cell cooperation, Bretscher and colleagues utilized the immune response to various peptides derived from the protein hen egg lysozyme (HEL). Firstly, they analysed the repertoire of HEL-specific cytokine-producing CD4+ T cells generated upon immunizing BALB/c mice with HEL (150) and determined that the repertoire was dominated by CD4+ T cells specific for the peptide $HEL_{105-120}$. This peptide was therefore termed the "major peptide", and the remainder peptides were termed "minor peptides". They then ablated, in naïve BALB/c mice, CD4+ T cells specific for the major peptide by a protocol, developed by Jenkins and colleagues, that is known to cause the antigen-specific deletion of CD4+ T cells (151, 152). The mice pretreated with such a protocol were then challenged with HEL and were found to have a dramatically reduced number of cytokine-producing CD4+ T cells specific for the minor peptides (153). Therefore, it appears that CD4+ T cells specific for the major peptide were required for the generation of cytokine-producing CD4+ T cells specific for the minor peptides, consistent with the Quorum Hypothesis. Furthermore, challenging the pretreated mice with HEL coupled to ovalbumin (OVA) instead of HEL alone generated cytokine-producing CD4+ T cells specific for the minor peptides. Therefore, it appears that OVA-specific CD4+ T cells cooperated sufficiently with CD4+ T cells specific for the minor peptides, thereby supporting their activation. A feature of this last experiment worth underscoring is the need for HEL to be coupled to OVA, which would ensure their presentation by the same antigen-specific B cells, thereby suggesting that B cells may play a critical role in mediating the cooperation between CD4+ T cells

specific for HEL and those specific for OVA. To further analyse the role of B cells in mediating CD4+ T cell cooperation, Bretscher and colleagues employed peptide-pulsed APCs (154). Splenocytes were either decorated with $HEL_{105-120}$, $OVA_{323-339}$, or both and were then used to immunize naïve BALB/c mice and cytokine-producing CD4+ T cells specific for $HEL_{105-120}$ were enumerated nine days post-injection. More $HEL_{105-120}$-specific cytokine-producing CD4+ T cells were generated if the mice were immunized with double-coated splenocytes than those immunized with splenocytes coated with only $HEL_{105-120}$ or those immunized with two populations of splenocytes, one coated with $HEL_{105-120}$ and the other coated with $OVA_{323-339}$. Therefore, it appears that CD4+ T cells specific for $OVA_{323-339}$ cooperated with those specific for $HEL_{105-120}$, resulting in the enhanced generation of cytokine-producing cells. Furthermore, this cooperation occurred by linked recognition, as the two peptides had to have been on the same splenocyte for the cooperation to take place. Interestingly, using purified B cells or DCs as APCs, Bretscher and colleagues reported that only B cells supported such a cooperation, as no enhancement in the generation of cytokine-producing cells was observed when DCs were used as APCs (154).

The findings described above are difficult to explain in context of the Danger Model. The different treatment groups were otherwise identical and, therefore, one could assume that the treatments occurred under similarly dangerous circumstances. This serves as a prelude for the next section, where I discuss whether the Danger Model and the Quorum Hypothesis could be incorporated into a broad view of how immune responses are regulated. However, I think it worthwhile discussing the phenomenon of epitope spreading, observed in some autoimmune diseases, prior to transitioning to the next section. When an autoimmune disease is first initiated, it is usually against one or a few epitopes of an antigen. As the disease progresses, the repertoire of specificity increases to include more epitopes of that antigen, and later, epitopes on other antigens. This phenomenon was demonstrated using non-obese diabetic (NOD) mice (155), an inbred murine strain that spontaneously develops insulin dependent autoimmune diabetes. At the initial stage of the disease, the CD4+ T cell repertoire was restricted to a certain region of glutamic acid decarboxylase (GAD). As the disease progressed, the repertoire of CD4+ T cell specificities ‘spread’ to include other regions of GAD and later to include other antigens in the beta islets of the pancreas. Interestingly, making NOD mice tolerant to the initial epitope of GAD prevents the generation of autoreactive

CD4+ T cells specific for other beta islet antigens, and prevents the development of autoimmune diabetes in NOD mice. This sequential expansion of the repertoire of CD4+ T cell specificities seems likely to occur due to lymphocyte cooperation, leading to activation of lymphocytes specific for new epitopes. Furthermore, the observation that such expansion is more readily involves linked epitopes rather than epitopes on unrelated antigens supports a role for B cells in mediating CD4+ T cell cooperation.

1.9 Are the Danger Model and the Quorum Hypothesis mutually exclusive?

I explained above that I favour the Quorum Hypothesis as a framework for understanding how the immune system discriminates between self and nonself due to its ability to account for diverse observations in literature, some of which were outlined above, that seem paradoxical in context of the Danger model. I would like to further elaborate on my comment. According to the Danger Model, the critical factor determining whether a lymphocyte becomes activated or inactivated is the presence or absence of danger signals, respectively. However, as I discussed above, this does not seem to be the case. Nevertheless, plenty of evidence exists supporting the importance of danger signals in initiating immune responses. How can this be reconciled? I do not believe the Quorum Hypothesis and the Danger Model to be mutually exclusive. However, I do believe the Quorum Hypothesis to act upstream to the Danger Model. In other words, I believe that danger signals are not critical for the initiation of immune responses, but contribute to the quorum threshold needed for lymphocyte activation. Therefore, fewer lymphocytes might be sufficient for antigen to induce an immune response in the presence of danger than in its absence. This could be achieved through the upregulation on APCs of costimulatory molecules, such as B7, leading to increased proliferation of CD4+ T cells, thereby increasing the intensity of the cooperation. Similarly, blockade of CTLA-4 and PD-1 action can lead to increased CD4+ T cell proliferation, facilitating the generation of immune responses (156). Lastly, lymphopenia is known to cause autoimmunity under some circumstances. We recently published an article discussing how the Quorum Hypothesis might account for this phenomenon (85), as lymphopenia induces lymphocyte proliferation, which again facilitates lymphocyte cooperation leading to the activation of autoreactive lymphocytes and autoimmunity.

CHAPTER 2 - RATIONALE AND OBJECTIVES

When I sought to examine whether the Quorum Hypothesis provides a better framework for understanding the circumstances leading to CD4+ T cell activation/inactivation, I thought it best to first breakdown the Quorum Hypothesis into its salient features to facilitate such an examination. Therefore, my hypotheses were:

1) CD4+ T cell activation by antigen requires a minimum number of antigen-specific CD4+ T cells
2) Quorum sensing is mediated by antigen-specific B cells
3) Antigen-specific B cells are required for the generation of cytokine-producing CD4+ T cells
4) In the absence of sufficient cooperation, single naïve CD4+ T cells are inactivated by antigen

While we have previously published several observations in support of the Quorum Hypothesis, I hope that my experimental approach will provide a more quantitative analysis using an *in vitro* system developed in our lab.

It is worthwhile noting that the examination of the four hypotheses listed above is largely dependent on the development of an experimental system to test the first, as well as the demonstration that the activation of CD4+ T cells requires a minimum number of antigen-specific CD4+ T cells. Naturally, a large body of this thesis will be dedicated to that end.

CHAPTER 3 - MATERIALS AND METHODS

3.1 Mice

Female recombination activating gene (RAG)-sufficient DO11.10 mice (purchased from Jackson Laboratories) and female RAG-deficient, CD45.1+ Marilyn mice (generously provided by Dr. Colin Anderson, UofA, Canada) were bred at the Lab Animal Service Unit at the University of Saskatchewan and were used as sources of CD4+ T cells. DO11.10 mice are transgenic for a T cell receptor (TCR) that recognizes the peptide $OVA_{323-339}$ in context of the $I\text{-}A^d$ restriction element, while Marilyn mice are transgenic for a TCR that recognize the male H-Y peptide (also known as $DBY_{608-622}$) in context of $I\text{-}A^b$. RAG-sufficient OT-II mice, purchased from Jackson Laboratories, were used as a source of CD4+ T cells expressing a TCR that recognizes $OVA_{323-339}$ in context of the $I\text{-}A^b$ restriction element. Wild-type female BALB/c and C57Bl/6 mice were used as sources of antigen presenting cells (APCs). All mice used were 4 – 12 weeks of age, unless otherwise stated.

3.2 Media and solutions

3.2.1 Carbonate/bicarbonate buffer

Carbonate/bicarbonate buffer (pH 9.6), used to for coating ELISPOT plates with antibodies, was prepared by mixing 16mL 0.2M Na_2CO_3 with 34 mL 0.2M $NaHCO_3$ and 150mL ddH_2O.

3.2.2 Phosphate buffered saline (PBS)

PBS was prepared as a 10X stock in 20L batches containing 80g/L NaCl, 4g/L KCl, 11.5g/L Na_2PO_4, and 4g/L KH_2PO_4 in ddH2O. Working 1X PBS solutions were prepared by diluting the stock 1/10 in ddH_2O. Sterile 1X PBS solutions were obtained by autoclaving the 1X PBS solutions.

3.2.3 PBS-Tween 20 (PBST)

PBST was used as a washing buffer for ELISPOT plates and was prepared in 20L batches by mixing 20L PBS with 20mL Tween 20 (0.1% v/v).

3.2.4 Substrate buffer

Substrate buffer was used for the development of ELISPOT plate and served as a buffer solution for alkaline phosphatase to catalyse the breakdown of NBT (nitro-blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt) to form an insoluble black precipitate. The substrate buffer consisted of 0.1M Tris-HCl, 0.1M NaCl and 0.05M $MgCl_2$.

3.2.5 MACS buffer

Magnetic-activated cell sorting (MACS) buffer was used to cell isolation and for flowcytometry. MACS buffer consisted of PBS supplemented with 2mM EDTA and 2% fetal bovine serum (FBS; Hyclone)

3.2.6 RPMI and RPMI complete media

RPMI 1640 media supplemented with L-glutamine and Leibovitz (Gibco, Grand Island, NY, USA) was prepared from powdered stocks according to the manufacturer's instructions. Sterility was achieved by filtration through a 0.22μM filter. The filtered media was then incubated overnight at 37^{o}C to assess their sterility and then stored at 4^{o}C.

RPMI complete media, used for tissue cultures and ELISPOT, consisted of RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin-streptomycin (Gibco), 0.8% sterile sodium pyruvate and 50 μM β-mercaptoethanol.

3.3 Peptide antigens

OVA323-339 (ISQAVHAAHAEINAAGR) and H-Y (NAGFNSNRANSSRSS) were purchased from GenScript at >90% purity. Both were stored in powdered form at -20^{o}C and reconstituted in ddH_2O when needed, at which point they were stored at 4 ^{o}C for short term storage.

3.4 Cell isolation

3.4.1 Preparation of single-cell suspensions

Spleens were removed aseptically from mice euthanized by cervical dislocation into 10mL of cold PBS. The spleens were then cut into a paste using sterile scissors and gently ground on a sterile steel mesh using a sterile glass rod. The resulting suspension was then "de-clumped" by transferring to a 15 mL centrifuge tube and allowing large aggregates to settle for approximately two minutes, after which 9 mL were then transferred to a new centrifuge tube. The de-clumped suspension was then centrifugated for 10 minutes at 300 x g. The pellet was then resuspended in MACS buffer in preparation for isolation or into RPMI complete for use in the ELLISPOT assay. Viable cells were counted using a hemocytometer by virtue of their ability to exclude trypan blue (Sigma).

For isolation of DCs, the spleen was initially removed from PBS, cut into a paste, and transferred to a spleen dissociation medium (STEMCELL Technologies).

3.4.2 CD4+ T cells

CD4+ T cells were isolated from a single-cell splenocyte suspension using EasySep™ Mouse CD4+ T Cell Isolation Kit from STEMCELL Technologies (#19852), according to manufacturer's instructions. This kit isolates CD4+ T cells by negative selection.

3.4.3 Naïve CD4+ T cells

Naïve CD4+ T cells were isolated from a single-cell splenocyte suspension using EasySep™ Mouse Naive CD4+ T Cell Isolation Kit from STEMCELL Technologies (#19765), according to manufacturer's instructions. This kit isolates CD25- CD44- CD4+ T cells by negative selection.

3.4.4 B cells

B cells were isolated from a single-cell splenocyte suspension using EasySep™ Mouse B Cell Isolation Kit from STEMCELL Technologies (#19854), according to manufacturer's instructions. This kit isolates CD43- B cells (i.e., resting B2 B cells) by negative selection.

3.4.5 Dendritic cells

Dendritic cells (DCs) were isolated from a single-cell splenocyte suspension using EasySep™ Mouse CD11c Positive Selection Kit II from STEMCELL Technologies (#18780), according to manufacturer's instructions.

3.4.6 T cell depletion

T cells where depleted from a single-cell splenocyte suspension using EasySep™ Mouse CD90.2 Positive Selection Kit II from STEMCELL Technologies (#18951), according to manufacturer's instructions. This kit depletes Thy1.2+ cells by positive selection.

3.5 In vitro cultures

Purified CD4+ T cells were isolated from splenocytes of DO11.10 or Marilyn mice, as described above, and cultured at 37°C and 5% CO_2 in RPMI complete media for five days in tissue culture treated 96-well V-bottom plates (Corning, #3894) with $3x10^4$ APCs. Peptide was used as antigen (added at 0.3 μM for $OVA_{323-339}$ or 1 μM for H-Y) with or without addition of 1 μg/mL LPS (*Escherichia coli* serotype 0111:B4; Sigma). The number of antigen-dependent cytokine-producing cells was determined on day 5 by a modified ELISPOT assay (157).

3.6 ELISPOT

Antigen-specific cytokine-producing cells were enumerated using a modified ELISPOT assay (157). Non-sterile ELISPOT plates (96-well plates with 0.45 μm surfactant free mixed cellulose ester membranes at the bottom, purchased from Merck Millipore) were coated by incubation at

4°C with 1 µg/mL purified anti-IL-2 (JES6-1A12, BD Biosciences), 2 µg/mL purified anti- IFN-γ (R4-6A2, BD Biosciences), or 1 µg/mL purified anti-IL4 (11B11, BD Biosciences) in 1M bicarbonate buffer (pH 9.6). The following day, the coated plates were washed twice with 200 µL/well of PBS and then blocked by incubation with 200 µL/well of RPMI complete at 37°C and 5% CO_2 for at least one hour.

Cultures were harvested and washed with 5 mL of PBS and centrifugated at 300 x g for 10 minutes. The pellets were resuspended in RPMI complete and transferred to the blocked plates with 10^6 splenocytes, as APC, isolated from naïve BALB/c or C57/BL mice in a total volume of 200 µL. To exclude antigen-independent spots, some wells were supplemented with peptides at the concentrations mentioned above while others were not. The plates were then incubated at 37°C with 5% CO_2 for 8 hours and afterwards washed twice with PBS, twice with ddH_2O, four times with PBST, then once with PBS. After the plates were washed, they were incubated overnight at 4°C with 1 µg/mL biotinylated anti-IL-2 (JES6-5H4, BD Biosciences), biotinylated anti- IFN-γ (XMG1.2, BD Biosciences), or biotinylated anti-IL4 (BVD6-24G2, BD Biosciences).

The next day, the plates were washed seven times with PBST to remove the secondary antibodies, followed by one wash with PBS. The wells were incubated with 20 ng of streptavidin conjugated alkaline phosphatase (SAP, Cedarlane) for ninety minutes, at room temperature. The plates were then washed seven times with PBST, to remove unbound SAP, followed by one wash with PBS, and developed in the dark for at least five minutes by the addition of 100 µL of NBT/BCIP (Roche) diluted 1:50 in substrate buffer. The reaction was stopped by rinsing eight times with distilled water. The number of spots per well was determined by manual counting using a dissection microscope.

3.7 Cell proliferation analysis by flow cytometry

Purified CD4+ T cells were stained with CellTrace® Violet (CTV; ThermoFisher Scientific) as recommended by the manufacturer. Stained cells were then resuspended in RPMI complete and cultured as described above. On day 4 post-culture, the cultures were washed once by centrifugating the 96-well plate for 10 minutes at 300 x g and resuspending the pellet in MACS buffer. The cells were then stained with PE-anti-CD3 (17A2, BioLegend), APC/Fire-anti-CD4+

(RM4-5, BioLegend) and the viability dye Helix NP NIR (BioLegend) for 10-15 minutes at 4°C, washed three times with PBS and resuspended in MACS buffer. The gating strategy followed was to first gate on lymphocytes, then excluding dead cells that stain positive with Helix NP NIR, then gaiting CD3+ and CD4+ cells, followed by a plot of the intensity of CTV on CD3+ CD4+ cells. All antibodies and dyes were titrated to obtain the optimal concentration and gates were established using the fluorescence minus one technique (FMO) when appropriate.

3.8 Preparation of peptide-pulsed APCs

3.8.1 Single-pulsed APCs

T cell-depleted splenocytes were isolated as described above and resuspended in RPMI complete media at 10^7/mL. The cell suspension was then incubated at 37°C with 5% CO_2 with either 10 μM H-Y for 3 hours or with 60 μM $OVA_{323-339}$ for 24 hours. The cells were then washed 3x with PBS and resuspended in RPMI complete at the desired concentration prior to transfer to cultures.

3.8.2 Double-coated APCs

T cell-depleted splenocytes were isolated as described above. These cells were not pulsed. Instead, $3x10^4$ of the T cell-depleted splenocytes were transferred to cultures, along with 1 μM H-Y and 0.9 μM $OVA_{323-339}$, unless otherwise specified. These APCs were not washed after addition of peptides.

3.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism 9, version 9.2.0 for Mac OS, licenced for research use for Ghassan Al-Yassin.

CHAPTER 4 - DEVELOPING AN IN VITRO SYSTEM FOR TESTING THE QUORUM HYPOTHESIS USING DO11.10 CD4+ T CELLS

4.1 The original in vitro system

We have previously established an in vitro system to examine the circumstances affecting the Th1/Th2 differentiation of CD4+ T cells. We employed TCR-transgenic CD4+ T cells in our studies to examine the effect of the number of cultured antigen-specific CD4+ T cells on the Th1/Th2 phenotype of the effector T cells generated. Briefly, we cultured between 1000 to $3x10^4$ DO11.10 CD4+ T cells, specific for the peptide $OVA_{323-339}$ in context of $I\text{-}A^d$, in 96-well V-bottom plates with $3x10^4$ T cell-depleted (T-depleted) splenocytes as APCs, 0.3 μM $OVA_{323-339}$ and 1 μg/mL LPS. On day 4 post-culture, peptide-specific cytokine-producing cells were enumerated by the ELISPOT assay. Our in vitro system is robust in that it resulted in the generation of many more cytokine-producing cells per input CD4+ T cells compared to other in vitro systems that involve the of culture large numbers of CD4+ T cells per well (158). Importantly, we were able to show that CD4+ T cells cultured at low densities (i.e., 1000 input CD4+ T cells/per well) primarily gave rise to Th1 cells, while those cultured at the higher densities predominantly gave rise to Th2 cells.

4.2 Modifying the original in vitro system to test the Quorum Hypothesis

We wished to use our in vitro system to determine whether antigen activates/inactivates antigen-specific CD4+ T cells by a quorum-dependent mechanism. To that end, we further lowered the number of input CD4+ T cells and titrated them from 6 to 100, hoping that CD4+ T cells cultured at very low densities would fail to generate cytokine-producing cells, as anticipated by the Quorum Hypothesis. Furthermore, to improve our ability to detect cytokine-producing cells from low density cultures, we extended the culture duration to a minimum of 5 days. However, as seen in figure 4-1 A and D, all low-density cultures generated a robust Th1 response. We wondered if these CD4+ T cells were producing cytokines only transiently and so decided to enumerate cytokine-producing cells on days 6 and 7 post-culture. While the number of cytokine-producing cells declined slightly in some cultures, the size of the response remained high. Two features of these observations are noteworthy. We plotted the number of cytokine-producing cells per culture

as well as the number per input transgenic T cell. If there is cooperation between the T cells in the generation of the cytokine-producing cells, one would expect that, when cooperation is limiting the generation of cytokine-producing T cells, the value of the number of cytokine-producing cells/input T cell would increase with increasing number of transgenic cells per well. Such a dependence was not evident in the observations recorded in figure 4-1. Secondly, the number of cytokine-producing cells per input transgenic cell is large, of the order of a hundred. This represents much more efficient generation of cytokine-producing cells than when transgenic cells were cultured under similar conditions but at a much higher number per well. We hypothesised that, should a quorum of CD4+ T cells be required for activation, whereas single CD4+ T cells can be inactivated by antigen, the number of CD4+ T cells in our cultures was well above the quorum threshold. The transgenic mice used in these experiments were RAG sufficient and about 8 weeks old. It is known that some of the CD4+ T cells of such mice are partially activated. We hypothesized that the quorum threshold would be higher if the CD4+ T cells were all in a naïve state. Therefore, we opted to use CD25- CD44- CD4+ DO11.10 T cells, purified as described above, and explored whether they would fail to generate cytokine-producing cells when cultured at low densities. Furthermore, since our cultures consistently generated a robust Th1 response but only very low numbers of IL-4-producing cells, we decided to discontinue the enumeration of IL-4-producing cells. Previous in vivo studies had led our lab to propose that a smaller quorum of CD4+ T cells is required to generate IL-2-producing than IFN-γ-producing effector cells (153). We therefore also enumerated IL-2-producing cells. As seen in figure 4-2, CD25- CD44- CD4+ T cells generated a robust Th1 response at all culture densities, prompting us to further decrease the number of input CD25- CD44- DO11.10 CD4+ T cells to an average of 1 and 3 cells/well. It is worthwhile noting that the quality of purification of CD25- CD44- CD4+ T cells was not formally assessed by flow cytometry. Therefore, it remains possible that the lack of apparent CD4+ T cell cooperation was due to the presence of activated or partially activated CD4+ T cells that were not removed by MACS. This possibility was not addressed experimentally and should be kept in mind when interpreting further observations. Typically, we set up 3 cultures for each CD4+ T cell density in our experiments. However, the coefficients of variance of the number of input cells and of the size of the response increase when the average number of input CD4+ T cells per culture decreases.

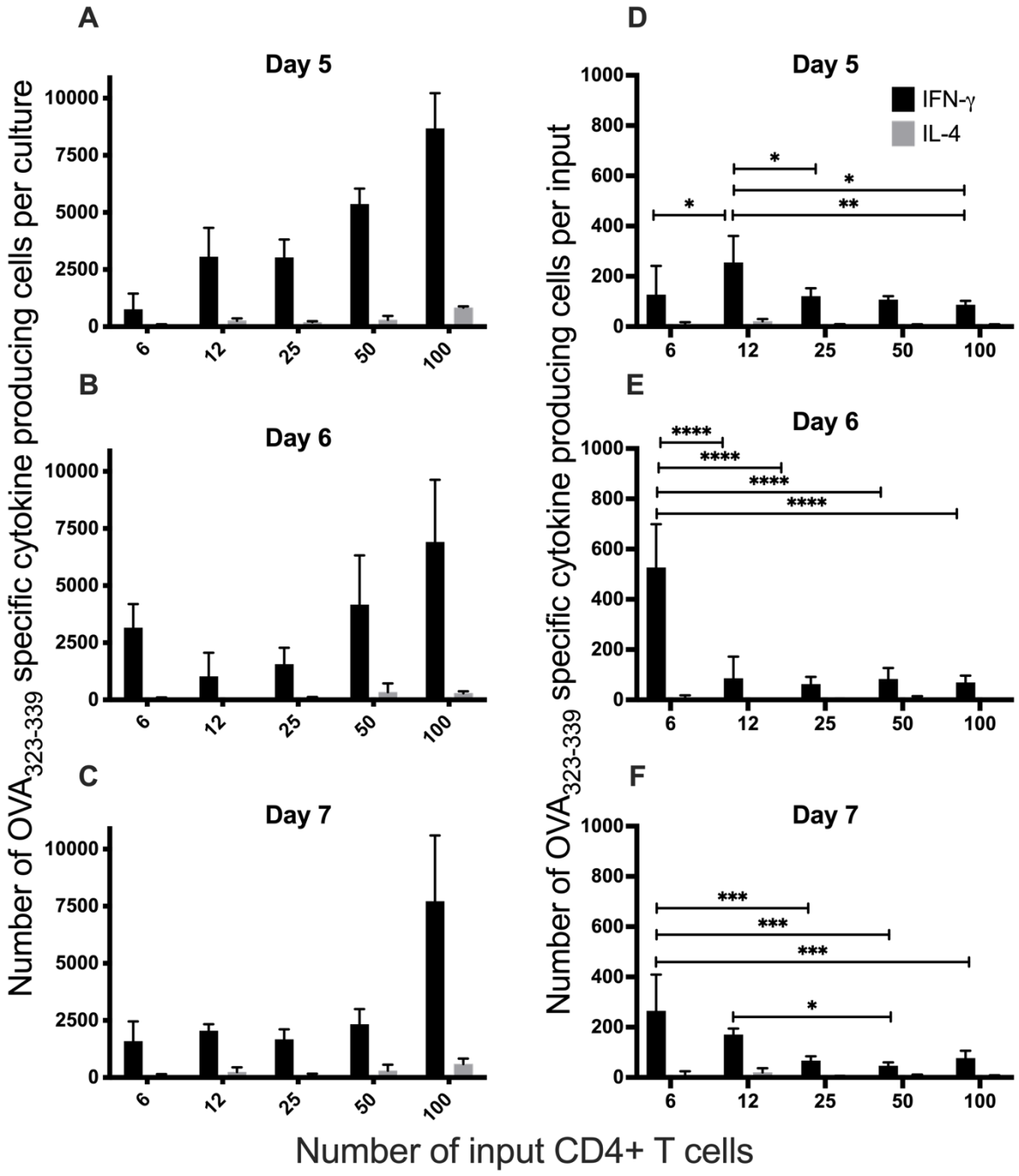
A
Day 5
B
Day 6
C
Day 7
D
Day 5
IFN-γ
IL-4
E
Day 6
F
Day 7
Number of OVA323-339 specific cytokine producing cells per culture
Number of OVA323-339 specific cytokine producing cells per input
Number of input CD4+ T cells
6
12
25
50
100

Figure 4-1: Establishing low-density cultures of RAG-sufficient DO11.10 CD4+ T cells with added LPS using T cell depleted splenocytes as APCs. The indicated number of CD4+ T cells, isolated from adult RAG-sufficient DO11.10 TCR transgenic mice, were cultured for the indicated number of days with $3x10^4$ T cell depleted splenocytes as APCs, 0.3 μM $OVA_{323-339}$ peptide and 1 μg/mL LPS. The IFN-γ-producing cells and IL-4-producing cells were enumerated on days 5, 6 and 7 post-culture via the ELISPOT assay. Panels A, B and C display the number of cytokine-producing cells per culture, while panels D, E and F display the number of cytokine-producing cells per input CD4+ T cell. This figure is representative of one experiment. However, culturing the same number of RAG-sufficient DO11.10 CD4+ T cells for 5 days has been performed at least three times. Each bar represents the average of three cultures. Error bars represent standard deviation from the mean. Statistical significance was determined using two-way ANOVA, with P values adjusted for multiple comparison using Holm-Šídák's multiple comparison test. Significance for IFN-γ production is shown. The remainder of the comparisons, including those involving IL-4 production, were not statistically significant. * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001.

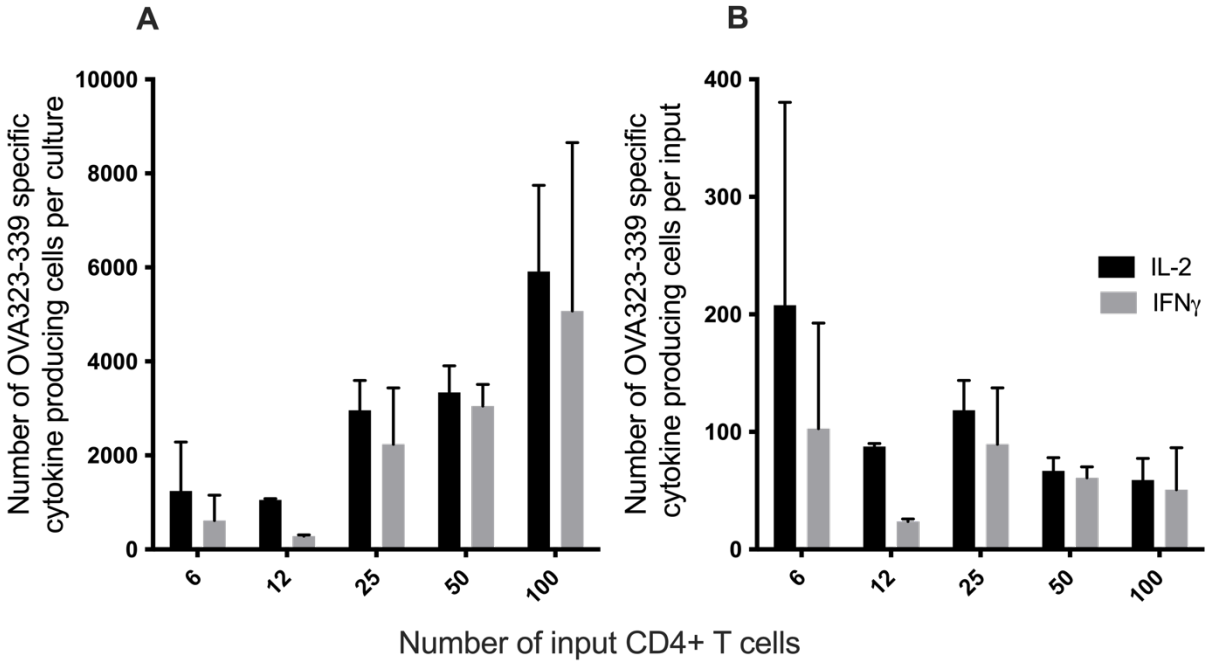
A
B
Number of OVA323-339 specific cytokine producing cells per culture
Number of OVA323-339 specific cytokine producing cells per input
10000
8000
6000
4000
2000
0
400
300
200
100
0
6
12
25
50
100
IL-2
IFNγ
Number of input CD4+ T cells

Figure 4-2: Establishing low-density cultures of naïve RAG-sufficient DO11.10 CD4+ T cells with added LPS using T cell depleted splenocytes as APCs. The indicated number of CD25- CD44- CD4+ T cells, isolated from adult RAG-sufficient DO11.10 TCR transgenic mice, were cultured for the indicated number of days with $3x10^4$ T cell depleted splenocytes as APCs, 0.3 μM $OVA_{323-339}$ peptide and 1 μg/mL LPS. The IL-2-producing cells and IFN-γ-producing cells were enumerated on day 5 post-culture via the ELISPOT assay. Panel A displays the number of cytokine-producing cells per culture, while panel B displays the number of cytokine-producing cells per input CD4+ T cell. This figure is representative of two identical experiments, each bar represents the average of three identical cultures, and error bars represent standard deviation from the mean. Data represented in panel B was analysed using two-way ANOVA, with P values adjusted for multiple comparison using Holm-Šídák's multiple comparison test. No statistically significant differences were observed when comparing the different densities.

Therefore, we expected a great degree of variability in the size and cytokine profile of the response generated by CD4+ T cells cultured at an average of 1 or 3 per culture. We sought to improve the power of the experiment by establishing 48 cultures for each density. Interestingly, of the 48 cultures containing an average of 1 CD25- CD44- DO11.10 CD4+ T cell, only 34 had detectable cytokine-producing cells by day 5 post culture. According to the Poissonian distribution, there is a 63% probability that a culture would have at least 1 input CD25- CD44- DO11.10 CD4+ T cell. In other words, approximately 30 of the 48 were expected to have at least 1 such T cell, meaning that we were able to detect cytokine-producing cells from cultures containing as low as 1 input CD4+ T cell. Importantly, while these cultures generated detectable IL-2-producing cells, the majority failed to generate detectable IFN-γ-producing cells. CD4+ T cells that produce IL-2, in the absence of Th1 or Th2 cytokines, are thought to represent a population of CD4+ T cells that are early in their activation stage and have not yet committed to a helper phenotype (159, 160). When the average number of input DO11.10 CD4+ T cells per culture was increased to 3, almost all of the cultures generated detectable cytokine-producing cells by day 5, which is not surprising since there is a 95% probability that a culture would contain at least 1 input DO11.10 CD4+ T cell. Importantly, the large majority of cultures containing an average of 3 DO11.10 CD4+ T cells supported the generation of IFN-γ-producing cells. These results are shown in figure 4-3. Since the DO11.10 CD4+ T cells in both conditions were isolated from the same animal, and so were the T-depleted splenocytes, we propose that the generation of effector, IFN-γ-producing DO11.10 CD4+ T cells, and their full activation, occurs by a quorum-dependent mechanism. Another remarkable feature of these observations will be the subject of later discussion. We note for now that as cultures with only an average of one CD4+ T cell per well give rise to about a hundred cytokine-producing cells in 5 days, the T cell or T cells must go through several rounds of cell-division. Cultures with an average of 3 rather than 1 T cell per well at the initiation of culture support more readily the generation of IFN-γ-producing cells. So why do the cultures with initially only one T cell per well not give to IFN-γ-cells after they have divided to produce many more than three T cells in the culture?

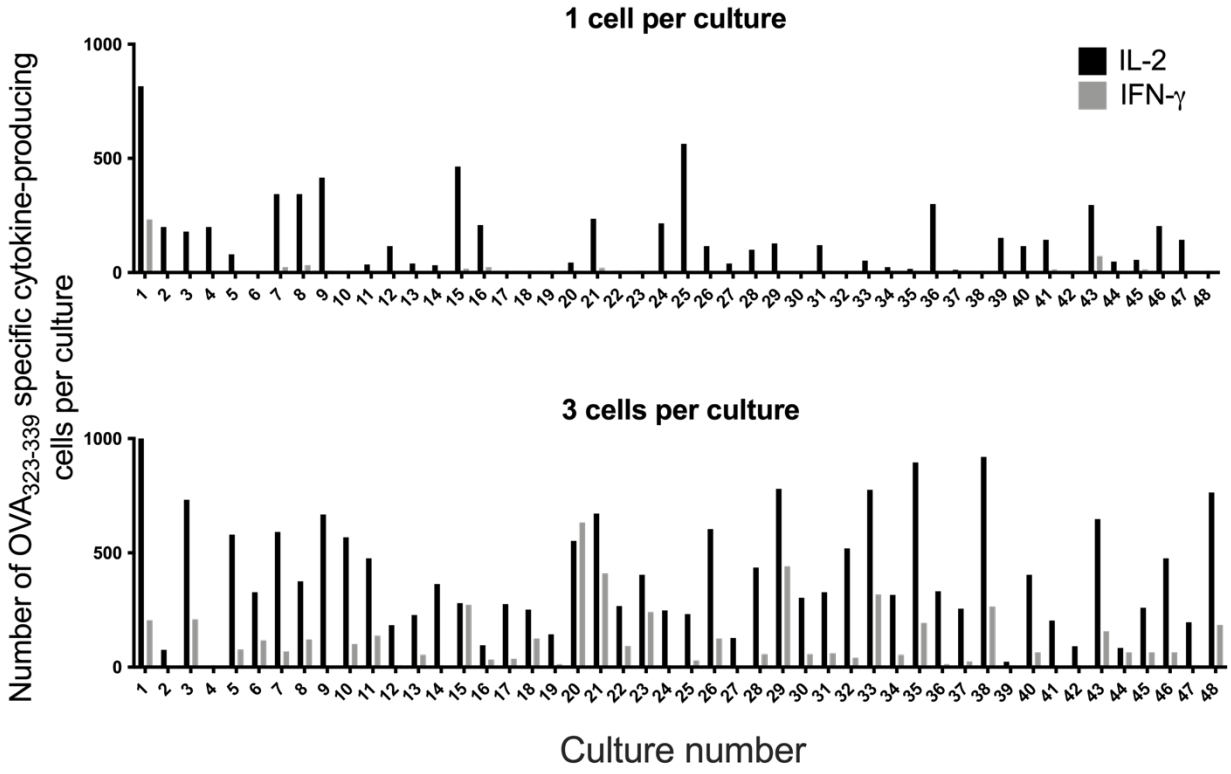
1 cell per culture
IL-2
IFN-γ
1000
500
0
3 cells per culture
1000
500
0
Number of OVA323-339 specific cytokine-producing cells per culture
Culture number

Figure 4-3: Culturing an average of 1 or 3 naïve RAG-sufficient DO11 with LPS using T cell depleted splenocytes as APCs. IL-2-producing cells and IFN-γ-producing cells from 48 cultures, containing an average of 1 (top panel) or 3 (bottom panel) CD25- CD44- RAG-sufficient DO11.10 CD4+ T cells, were enumerated by ELISPOT on day 5 post-culture. The CD4+ T cells were cultured with 3 x 10^4 T-depleted splenocytes as APCs, 0.3 μM $OVA_{323-339}$ peptide and 1 μg/mL LPS. This figure is representative of two identical experiments.

4.3 Attempting to raise the quorum threshold by lowering antigen dose

While the results from the previous experiment, and summarized in figure 4-3, seem very interesting, there is a difficulty in employing this system to test the idea that antigen can inactivate CD4+ T cells if present below the quorum required to achieve activation. It appears that single CD4+ T cells present in a culture well can be activated. This implies the Quorum Hypothesis is incorrect, or the in vitro system may not reflect the in vivo situation in a manner such that quorum is not required to achieve the activation that results in IL-2-producing cells. For example, the presence of LPS in our cultures could undermine the need for quorum to achieve the activation seen. Another possibility we were mindful of is that the CD4+ T cells, despite the way there were purified, were partially activated. We decided to explore a different approach to see if antigen could inactivate CD4+ T cells under conditions were insufficient CD4+ T cell collaboration took place to achieve antigen-dependent CD4+ T cell activation. As discussed above, cooperation between CD4+ T cells is influenced by the antigen dose (161). Therefore, we sought to limit the ability of CD4+ T cells to cooperate, and thereby raise the quorum threshold, by reducing the concentration of $OVA_{323-339}$. To that end, an average of 27 CD25- CD44- DO11.10 CD4+ T cells per well were cultured at different dilutions of the standard 0.3 μM $OVA_{323-339}$ peptide concentration. As shown in figure 4-4, decreasing the peptide concentration dramatically affected the generation of IL-2 and IFN-γ-producing CD4+ T cells. Interestingly, the generation of IFN-γ-producing cells appeared to be more susceptible to lowering peptide dose than the generation of IL-2-producing cells. This seemed consistent with the prediction of the quorum hypothesis.

However, we were concerned that the effect of antigen dose, recorded in figure 4-4, might have been due to fewer CD4+ T cells entering proliferation at lower peptide concentrations, thereby resembling low density cultures and/or that the lower concentration of peptide not only affected the proliferation of CD4+ T cells but resulted in insufficient generation of signal 1. This is important because CD4+ T cells must receive signal 1 prior to their inactivation, and in our system, we anticipate proliferation to be a surrogate marker for signal 1 in conjunction with a constitutive costimulatory signal, as proposed in step one of the Two Step, Two Signal Model of CD4+ T cell activation. Indeed, T cells have been shown to proliferate prior to their inactivation by antigen (151, 162). Therefore, if we were to assay for inactivated CD4+ T cells, an objective of this project,

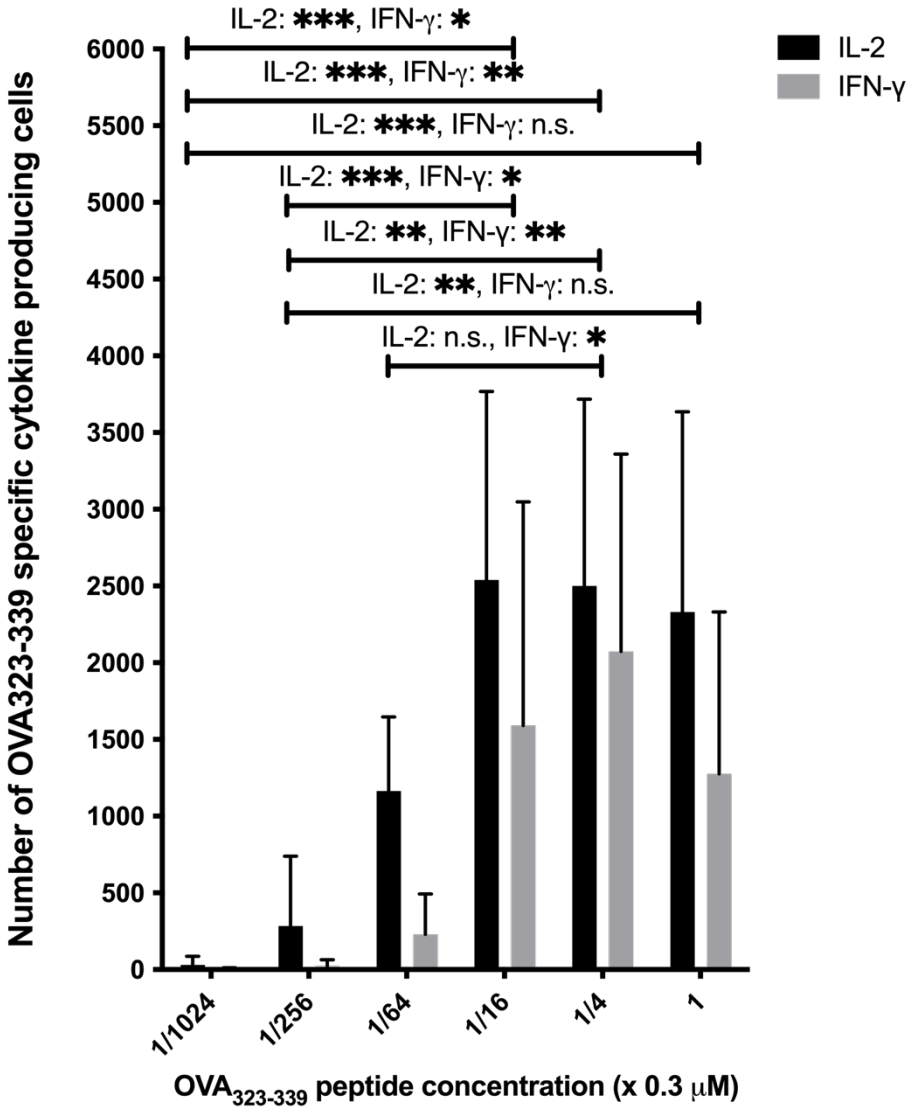
IL-2: ***, IFN-γ: *
IL-2: ***, IFN-γ: **
IL-2: ***, IFN-γ: n.s.
IL-2: ***, IFN-γ: *
IL-2: **, IFN-γ: **
IL-2: **, IFN-γ: n.s.
IL-2: n.s., IFN-γ: *
IL-2
IFN-γ
Number of OVA323-339 specific cytokine producing cells
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
1/1024
1/256
1/64
1/16
1/4
1
OVA323-339 peptide concentration (x 0.3 μM)

Figure 4-4: Attempting to limit CD4+ T cell cooperation by titrating down the peptide concentration. 27 CD25- CD44- RAG-sufficient DO11.10 CD4+ T cells were cultured with 3 x 10^4 T-depleted splenocytes as APCs, 1 μg/mL LPS, and the indicated concentration of $OVA_{323-339}$ peptide. The number of IL-2-producing cells and IFN-γ-producing cells was determined on day 5 by the ELISPOT assay. Each bar represents the average of six identical cultures and error bars represent standard deviation from the mean. Two identical experiments were later performed using the peptide concentrations of 1/4, 1/64 and 1/256 to confirm these findings. Statistical significance was determined using two-way ANOVA, with P values adjusted for multiple comparison using Holm-Šídák's multiple comparison test. Comparisons not indicated on the figure means they were not statistically significant. * < 0.05, ** < 0.01, *** < 0.001.

the majority of input CD4+ T cells should receive signal 1. Hence, we analysed the proliferation of 27 CD25- CD44- DO11.10 CD4+ T cells stimulated with 1/4, 1/64 and 1/256 of the standard 0.3 μM $OVA_{323-339}$ peptide concentration. As the peptide concentration decreased, the number of non-proliferating cells increased. The results of this experiment are shown in figure 4-5.

4.4 Concluding remarks

We attempted to determine whether the generation of cytokine-producing CD4+ T cells requires the presence of a minimum number, i.e. a quorum, of input CD4+ T cells. To that end, we cultured CD25- CD44- DO11.10 CD4+ T cells at very low densities and observed that the cytokine profile of the response on day 5 post-culture depended on the number of input CD4+ T cells. A drawback of establishing very low-density cultures is the high degree of inter-culture variability, which makes it challenging to conduct further analysis. We postulated that a lower peptide concentration would limit the ability of CD4+ T cells to cooperate, thereby increasing the quorum threshold, which may allow us to study the quorum effect at higher densities. However, we observed that by decreasing the peptide concentration, fewer cells proliferated, and possibly received insufficient signal 1, which makes such studies inappropriate for our objectives. We were concerned, as explained above, that our T cells were not fully naïve. Therefore, we decided to employ a different TCR transgenic system, which would allow us to conduct more sophisticated analyses. This system is discussed in chapter 5 below.

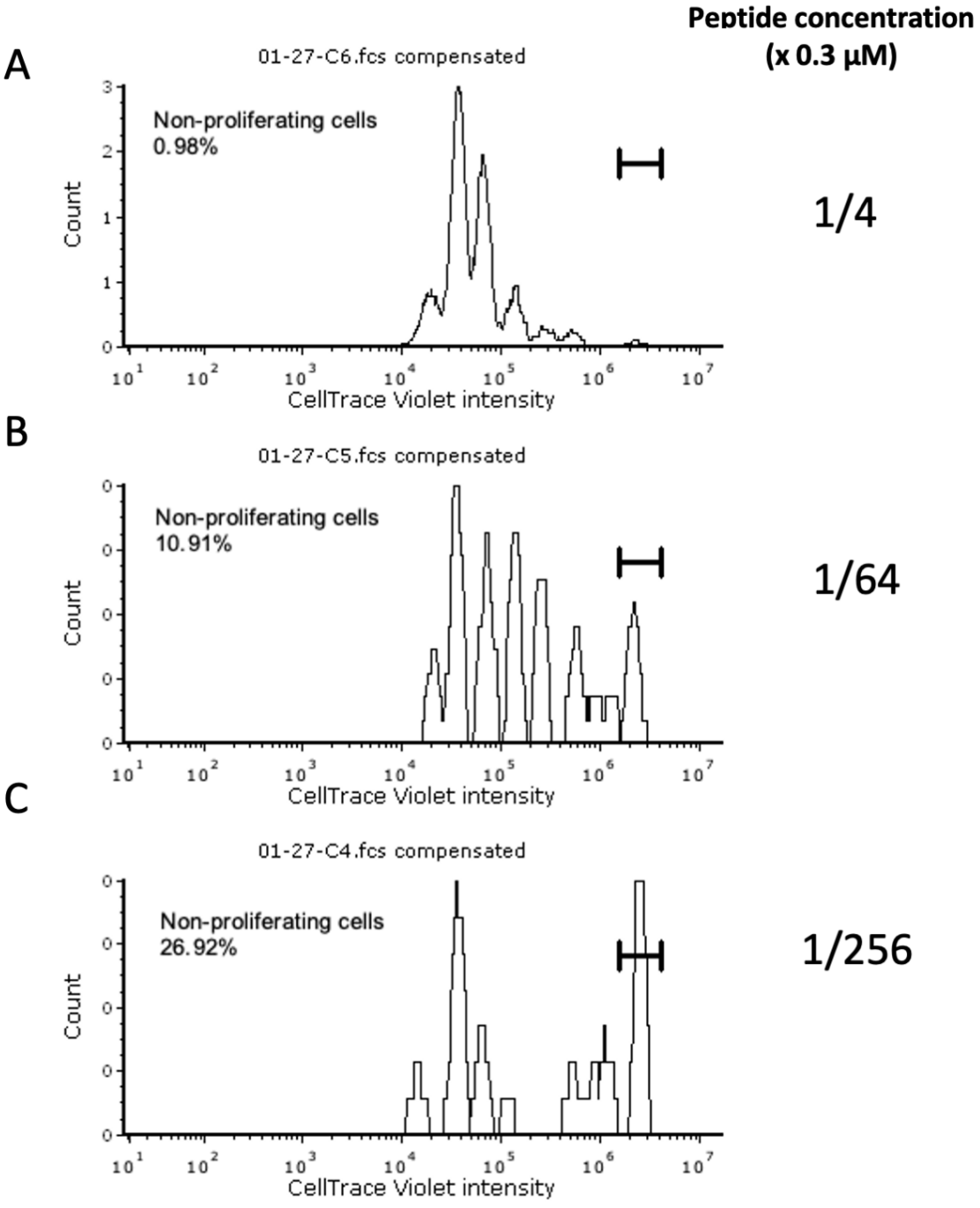
Peptide concentration
(x 0.3 µM)
A
01-27-C6.fcs compensated
Non-proliferating cells
0.98%
Count
CellTrace Violet intensity
1/4
B
01-27-C5.fcs compensated
Non-proliferating cells
10.91%
Count
CellTrace Violet intensity
1/64
C
01-27-C4.fcs compensated
Non-proliferating cells
26.92%
Count
CellTrace Violet intensity
1/256

Figure 4-5: Determining whether lower peptide concentration affects the proliferative capacity of CD4+ T cells. CD25- CD44- CD4+ RAG-sufficient DO11.10 CD4+ T cells were cultured at 27 cells/well with 3 x 10^4 T-depleted splenocytes as APCs, 1 μg/mL LPS, and the indicated concentration of $OVA_{323-339}$ peptide (Panel A, 1/4 x standard peptide concentration; Panel B, 1/64 x standard peptide concentration; Panel C, 1/256 x standard peptide concentration). Four cultures for each condition were established that were then pooled on day 4 post-cultured and assayed for proliferation by flow cytometry. The proportion of non-proliferating cells is displayed. This figure is representative of two identical experiments.

CHAPTER 5 - DEVELOPING AN IN VITRO SYSTEM FOR TESTING THE QUORUM HYPOTHESIS USING MARILYN CD4+ T CELLS

5.1 The advantages of using the Marilyn system

DO11.10 CD4+ T cells are recombination-activating gene (RAG)-sufficient, meaning that they do not fully suppress the endogenous recombination of the genes coding for the alpha chain of the TCR (163). This can result in the generation of bispecific CD4+ T cells that may be partially activated by environmental antigens and so are not in a naïve state. The generation of such bi-specific T cells can be circumvented by the use of RAG-deficient, TCR transgenic mice, which are known to have a more homogeneous, naive population of CD4+ T cells (164). An example of such a murine strain is Marilyn. Marilyn CD4+ T cells express a TCR specific for the peptide $DBY_{608-622}$ of the male, H-Y antigen, expressed on the Y chromosome, recognised in context of $I\text{-}A^b$. This H-Y peptide appears to have minimal, if any, cross-reaction with environmental antigens (165, 166). Therefore, Marilyn CD4+ T cells are likely to be in a more naïve state of activation for this reason, and because they all are monospecific, than DO11.10 CD4+ T cells.

A practical advantage of using Marilyn CD4+ T cells pertains to their being of C57BL/6 murine background. A variety of TCR transgenic mice exist that are of the C57BL/6 background, which makes it possible to explore whether CD4+ T cells specific for H-Y could cooperate with TCR transgenic CD4+ T cells specific for an unrelated peptide. We exploited this possibility in chapter 6 below. Given these advantages of the Marilyn system, we decided to explore its use to further test the Quorum Hypothesis.

5.2 Using Marilyn CD4+ T cells allows the study of CD4+ T cell activation/inactivation without the need to add LPS

We compared the response of Marilyn CD4+ T cells to that of DO11.10 CD4+ T cells by culturing Marilyn cells under the same conditions as we had cultured the DO11.10 CD4+ T cells. We stimulated 6 to 100 Marilyn CD4+ T cells with 1 μM H-Y peptide with and without added LPS

for 5 days. It is interesting to compare the responses generated in the presence of LPS in the DO11.10 and Marilyn systems, see figures 2 and 6. Interestingly, there is no clear evidence for a quorum effect in either system, i.e. the generation of IL-2 and of IFN-γ-producing cells per input T cell does not increase with the number of transgenic T cells per cell.

Up to this stage, we had been adding LPS to the cultures to ensure a consistency in the response. Without LPS, neither the size of the response, nor the cytokine profile, correlated well with the number of cultured DO11.10 CD4+ T cells (Al-Yassin and Rudulier, unpublished observations). The reason(s) for the variability, and for their minimization by adding LPS, are unclear. However, murine CD4+ T cells express TLR4, and the stimulation, by LPS, of TLR4 on CD4+ T cells has been shown to have various effects on CD4+ T cells (167, 168). Therefore, we were concerned that the continuous presence of these higher levels of LPS in our cultures might not reflect a physiological environment for studying the circumstances under which antigen activates/inactivates CD4+ T cells. Given that Marilyn CD4+ T cells are more homogeneous than DO11.10 CD4+ T cells, we deemed it worthwhile culturing Marilyn CD4+ T cells without adding LPS.

When Marilyn CD4+ T cells were cultured without added LPS, the generation of cytokine-producing cells was dramatically impeded, as shown in figure 5-1. The inability of Marilyn CD4+ T cells cultured at low densities without added LPS could be attributed to one of two possibilities: 1) LPS is absolutely required for the generation of cytokine-producing cells, or 2) LPS results in the apparent reduction of the quorum threshold required for CD4+ T cells to differentiate into cytokine-producing cells. The Danger Model predicts the former while the Quorum Hypothesis predicts the latter. Therefore, we were interested in determining which possibility was more likely. To that end, we cultured Marilyn CD4+ T cells at higher densities without added LPS, as shown in figure 5-2 A and B. Indeed, increasing culture density dramatically improved the efficiency of the response. This can be seen in figure 5-2 B, where the number of cytokine-producing cells per input CD4+ T cell was greater when 1000 CD4+ T cells were cultured per well, supporting the existence of a quorum dependent mechanism for the generation of cytokine-producing cells.

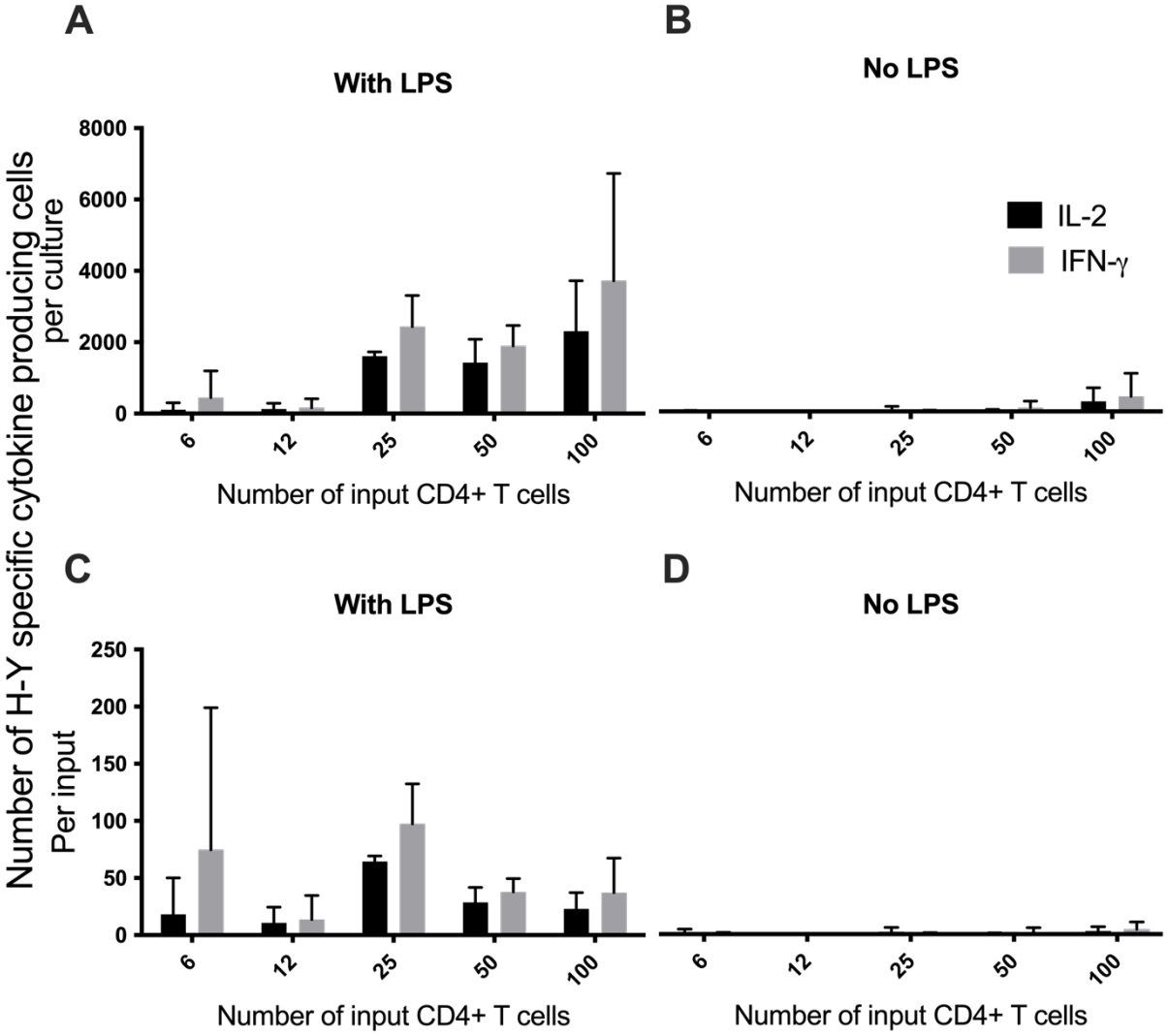
A
With LPS
B
No LPS
IL-2
IFN-γ
Number of H-Y specific cytokine producing cells per culture
8000
6000
4000
2000
0
6
12
25
50
100
Number of input CD4+ T cells
C
With LPS
D
No LPS
Number of H-Y specific cytokine producing cells Per input
250
200
150
100
50
0
Number of input CD4+ T cells

Figure 5-1: Establishing low-density cultures of Marilyn CD4+ T cells with and without added LPS using T cell depleted splenocytes as APCs. The indicated number of Marilyn CD4+ T cells were cultured with 3 x 10^4 T-depleted splenocytes as APCs and 1 μM H-Y peptide, with (A and C) or without (B and D) 1 μg/mL LPS. The number of IL-2 and IFN-γ-producing cells was determined on day 5 by the ELISPOT assay. Panels A and B display the number of cytokine-producing cells per culture, while panels C and D display the number of cytokine-producing cells per input CD4+ T cell. Each bar represents the average of three identical cultures and error bars represent standard deviation from the mean. This figure is representative of two identical experiments. The data reported in this figure were analyzed using two-way ANOVA, with P values adjusted for multiple comparisons using Holm-Šídák's multiple comparison test. This analysis was used to assess the effect of LPS on the number of cytokine-producing cells per input (i.e. to compare panels C and D). Overall, the effect of LPS on the number of IL-2 producing cells per input had a P value of < 0.0001, while its effect on the number of IFN-γ-producing cells per input had a P value of 0.004.

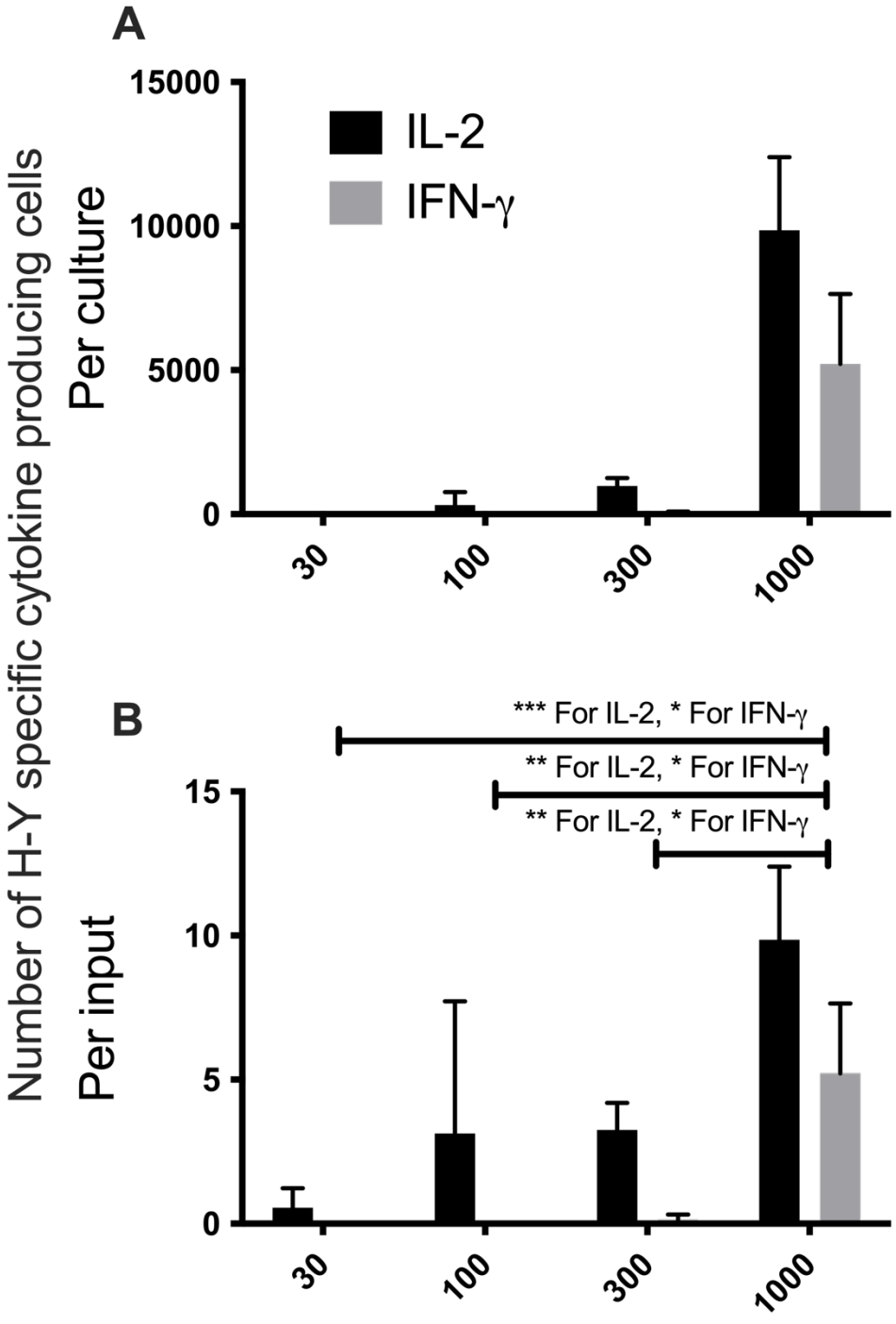
A
15000
10000
5000
0
IL-2
IFN-γ
Number of H-Y specific cytokine producing cells
Per culture
30
100
300
1000
B
*** For IL-2, * For IFN-γ
** For IL-2, * For IFN-γ
** For IL-2, * For IFN-γ
15
10
5
0
Per input
30
100
300
1000

Figure 5-2: The number of cultured Marilyn CD4+ T cells without added LPS affects their ability to generate cytokine-producing cells. The indicated number of Marilyn CD4+ T cells were cultured with 3 x 10^4 T-depleted splenocytes as APCs and 1 μM H-Y peptide, without adding LPS. The number of IL-2 and IFN-γ-producing cells was determined on day 5 by the ELISPOT assay. Panels A and B display the number of cytokine-producing cells per culture and per input, respectively. Each bar represents the average of three identical cultures and error bars represent standard deviation from the mean. This figure is representative of two identical experiments. However, several independent experiments were performed where cultures containing 30 and 1000 Marilyn CD4+ T cells were established under conditions identical to that described in this figure. The numbers of cytokine-producing cells generated in such experiments were similar to those represented in this figure. Statistical significance was determined using two-way ANOVA, with P values adjusted for multiple comparison using Holm-Šídák's multiple comparison test. * < 0.05, ** < 0.01, *** < 0.001.

5.3 Marilyn CD4+ T cells cultured at low and high numbers per well similarly proliferate in response to peptide

It remained possible that the difference in the ability to generate cytokine-producing cells from Marilyn CD4+ T cells cultured at low and high densities was due to a significant difference in their proliferative capacity. Therefore, we analyzed the proliferation of Marilyn CD4+ T cells cultured at 30 and 1000 input CD4+ T cells per culture. As shown in figure 5-3, there were no significant differences in the proliferation of CD4+ T cells cultured at the different densities, albeit CD4+ T cells cultured at 1000 input cells per culture appearing to have proliferated slightly better. This is not surprising as such cells produced significantly more IL-2 per input, as was shown in figure 5-2. Therefore, we were satisfied that the difference in the number of cytokine-producing cells per input was due to the relative failure of Marilyn CD4+ T cells in low density cultures to differentiate into cytokine-producing cells, rather than to proliferate. These observations demonstrate again that different conditions support the antigen-dependent proliferation of T cells and the proliferation associated with their differentiation into cytokine-producing cells.

These observations are significant, as they demonstrate that CD4+ T cells in low density cultures have received the signals required to proliferate, including signal 1, a prerequisite for their potential inactivation. Indeed, evidence exists that T cells proliferate prior to their inactivation by antigen (151, 162). Our observations paved the road for us to attempt to establish an in vitro model for the antigen-mediated inactivation of CD4+ T cells.

5.4 Concluding remarks

Marilyn CD4+ T cells are more homogeneous and more naïve than DO11.10 CD4+ T cells. This is likely the reason why we were able to demonstrate the quorum effect when Marilyn CD4+ T cells were cultured at different densities without adding LPS. Briefly, low density cultures failed to generate a robust response, as the number of cytokine-producing cells was less than 1 per input CD4+ T cell. On the other hand, higher density cultures generated a robust response, reaching upwards of 10 cytokine-producing cells per input. This difference was largely due to a decrease in the differentiation of CD4+ T cells in low density cultures rather than a decrease in their

proliferation. In other words, we demonstrated that a quorum of CD4+ T cells is required for the generation of IL-2 and IFN-γ-producing CD4+ T cells, with the quorum being smaller for the former than the latter cytokine-producing cells.

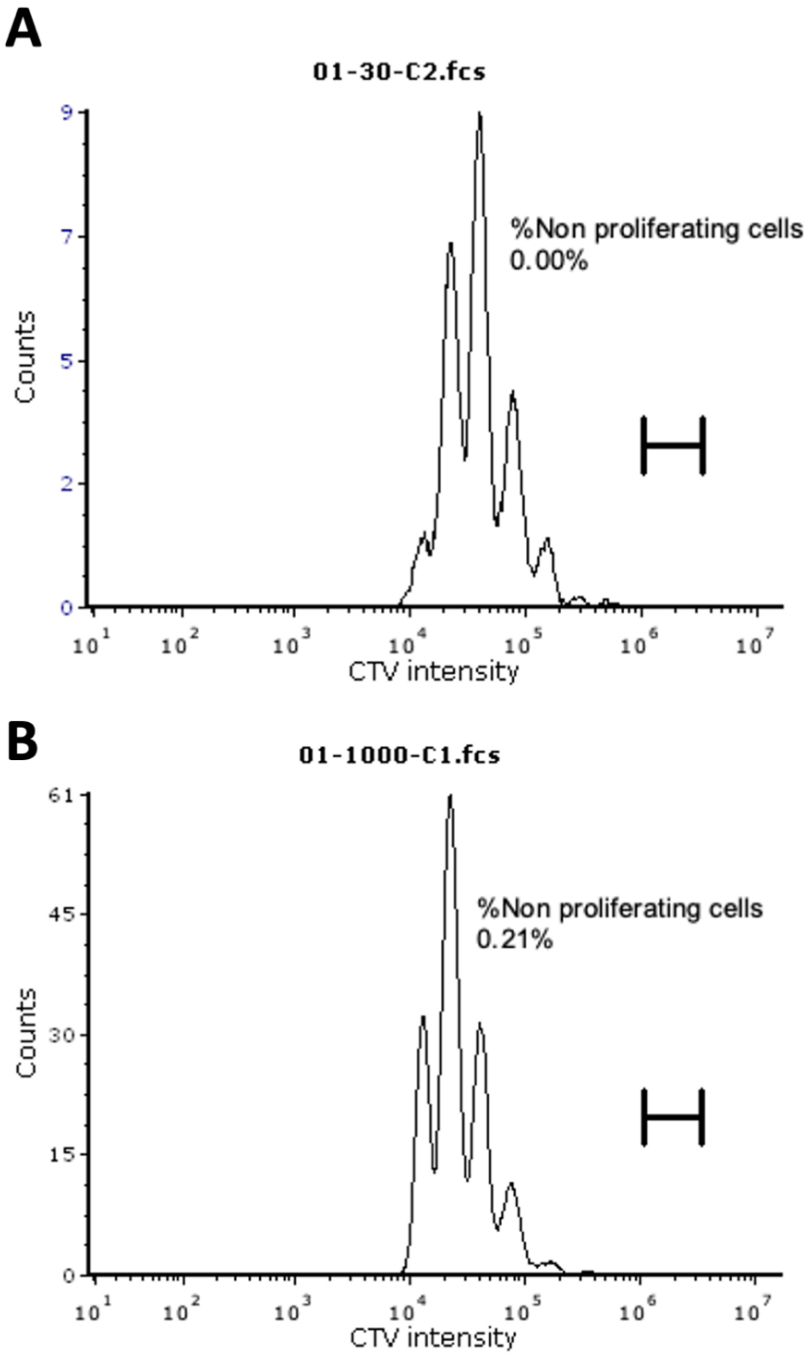
A
01-30-C2.fcs
%Non proliferating cells
0.00%
Counts
CTV intensity
B
01-1000-C1.fcs
%Non proliferating cells
0.21%
Counts
CTV intensity

Figure 5-3: Culturing low number of Marilyn CD4+ T cells does not impede their ability to proliferate. Marilyn CD4+ T cells were cultured at 30 or 1000 cells/well with 3 x 10^4 T-depleted splenocytes as APCs, and 1 μM H-Y peptide, without adding LPS. 48 cultures containing 30 cells/well and 5 cultures containing 1000 cells per well were established that were then independently pooled on day 4 post-cultured and assayed for proliferation by flow cytometry. Panel A represents the proliferation profile of the pooled cultures containing 30 cells/well and panel B represents the proliferation profile of the pooled cultures containing 1000 cells/well. The proportion of non-proliferating cells is displayed in each panel.

CHAPTER 6 - DETERMINING WHETHER THE ACTIVATION OF MARILYN CD4+ T CELLS REQUIRES CD4+ T CELL COOPERATION MEDIATED BY A LINKED OR AN UNLINKED MECHANISM

6.1 Developing an in vitro system to study CD4+ T cell cooperation

We discussed in chapter 1 the merits of requiring a linked mechanism for the antigen-mediated CD4+ T cell cooperation leading to the full activation of CD4+ T cells. Having provided several observations consistent with the Quorum Hypothesis, we sought to determine whether the antigen-mediated CD4+ T cell cooperation occurred by a linked or an unlinked mechanism. This is important because the Quorum Hypothesis predicts this cooperation to occur by a linked mechanism. Therefore, we first had to establish an in vitro system where "target" TCR transgenic CD4+ T cells specific for a peptide, cultured at limiting densities, receive "help" from TCR transgenic CD4+ T cells specific for a non-crossreacting peptide. The two sources of transgenic T cells were chosen to come from TCR transgenic mice with the same H-2 and genetic background. We examined whether the "helper" TCR transgenic cells would help when added at numbers/well above their quorum, i.e. where antigen activated them to generate cytokine-producing cells. Therefore, we used 1000 CD25- CD44- OT-II CD4+ T cells (referred to from here on as OT-II CD4+ T cells), specific for $OVA_{323-339}$ in context of $I\text{-}A^b$, to help 30 Marilyn CD4+ T cells generate cytokine-producing cells. We note we used CD4+ T cells from Marilyn as the target cells as the source of the other transgenic CD4+ T cells came from a RAG sufficient mouse.

Firstly, we explored whether H-Y and $OVA_{323-339}$ crossreact. We stimulated Marilyn and OT-II CD4+ T cells with their cognate peptides for 5 days. In the ELISPOT assay, we stimulated them separately with each of the two peptides. While each strain responded well to its cognate peptide, there was barely any detectable response to the other peptide (data not shown). Therefore, we were confident that the two peptides do not significantly crossreact.

We then wished to co-culture 1000 OT-II CD4+ T cells with 30 Marilyn CD4+ T cells, and stimulate them with peptides to determine the circumstances under which the OT-II CD4+ T cells were able to help Marilyn CD4+ T cells generate cytokine-producing cells. A major challenge we faced stemmed from the fact that the two peptides bind to the same restriction element, namely I-A^b. Therefore, we titrated both peptides until optimal concentrations were established. The observations summarized in figure 6-1 clearly show the ability of OT-II CD4+ T cells to help Marilyn CD4+ T cells generate cytokine-producing cells. While culturing 30 Marilyn CD4+ T cells alone generated ~~a~~ few, if any, cytokine-producing cells, co-culturing the Marilyn CD4+ T cells with 1000 OT-II CD4+ T cells resulted in the robust generation of cytokine-producing Marilyn CD4+ T cells, more than 10 per input Marilyn CD4+ T cell.

6.2 Using peptide-pulsed APCs to characterize CD4+ T cell cooperation

In the previous experiments, the peptides were added at the time of setting up the cultures and were left in the media for the duration of the culture. This resulted in individual APCs being decorated with both peptides (figure 6-2 C). However, it remained possible that the cooperation between OT-II and Marilyn CD4+ T cells observed above occurred by an unlinked mechanism. To rule out a significant role for an unlinked mechanism, OT-II and Marilyn CD4+ T cells should be co-cultured under circumstances where the peptides decorate separate APCs so that no individual APC presented both peptides (figure 6-2 A and B). The use of peptide pulsed APCs makes creating such a circumstance possible. APCs were incubated with either H-Y or $OVA_{323-339}$, but not both, washed extensively, then used to stimulate CD4+ T cells. We co-cultured 1000 OT-II with 30 Marilyn CD4+ T cells and stimulated them with a 1:1 mixture of singly coated APCs. As seen in figure 6-3, such a stimulation resulted in a small number of IL-2-producing cells, but very few, if any, IFN-γ-producing cells, being generated by Marilyn CD4+ T cells. We believe that our observations are consistent with our proposal that CD4+ T cells cooperate by a linked mechanism. Not shown in the figure is the number of OT-II cytokine-producing cells, which was well above 5 per input for both IL-2 and IFN-γ. The small number of cytokine-producing Marilyn CD4+ T cells generated was not unexpected, as the many OT-II CD4+ T cells present in the culture produce cytokines, such as IL-2, that play a role in CD4+ T cell proliferation and differentiation, which accumulate in the in vitro cultures and act in an unlinked manner.

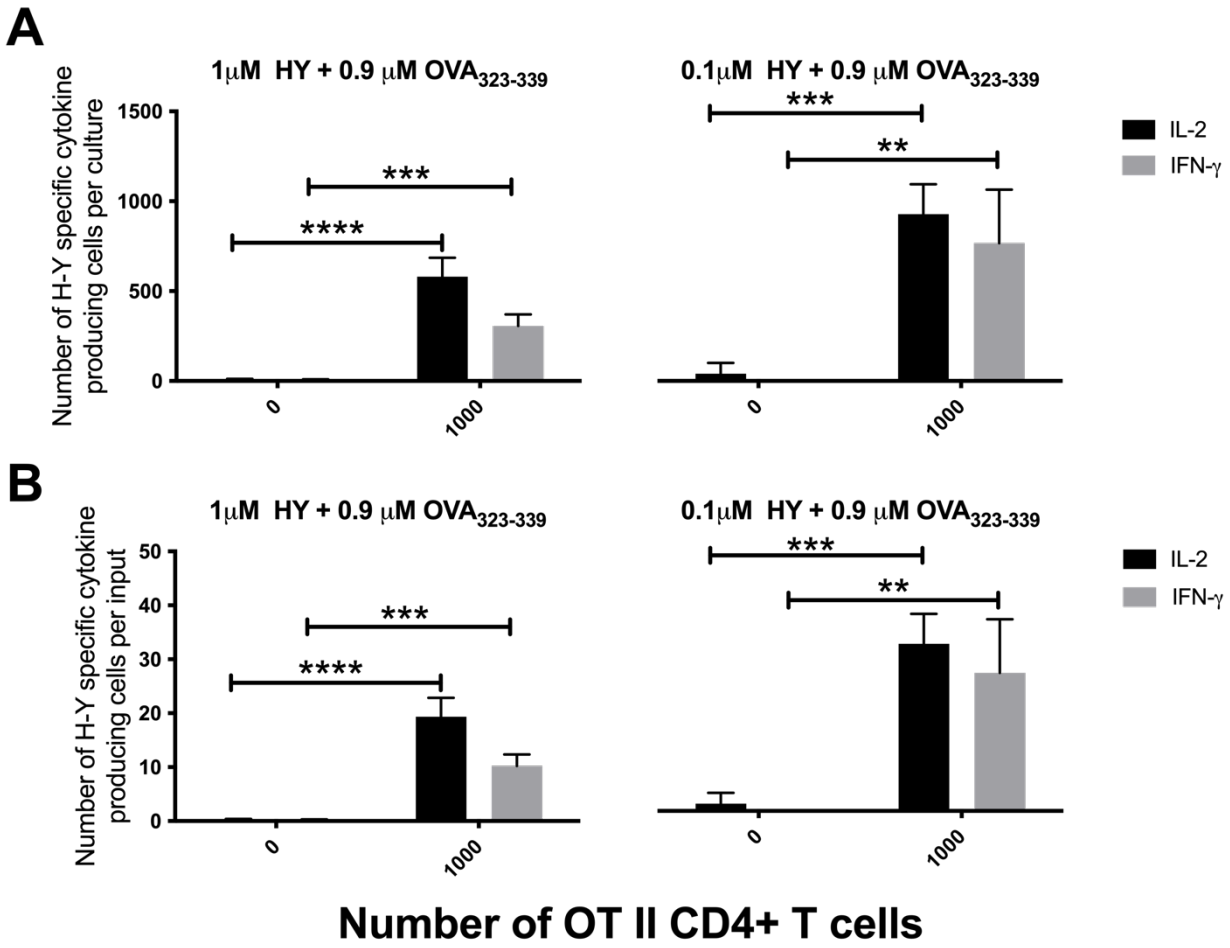
A
1μM HY + 0.9 μM OVA323-339
0.1μM HY + 0.9 μM OVA323-339
Number of H-Y specific cytokine producing cells per culture
1500
1000
500
0
***
****
**
0
1000
IL-2
IFN-γ
B
1μM HY + 0.9 μM OVA323-339
0.1μM HY + 0.9 μM OVA323-339
Number of H-Y specific cytokine producing cells per input
50
40
30
20
10
0
***
****
**
0
1000
IL-2
IFN-γ
Number of OT II CD4+ T cells

Figure 6-1: Using OT II CD4+ T cells to facilitate the generation of Marilyn cytokine-producing cells through CD4+ T cell cooperation. 30 Marilyn CD4+ T cells were cultured with 1000 CD25- CD44- RAG-sufficient OT II CD4+ T cells, $3x10^4$ T-depleted splenocytes as APCs and the indicated concentrations of H-Y and $OVA_{323-339}$ peptides. On day 5 post-culture, ELISPOT assay was used to enumerate IL-2 and IFN-γ-producing Marilyn CD4+ T cells per culture (A) and per input (B). Each bar represents the average of 3 cultures and error bars represent standard deviation from the mean. This figure is representative of two experiments. Statistical significance was determined using two-way ANOVA, with P values adjusted for multiple comparison using Šídák's multiple comparison test. ** < 0.01, *** < 0.001, **** < 0.0001.

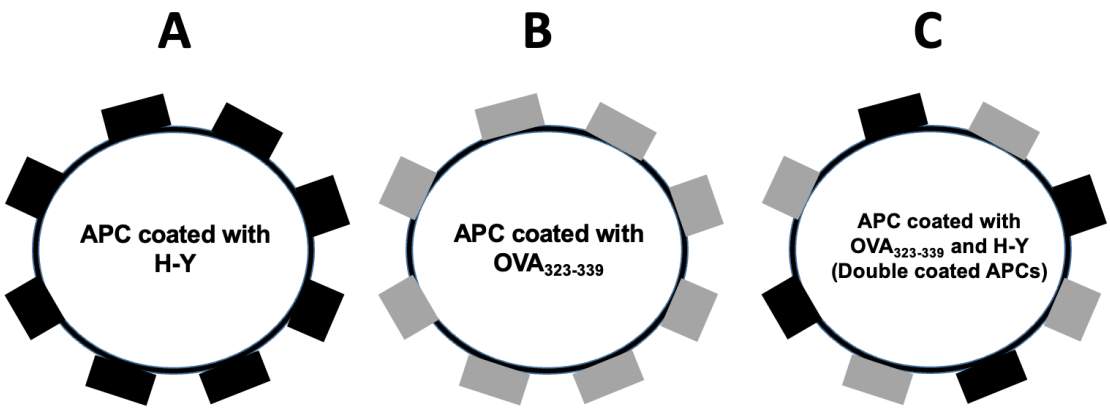
A
B
C
APC coated with H-Y
APC coated with OVA323-339
APC coated with OVA323-339 and H-Y (Double coated APCs)

Figure 6-2: A visual representation of the different APC populations used to determine whether the cooperation between Marilyn and OT II CD4+ T cells occurred by a linked or unlinked mechanism. Population A represents APCs decorated with H-Y peptide, population B represents APCs decorated with $OVA_{323-339}$, while population C represents APCs decorated with both H-Y peptide and $OVA_{323-339}$.

We have attempted to demonstrate linked recognition using double pulsed APCs (i.e., pulsed with H-Y and $OVA_{323-339}$ simultaneously), but with no success. We speculated this to be due to the two peptides competing for binding to the $I\text{-}A^b$ restriction element. We were only able to conduct limited titrations before the lab was temporarily closed due to the lockdown. Nevertheless, we were reassured by the ability of single pulsed APCs to potently stimulate the corresponding strain of CD4+ T cells. Prior to attempting the experiment above, we first assessed the ability of pulsed APCs to support the generation of cytokine-producing cells from 1000 Marilyn or 1000 OT-II CD4+ T cells. We titrated the peptide concentration and duration of the incubation and established that APCs pulsed with 10 μM H-Y for 3 hours or 60 μM $OVA_{323-339}$ overnight were potent stimulators of their corresponding CD4+ T cell strain, with at least 5 cytokine-producing cells per input generated, similar to what we would expect when culturing 1000 CD4+ T cells without LPS (data not shown). Therefore, we were convinced that the impaired generation of cytokine-producing Marilyn CD4+ T cells reported in figure 6-3 was due to lack of cooperation and not due to the inability of pulsed APCs to stimulate CD4+ T cells. Nevertheless, the experiment above should be repeated using double pulsed APCs to confirm our conclusions.

6.3 Concluding remarks

We developed an in vitro system whereby TCR transgenic CD4+ T cells, specific for non-crossreacting peptides, recognized in context of the same restriction element, cooperate in the generation of cytokine-producing T cells from the "target" CD4+ T cells. We have previously seen that culturing 30 Marilyn CD4+ T cells generates only a few, if any, cytokine-producing cells. However, culturing 30 Marilyn CD4+ T cells with 1000 OT-II CD4+ T cells and their cognate peptides results in the generation of many cytokine-producing cells derived from Marilyn CD4+ T cells, upwards of 20 effector cells per input Marilyn CD4+ T cell. Using singly pulsed APCs, we were able to demonstrate that the optimal cooperation between Marilyn and OT-II CD4+ T cells requires the two peptides be present on the same APC. When the two peptides were exclusively present on separate APCs, the generation of cytokine-producing Marilyn CD4+ T cells was almost an order of magnitude less efficient in terms of the number of cytokine-producing cells per input Marilyn cell. Given that pulsed APCs efficiently supported the generation of cytokine-

producing cells from high density cultures of their corresponding CD4+ T cells, we concluded that CD4+ T cell cooperation occurs by a linked mechanism.

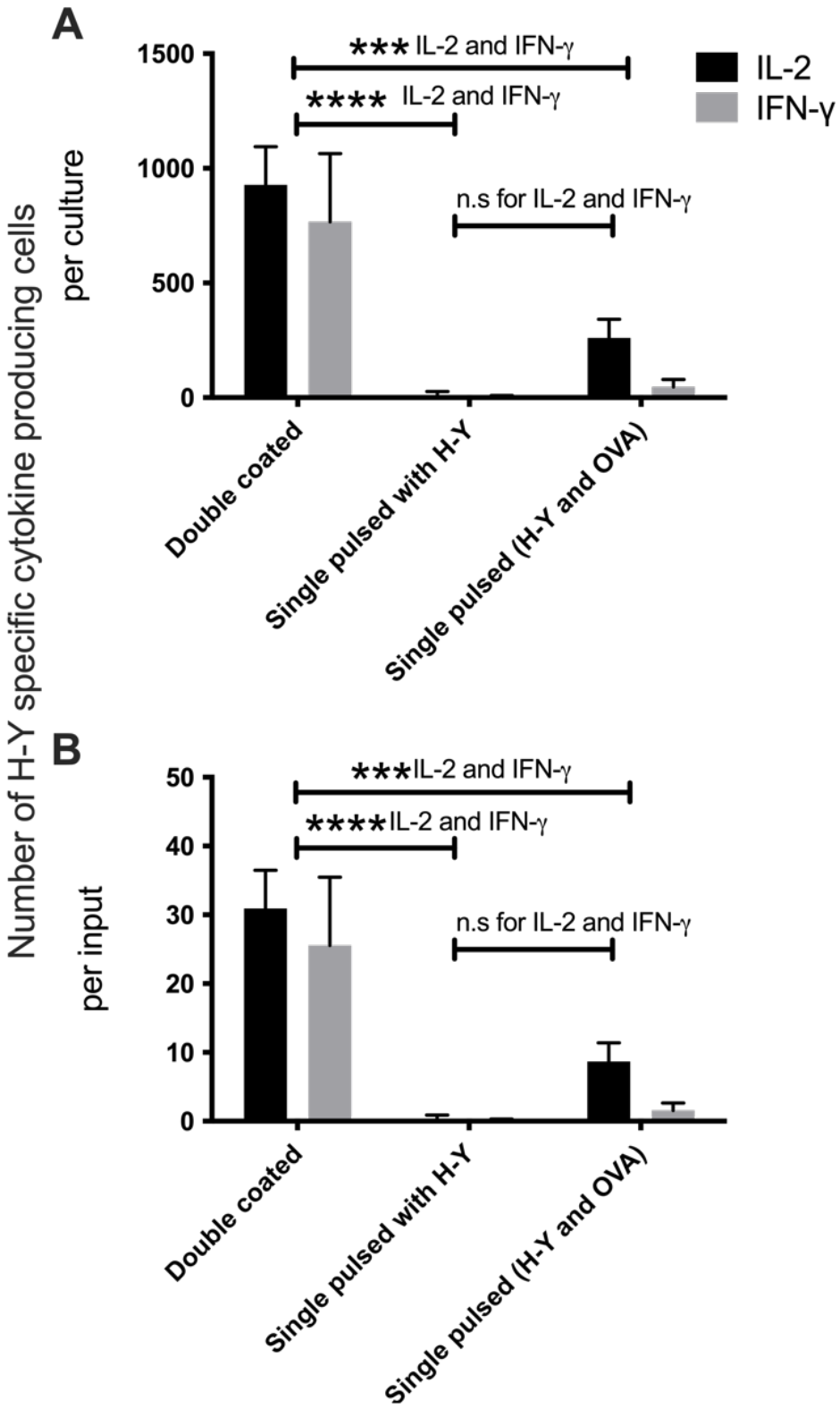
A
B
Number of H-Y specific cytokine producing cells
per culture
per input
IL-2
IFN-γ
*** IL-2 and IFN-γ
**** IL-2 and IFN-γ
n.s for IL-2 and IFN-γ
1500
1000
500
0
50
40
30
20
10
Double coated
Single pulsed with H-Y
Single pulsed (H-Y and OVA)

Figure 6-3: Optimal cooperation between Marilyn and OT II CD4+ T cells occurs by a linked mechanism. 30 Marilyn CD4+ T cells were cultured with 1000 CD25- CD44- RAG-sufficient OT II CD4+ T cells in the presence of either $3x10^4$ T-depleted splenocytes and 0.1 μM H-Y and 0.9 μM $OVA_{323-339}$ (double coated), $3x10^4$ T-depleted splenocytes pulsed with H-Y (single pulsed with H-Y), or with a mixture of $1.5x10^4$ T-depleted splenocytes pulsed with H-Y and $1.5x10^4$ T-depleted splenocytes pulsed with $OVA_{323-339}$ (single pulsed H-Y and OVA). On day 5, ELISPOT assay was used to enumerate IL-2 and IFN-γ-producing Marilyn CD4+ T cells per culture (A) and per input (B). Each bar represents the average of 3 cultures and error bars represent standard deviation from the mean. Statistical significance was determined using two-way ANOVA, with P values adjusted for multiple comparison using Šídák's multiple comparison test. *** < 0.001, **** < 0.0001, n.s. = not significant.

CHAPTER 7 - DIFFERENT POTENTIAL ROLES OF B CELLS AND CD11C+ DENDRITIC CELLS IN QUORUM SENSING

7.1 An assessment of the activation of Marilyn CD4+ T cells in the presence of only B cells as APC

We titrated Marilyn CD4+ T cells with $3x10^4$ B cells as APCs with or without added LPS to assess their ability to support the generation of cytokine-producing cells. We opted to use $3x10^4$ B cells because we had previously demonstrated that $3x10^4$ APCs, in the form of T cell-depleted splenocytes, optimally support the generation of cytokine-producing cells in our *in vitro* system. Therefore, we opted to not change the number of B cells at this stage and focus only on the effect of the number of input CD4+ T cells per well. The results are summarized in figure 7-1. In the presence of LPS, B cells were efficient in their ability to support the generation of cytokine-producing cells (figure 7-1 A and B). Our observations contradict an early, widely-accepted report by Fuchs and Matzinger (84), namely that B cells, even those stimulated with LPS, inactivate "virgin" CD4+ T cells.

When we cultured Marilyn CD4+ T cells with B cells without adding LPS, a minimum of 300 Marilyn CD4+ T cells was required to reliably generate IL-2-producing cells and a minimum of 1000 was required to reliably generate IFN-γ-producing cells. There was a considerable degree of variability in lower density cultures. We noted that 1 of the 3 cultures in the 30 and 100 group made what we considered an unusually high response. Unfortunately, we did not repeat this experiment to confirm our findings, in part due to the shutdown of the laboratory.

Lastly, we would like to address the possibility that the B cell population was not pure and could therefore contain DCs that either synergised with B cells (as will be discussed in section 8.1 below) to activate CD4+ T cells, or directly activated the CD4+ T cells. While we have not formally confirmed the purity of the B cell population by flow cytometry, we think this possibility unlikely. If the B cell purification completely failed to purify B cells, we would expect there to be 1-2%

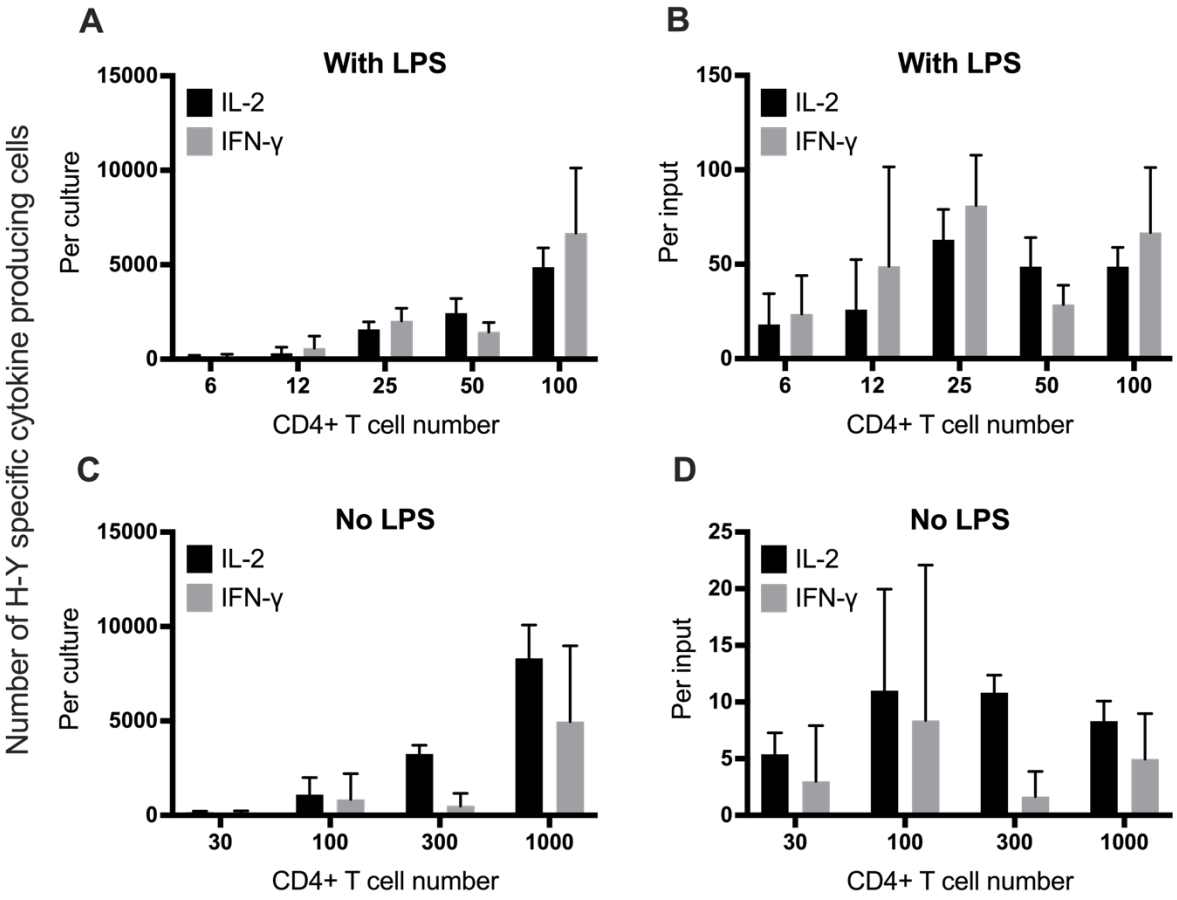
A
With LPS
IL-2
IFN-γ
Per culture
15000
10000
5000
0
6
12
25
50
100
CD4+ T cell number
B
With LPS
IL-2
IFN-γ
Per input
150
100
50
0
6
12
25
50
100
CD4+ T cell number
C
No LPS
IL-2
IFN-γ
Per culture
15000
10000
5000
0
30
100
300
1000
CD4+ T cell number
D
No LPS
IL-2
IFN-γ
Per input
25
20
15
10
5
0
30
100
300
1000
CD4+ T cell number
Number of H-Y specific cytokine producing cells

Figure 7-1: Establishing low-density cultures of Marilyn CD4+ T cells with and without added LPS using B cells as APCs. The indicated number of Marilyn CD4+ T cells were cultured with 3 x 10^4 B cells as APCs and 1 μM H-Y peptide, with (A and B) or without (C and D) adding LPS. The number of IL-2 and IFN-γ-producing cells was determined on day 5 by the ELISPOT assay. Panels A and C display the number of cytokine-producing cells per culture, while panels B and D display the number of cytokine-producing cells per input. Each bar represents the average of three identical cultures and error bars represent standard deviation from the mean. This figure is representative of two identical experiments. Data reported in panels B and D were individually analyzed using two-way ANOVA, with P values adjusted for multiple comparisons using Holm-Šídák's multiple comparison test. For each panel, comparing the numbers of cytokine-producing cells from each cell density to the other did not yield statistically significant results. However, the overall effect of cell density on the number of cytokine-producing cells per input in panel B had a P value of 0.0258, indicating statistical significance, while the overall effect of cell density on the number of cytokine-producing cells per input in panel D had a P value of 0.5308.

CD11c+ DCs, which represents the normal composition of murine splenocytes. Therefore, the $3x10^4$ "B cell population" would contain 300 – 600 DCs. If the MACS purification, which claims to isolate B cells at 94-98% purity, managed to isolate B cells at a purity of only 80%, a gross underestimation, we would expect the upper limit of the DCs to be 60 – 120 DCs among the $3x10^4$ B cells, with the upper limit being determined assuming the remaining 20% consists of normal splenocytes. Therefore, we believe that the purified B cell population likely contained too few DCs to have physiological significance.

7.2 An assessment of the activation of Marilyn CD4+ T cells with only CD11c+ dendritic cells as a source of APC

We then examined the ability of splenic CD11c+ DCs to support the antigen dependent generation of cytokine-producing CD4+ T cells from purified Marilyn CD4+ T cells. Interestingly, we noted a stark difference in the cytokine profile of Marilyn CD4+ T cells stimulated with $3x10^4$ DCs as APCs. DCs supported the generation of cytokine-producing cells from high density cultures, but not from low density cultures. Importantly, addition of LPS only increased the size of the response and did not allow DCs to support the generation of cytokine-producing cells from lower density cultures. These results are summarized in figure 7-2. Their significance will be a subject in the discussion. However, we would like to reflect on the difficulty in determining the purity of the CD11c+ DC population. The isolation kit claims to isolate CD11c+ DCs at "up to 95% purity". Since the kit relies on positive selection, the CD11c marker will be occupied by the primary antibodies used in the selection and will compete with fluorochrome-conjugated anti-CD11c antibodies used in detecting CD11c+ cells. We brought the issue to the manufacturer, STEMCELL, who recommended the use of a combination of the primary antibodies provided by the kit and fluorochrome-conjugated anti-CD11c antibodies. However, we worried that such a combination would compromise the quality of the isolation, so we did not pursue this option. Alternatively, we could use fluorochrome-conjugated anti-CD11c antibodies as primary antibodies, with the secondary antibodies being magnetic bead-conjugated anti-fluorochrome antibody. This would allow for the positive selection of CD11c+ DCs and the assessment of their purity. However, this isolation is essentially different from the isolation guaranteed by STEMCELL and would require the repeat of all prior experiments employing CD11c+ DCs

isolated by STEMCELL's kit. Therefore, we opted not to pursue this option either. As such, we were unable to determine the purity of the CD11c+ DCs, and it remains possible that the generation of cytokine-producing cells from high density cultures was facilitated by B cell contaminants.

7.3 Concluding remarks

In the presence of LPS, B cells were efficient in supporting the generation of cytokine-producing Marilyn CD4+ T cells, even from low density cultures. Without adding LPS, B cells only reliably supported the generation of cytokine-producing cells from higher density cultures. On the other hand, DCs only supported the generation of cytokine-producing cells from high density cultures, regardless of whether LPS was added or not. These findings were surprising, albeit interesting. We shall discuss below how our observations, if true, can be reconciled with the Quorum Hypothesis.

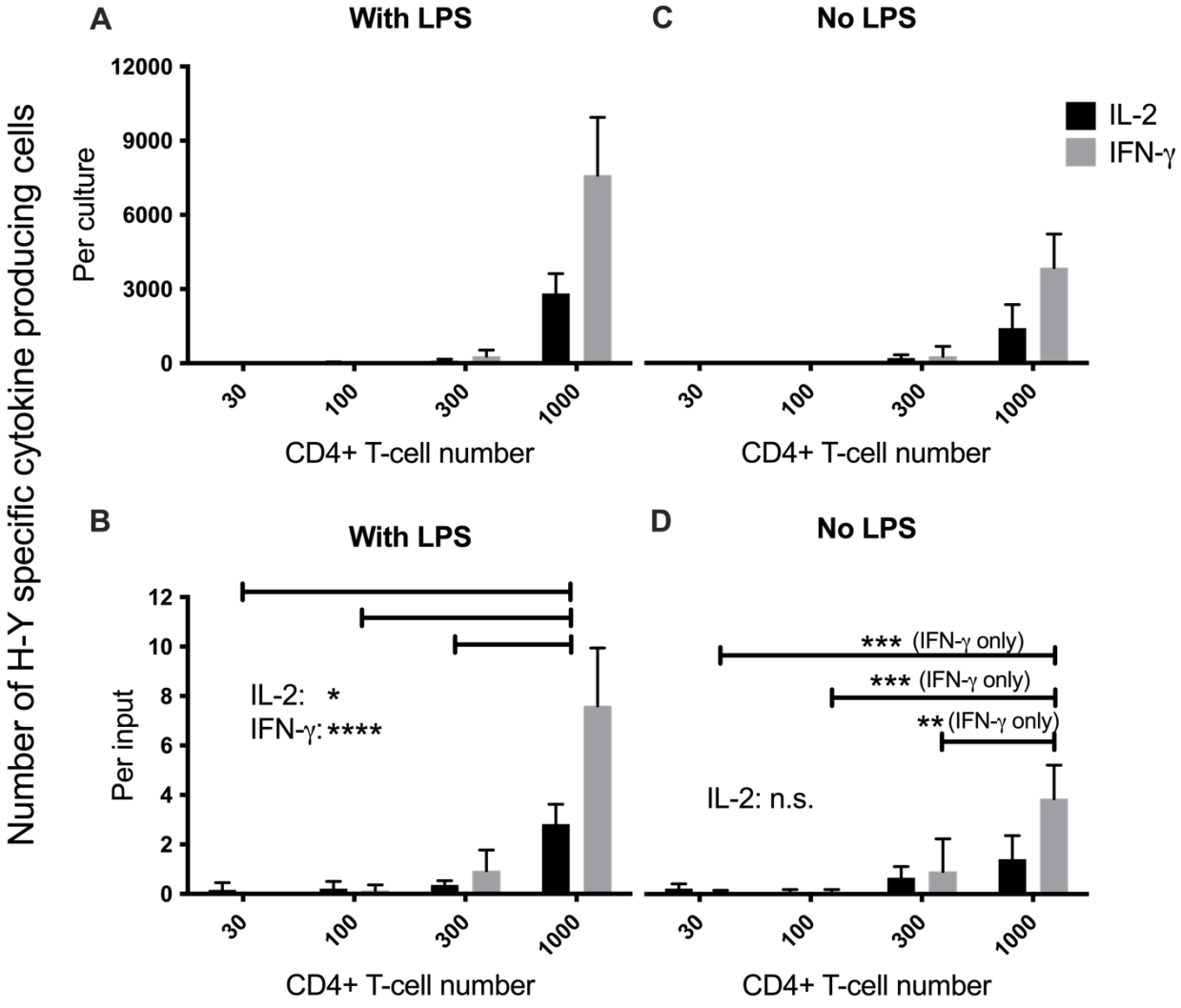
A
With LPS
C
No LPS
Number of H-Y specific cytokine producing cells
Per culture
12000
9000
6000
3000
0
IL-2
IFN-γ
30
100
300
1000
CD4+ T-cell number
B
With LPS
D
No LPS
Per input
12
10
8
6
4
2
0
IL-2: *
IFN-γ: ****
*** (IFN-γ only)
*** (IFN-γ only)
** (IFN-γ only)
IL-2: n.s.

Figure 7-2: Establishing low-density cultures of Marilyn CD4+ T cells with and without added LPS using DCs as APCs. The indicated number of Marilyn CD4+ T cells were cultured with 3 x 10^4 DCs as APCs and 1 μM H-Y peptide, with (A and B) or without (C and D) adding LPS. The number of IL-2 and IFN-γ-producing cells was determined on day 5 by the ELISPOT assay. Panels A and C display the number of cytokine-producing cells per culture, while panels B and D display the number of cytokine-producing cells per input. Each bar represents the average of three identical cultures and error bars represent standard deviation from the mean. Statistical significance was determined using two-way ANOVA, with P values adjusted for multiple comparison using Holm-Šídák's multiple comparison test. * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001, n.s. = not significant.

CHAPTER 8 - OTHER OBSERVATIONS WORTHY OF NOTE AND CONSIDERATION

The previous chapters were dedicated to providing a more coherent narrative that guides the reader through a series of experimental findings pertinent to the critical examination of the salient features of the Quorum Hypothesis. However, during my research journey, I have made some findings that, in my opinion, are worthy of consideration. However, I opted not to include such findings in the previous chapters, either because they were generated using experimental systems that I could no longer employ, or because they pertained to attempts to answer questions not related to the Quorum Hypothesis, as I describe below. In addition, in some cases they do not represent complete stories, but may nevertheless be of interest to researchers.

8.1 In pursuit of naïve CD4+ T cells: using RAG-deficient and two-week-old mice

Very early on in my career, I had access to RAG-deficient DO11.10 mice. However, we initially hesitated to use these mice because prior experiments in our lab (carried out by others) appeared to demonstrate an inability of RAG-deficient DO11.10 CD4+ T cells to differentiate into a Th2 phenotype when cultured at high densities, unlike their RAG-sufficient counterparts. To my mind, these findings did not make physiological sense, so I re-examined the ability of RAG-deficient DO11.10 CD4+ T cells to differentiate into a Th2 phenotype when cultured at high densities. Indeed, my experiments demonstrated such an ability, and these findings were published in the Journal of Immunology (161). Therefore, we were encouraged to employ these cells to attempt to establish an *in vitro* system where the circumstances leading to their activation/inactivation could be examined.

We first titrated the number of CD4+ T cells, in a similar fashion to that described in chapter 4, in an attempt to establish conditions where CD4+ T cell number is limiting. Since I performed these titrations early in my PhD, I had not yet considered culturing CD4+ T cells without adding LPS. Therefore, it is not surprising that low density cultures supported the generation of cytokine-producing cells and increasing culture density did not improve the generation of cytokine-producing cells, as shown in figure 8-1 A. However, culturing these CD4+ T cells under the same

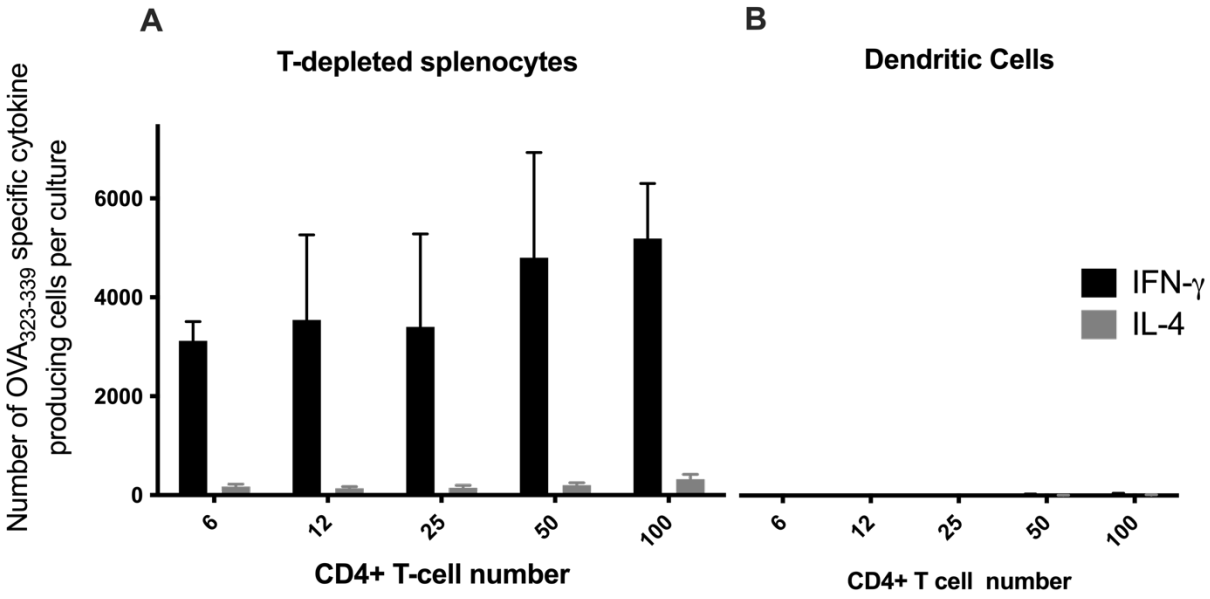
A
T-depleted splenocytes
Number of OVA323-339 specific cytokine producing cells per culture
6000
4000
2000
0
6
12
25
50
100
CD4+ T-cell number
B
Dendritic Cells
IFN-γ
IL-4
6
12
25
50
100
CD4+ T cell number

Figure 8-1: Establishing low-density cultures of RAG-deficient DO11.10 CD4+ T cells with added LPS using T cell depleted splenocytes as APCs. The indicated number of RAG-deficient DO11.10 CD4+ T cells were cultured with 0.3 μM $OVA_{323-339}$ peptide, 1 μg/mL LPS and either $3x10^4$ T cell depleted splenocytes (A) or $3x10^4$ DCs (B) as APCs. IFN-γ and IL-4-producing cells were enumerated on day 5 post-culture via the ELISPOT assay. This figure is representative of three identical experiments. Each bar represents the average of three identical cultures and error bars represent standard deviation from the mean. The data reported in this figure were analyzed using two-way ANOVA, with P values adjusted for multiple comparisons using Holm-Šídák's multiple comparison test. This analysis was used to assess the effect of APC type on the number of cytokine-producing cells. Individual comparisons at each density between T-depleted splenocytes and DCs were all statistically significant with a minimum P value of 0.0027. The overall effect of APC type on the number of IFN-γ producing cells had a P value of < 0.0001, and so did the overall effect of APC type on the number of IL-4 producing cells, with the P value being < 0.0001.

circumstances, but using DCs as a source of APCs failed to support the generation of detectable cytokine-producing cells from low density cultures, as shown in figure 8-1 B. We were excited by this finding, as it was consistent with the inferences drawn from the 2S2S model. Concerns were raised that the isolated DCs might not be functional, and so not able to support the generation of cytokine-producing cells. We therefore set up an experiment to test their functionality. BALB/c splenocytes contain approximately 1% CD11c+ cells, meaning that the $3x10^4$ T-depleted splenocyte population typically used in our system as APCs contains about 300 CD11c+ cells. We hypothesized that the T-depleted population resembles a more physiological APC population, and as such, supplementing B cells with DCs may result in a better generation of cytokine-producing cells compared to B cells alone. To that end, we cultured 50 RAG-deficient DO11.10 CD4+ T cells with $3x10^4$ B cells, 1 μg/mL LPS, and titrated DCs from 0 to 800 per culture. The results are summarized in figure 8-2. Despite the high variability, there was a dose dependent increase in the number of cytokine-producing cells generated when more DCs were added. This finding led to the conclusion that these DC alone cannot support the generation of cytokine-producing cells, despite the presence of LPS, but cooperate with B cells to result in the enhanced generation of cytokine-producing cells, consistent with expectations based on the 2S2S model.

Unfortunately, the company that bred the RAG-deficient DO11.10 mice had stopped breeding this strain, prompting us to look for alternatives. We opted to use two-week-old RAG-sufficient DO11.10 (2WO) mice, on the basis that these younger mice are less likely to have activated or partially activated CD4+ T cells than their adult counterparts.

We similarly titrated RAG-sufficient DO11.10 CD4+ T cells isolated from 2WO mice with either T-depleted splenocytes or DCs as APCs, with similar responses detected on day 5 post-culture to that previously noted in figure 8-1. These findings are shown in figure 8-3. We then examined whether a few DCs, added to $3x10^4$ B cells, were able to improve the generation of cytokine-producing cells when culturing RAG-sufficient DO11.10 CD4+ T cells isolated from 2WO mice. As shown in figure 8-4, DCs improved the generation of cytokine-producing cells in a dose dependent fashion, similar to that noted in figure 8-2.

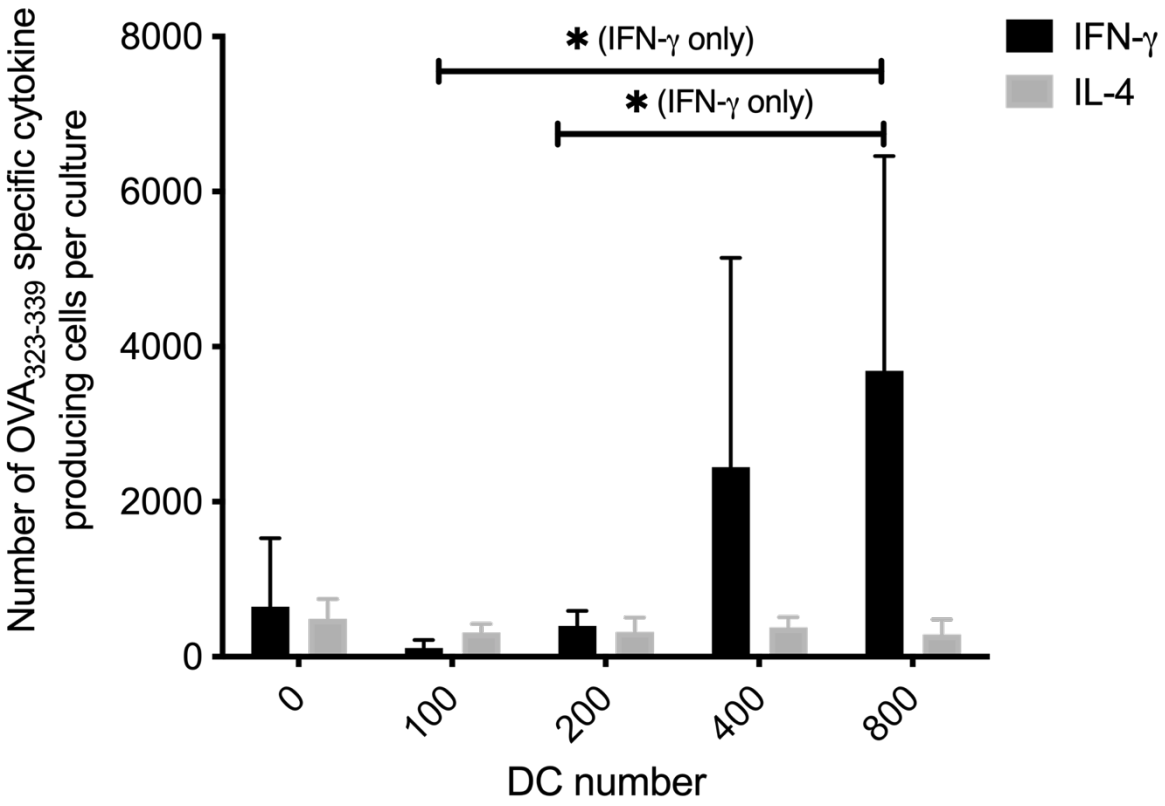
Number of OVA323-339 specific cytokine producing cells per culture
8000
6000
4000
2000
0
* (IFN-γ only)
* (IFN-γ only)
IFN-γ
IL-4
0
100
200
400
800
DC number

Figure 8-2: DCs and B cells synergise to support the generation of cytokine-producing cells from low-density cultures of RAG-deficient DO11.10 CD4+ T cells. 50 RAG-deficient DO11.10 CD4+ T cells were cultured with 0.3 μM $OVA_{323-339}$ peptide, 1 μg/mL LPS, $3x10^4$ B cells, and the indicated number of CD11c+ DCs. IFN-γ and IL-4-producing cells were enumerated on day 5 post-culture via the ELISPOT assay. Each bar represents the average of 3 identical cultures and error bars represent standard deviation from the mean. Statistical significance was determined using two-way ANOVA, with P values adjusted for multiple comparison using Holm-Šídák's multiple comparison test. * < 0.05. Exp 34.

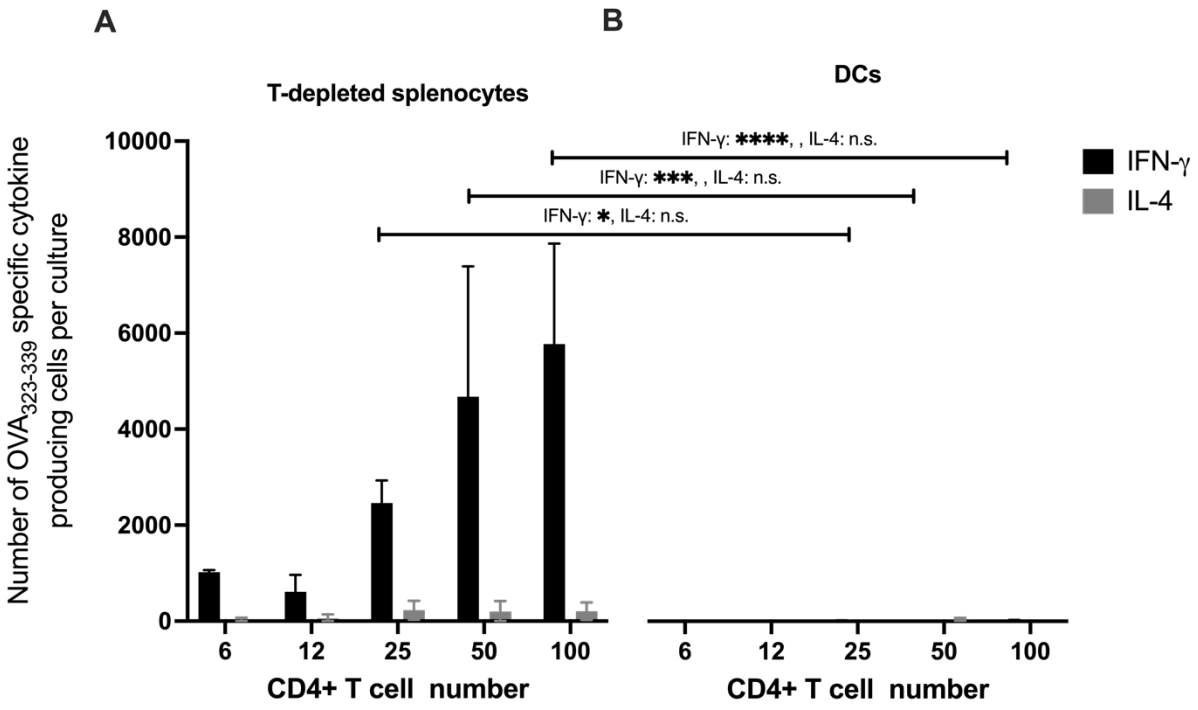
A
B
T-depleted splenocytes
DCs
IFN-γ: ****, , IL-4: n.s.
IFN-γ: ***, , IL-4: n.s.
IFN-γ: *, IL-4: n.s.
IFN-γ
IL-4
Number of OVA323-339 specific cytokine producing cells per culture
10000
8000
6000
4000
2000
0
6
12
25
50
100
CD4+ T cell number
6
12
25
50
100
CD4+ T cell number

Figure 8-3: Establishing low-density cultures of RAG-sufficient DO11.10 CD4+ T cells isolated from 2WO mice with added LPS using T cell depleted splenocytes as APCs. The indicated number of CD4+ T cells, isolated from 2WO RAG-sufficient DO11.10 mice, were cultured with 0.3 μM $OVA_{323-339}$ peptide, 1 μg/mL LPS and either $3x10^4$ T cell depleted splenocytes (A) or $3x10^4$ DCs (B) as APCs. IFN-γ and IL-4-producing cells were enumerated on day 5 post-culture via the ELISPOT assay. This figure is representative of three identical experiments. Each bar represents the average of three identical cultures and error bars represent standard deviation from the mean. Statistical significance was determined using two-way ANOVA, with P values adjusted for multiple comparison using Holm-Šídák's multiple comparison test. * < 0.05, *** < 0.001, **** < 0.0001, n.s. = not significant.

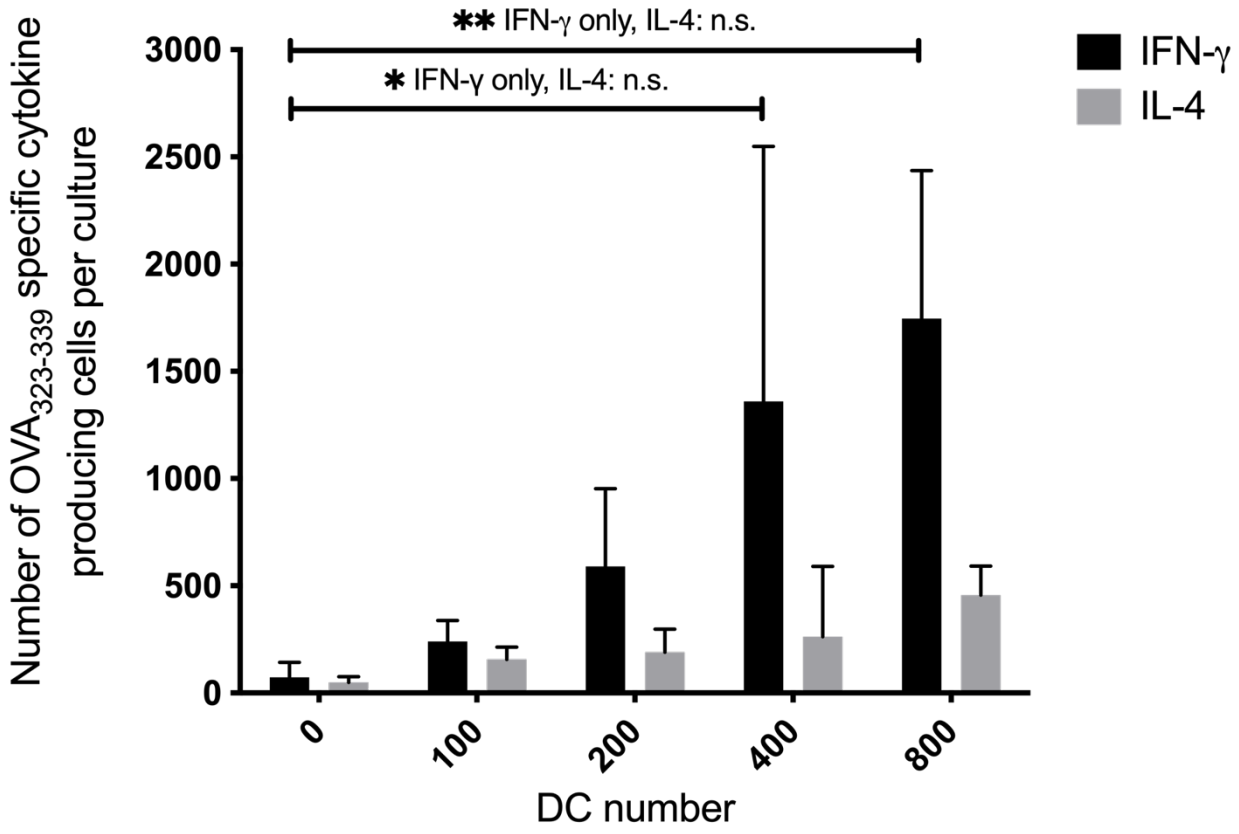
** IFN-γ only, IL-4: n.s.
* IFN-γ only, IL-4: n.s.
IFN-γ
IL-4
Number of OVA323-339 specific cytokine producing cells per culture
3000
2500
2000
1500
1000
500
0
0
100
200
400
800
DC number

Figure 8-4: DCs and B cells synergise to support the generation of cytokine-producing cells from low-density cultures of RAG-sufficient DO11.10 CD4+ T cells isolated from 2WO mice. 50 CD4+ T cells, isolated from 2WO RAG-deficient DO11.10 mice, were cultured with 0.3 μM $OVA_{323-339}$ peptide, 1 μg/mL LPS, $3x10^4$ B cells, and the indicated number of CD11c+ DCs. IFN-γ and IL-4-producing cells were enumerated on day 5 post-culture via the ELISPOT assay. Each bar represents the average of 3 identical cultures and error bars represent standard deviation from the mean. Statistical significance was determined using two-way ANOVA, with P values adjusted for multiple comparison using Holm-Šídák's multiple comparison test. * < 0.05, ** < 0.01, n.s. = not significant.

The above findings were indeed exciting and inspired further research to examine the different potential roles of B cells and DCs in CD4+ T cell activation. Unfortunately, we had to discontinue the use of 2WO mice for ethical/logistical reasons. The size of the murine liter far exceeded the number needed for our experiments. Furthermore, the narrow age range meant that unused animals could not be housed for later use, as they would be too old at that time. Unfortunately, this resulted in many mice being euthanized or donated. The continued used of this system could not be ethically justified. I therefore returned to the literature to search for other options and stumbled upon the "naïve" CD4+ T cell isolation kit from STEMCELL technologies that negatively selects for CD25- CD44- CD4+ T cells, and opted to use it while awaiting arrangements to obtain Marilyn mice from the University of Alberta to be bred at the University of Saskatchewan. Unfortunately, when attempting to demonstrate the cooperation between DCs and B cells using CD25- CD44- CD4+ T cells, the B cells alone were sufficient in supporting the generation of robust responses from low density cultures. Therefore, supplementation with a few DCs had no statistically significant effect (data not shown). This led to the hypothesis that isolated CD25- CD44- CD4+ T cells were not as naïve as CD4+ T cells isolated from 2WO or RAG-deficient mice. Therefore, we sought to examine other features of the Quorum Hypothesis, as outlined in the previous chapters.

8.2 The serendipitous establishment of an *in vitro* system to examine tonic signalling

As mentioned above, the proportion of DCs in a T-depleted splenocyte population is about 1-3%. Therefore, we were concerned that culturing low numbers of CD4+ T cells with $3x10^4$ DCs may be unphysiological and may explain why DCs failed to support the generation of cytokine-producing cells from low density cultures. Therefore, we stimulated CD4+ T cells with a variety of DC densities. We noted that culturing CD4+ T cells with only a few DCs was detrimental for the generation of cytokine-producing cells. I speculated that this was due to the short half-life of DCs in vitro, which would affect their ability to sustainably provide the tonic signals needed for T cell survival. Briefly, the TCRs of T cells were shown to be required to engage in interactions with non-cognate peptide/MHC complexes, which would deliver tonic signals to the T cells, thereby promoting their survival (169-171). Furthermore, MHC II expression on DCs alone was sufficient in promoting CD4+ T cell survival (169).

We designed a simple experiment to examine whether DCs were required for the sustained survival of CD4+ T cells. Briefly, 200 CD25- CD44- RAG-sufficient DO11.10 CD4+ T cells were cultured with 1000 CD11c+ DCs and 1 μg/mL LPS. On day 4, half of the cultures received another 1000 CD11c+ DCs, while the other half received only media. On day 8, all cultures were stimulated with $3x10^4$ T-depleted splenocytes and 0.3 μM $OVA_{323-339}$. On day 13, the number of cytokine-producing cells was determined by the ELISPOT assay. As shown in figure 8-5 A, many more cytokine-producing cells were generated from cultures that were supplemented with 1000 DCs on day 4. We then examined whether the decline in the number of cytokine-producing cells was due to inactivation or death by enumerating CD4+ T cells on day 13 by flowcytometry. As shown in figure 8-5 B, many more CD4+ T cells were detected on day 14 in cultures that were supplemented with DCs, leading to the conclusion that the difference in the number of cytokine-producing cells was due to the physical deletion of CD4+ T cells, likely due to insufficient DCs.

We were interested in elucidating the mechanism by which DCs promote the survival of CD4+ T cells. To test whether the mechanism involved tonic signalling, we sought to block TCR-MHC II interaction using anti-IA^d antibodies. We first determined the minimal antibody concentration required to block the stimulation of CD4+ T cells. Therefore, we cultured 300 DO11.10 CD4+ T cells with $3x10^4$ T-depleted splenocytes, 0.3 μM $OVA_{323-339}$, and 1 μg/mL LPS, and titrated the anti-IA^d antibody, as shown in figure 8-6, which led to a dose-dependent reduction in the number of cytokine-producing cells detected on day 5 by the ELISPOT assay. We then demonstrated that both B cells and DCs were able to promote CD4+ T cell survival by a mechanism that involves TCR-MHC II interaction. We cultured 200 CD4+ T cells with 3000 B cells or DCs for four days in the presence of 1 μg/mL LPS, and then stimulated them with $3x10^4$ T-depleted splenocytes and 0.3 μM $OVA_{323-339}$. The number of viable CD3+ CD4+ T cells was determined on day 4 post stimulation by flowcytometry. As shown in figure 8-7, both B cells and DCs were able to promote CD4+ T cell survival, although more CD4+ T cells survived when cultured with DCs than with B cells. Importantly, CD4+ T cell survival could be blocked by the addition of anti-IA^d antibodies, leading to the suggestion that TCR-MHC interaction were critical for CD4+ T cell survival. Unfortunately, further experiments led to the conclusion that the anti-IA^d antibodies led to non-

specific reduction in cell counts (data not shown). Therefore, we could no longer conclude that CD4+ T cell survival in our system required tonic signals.

While our findings were very interesting and had the potential to provide insight into a poorly understood mechanism, we thought it best to dedicate our resources toward the examination of the Quorum Hypothesis. We hope our analysis would be helpful in future studies.

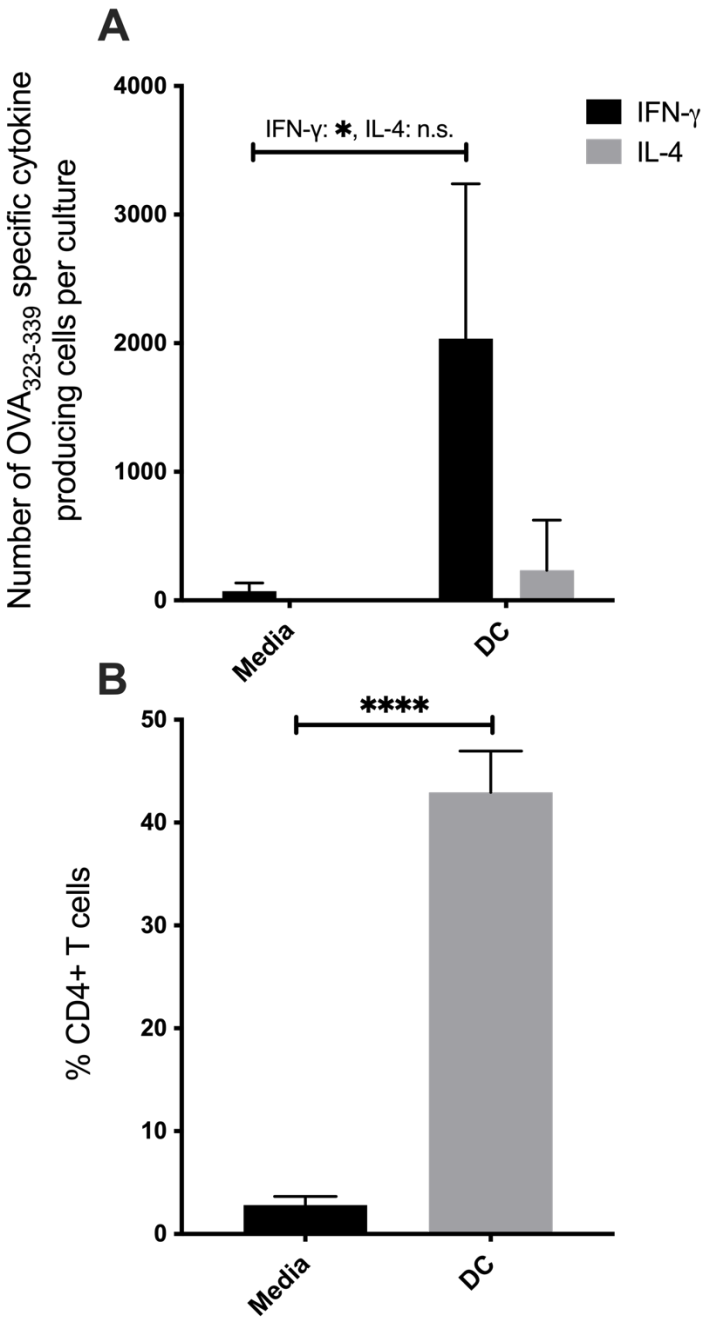
A
Number of OVA323-339 specific cytokine producing cells per culture
4000
3000
2000
1000
0
IFN-γ: ✱, IL-4: n.s.
IFN-γ
IL-4
Media
DC
B
% CD4+ T cells
50
40
30
20
10
0
****
Media
DC

Figure 8-5: An *in vitro* demonstration for the requirement of tonic signalling for CD4+ T cell responsiveness. 200 CD25- CD44- RAG-sufficient DO11.10 CD4+ T cells were cultured with 1000 CD11c+ DCs and 1 μg/mL LPS. On day 4, half of the cultures received another 1000 CD11c+ DCs, while the other half received only media. On day 8, all cultures were stimulated with $3x10^4$ T-depleted splenocytes and 0.3 μM $OVA_{323-339}$. On day 13, the number of cytokine-producing cells was determined by the ELISPOT assay (panel A) and the number of CD4+ T cells was determined by flow cytometry (panel B). Each bar represents the average of 3 identical cultures and error bars represent standard deviation from the mean. For panel A, statistical significance was determined using two-way ANOVA, with P values adjusted for multiple comparison using Šídák's multiple comparison test. For panel B, Statistical significance was determined unpaired, two-tailed t test. * < 0.05, **** < 0.0001.

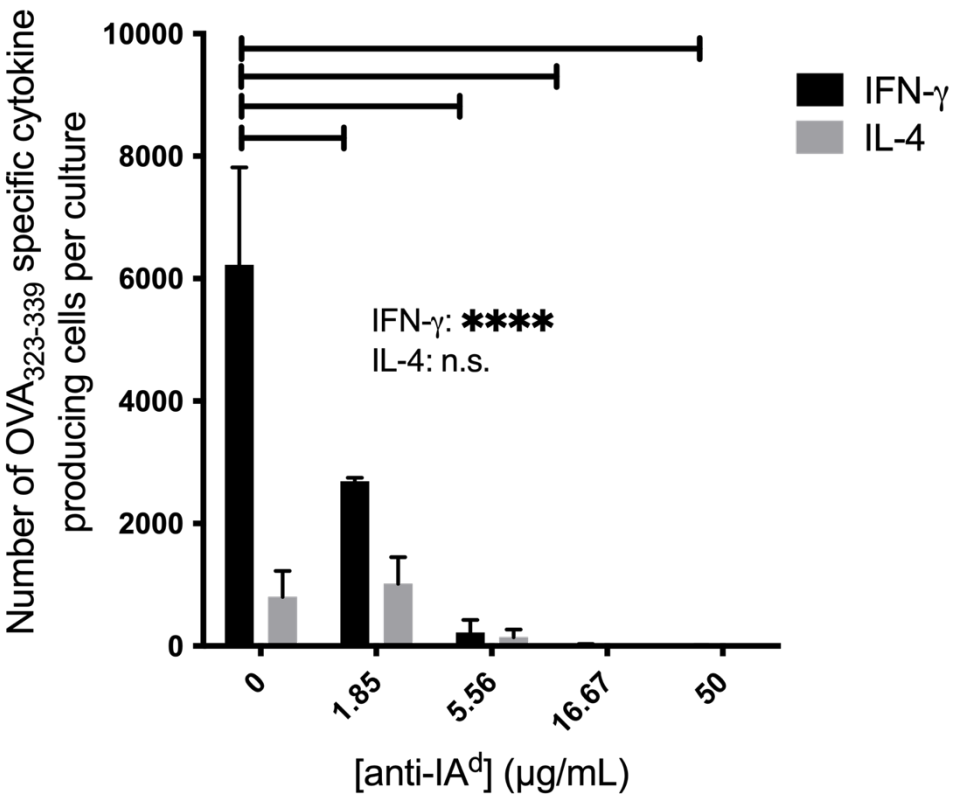
Number of OVA323-339 specific cytokine producing cells per culture
10000
8000
6000
4000
2000
0
IFN-γ
IL-4
IFN-γ: ****
IL-4: n.s.
0
1.85
5.56
16.67
50
[anti-IAd] (μg/mL)

Figure 8-6: Attempting to block the delivery of tonic signals to CD4+ T cells using anti-IA^d antibodies. 300 DO11.10 CD4+ T cells with $3x10^4$ T-depleted splenocytes, 0.3 μM $OVA_{323-339}$, 1 μg/mL LPS, and the indicated concentration of anti-IA^d antibodies. IFN-γ and IL-4-producing cells were enumerated on day 5 post-culture via the ELISPOT assay. Each bar represents the average of 3 identical cultures and error bars represent standard deviation from the mean. Statistical significance was determined using two-way ANOVA, with P values adjusted for multiple comparison using Holm-Šídák's multiple comparison test. **** < 0.0001, n.s. = not significant.

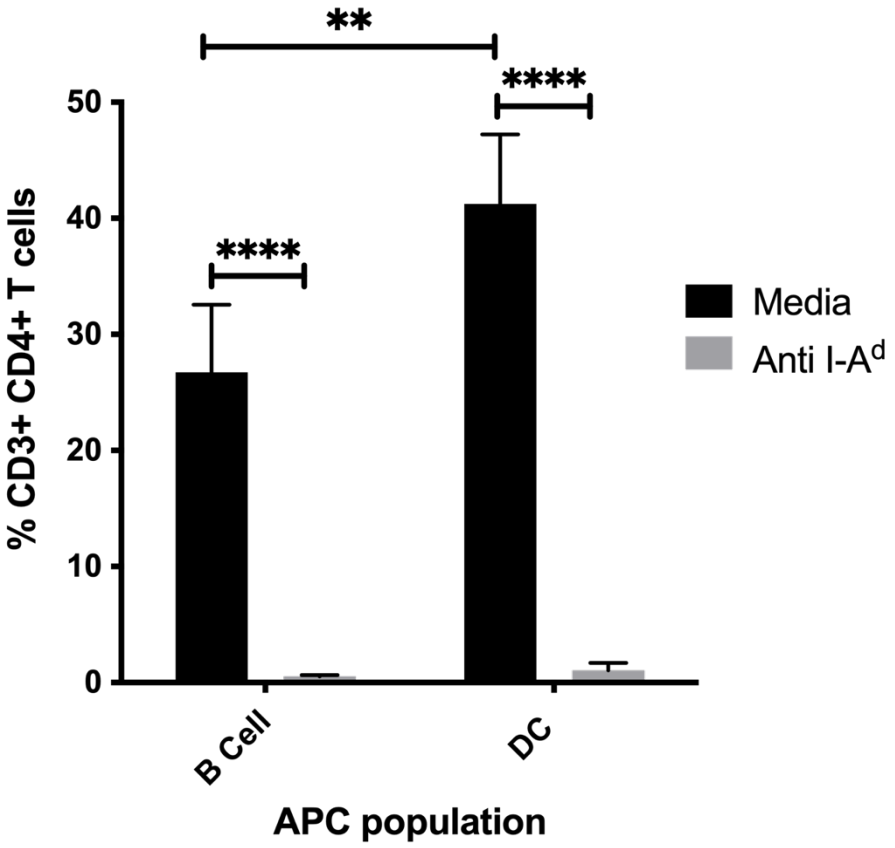
**
****
****
50
40
30
20
10
0
% CD3+ CD4+ T cells
Media
Anti I-Ad
B Cell
DC
APC population

Figure 8-7: Both B cells and DCs are capable of providing the tonic signals necessary for maintaining CD4+ T cell responsiveness. 200 DO11.10 CD4+ T cells were cultured with 3000 B cells or 3000 DCs for four days in the presence of 1 μg/mL LPS, and then stimulated with $3x10^4$ T-depleted splenocytes and 0.3 μM $OVA_{323-339}$. The number of viable CD3+ CD4+ T cells was determined on day 4 post stimulation by flow cytometry. Each bar represents the average of three identical cultures and error bars represent standard deviation from the mean. Statistical significance was determined using two-way ANOVA, with P values adjusted for multiple comparison using Holm-Šídák's multiple comparison test. ** < 0.01, **** < 0.0001.

CHAPTER 9 - DISCUSSION

9.1 The essential elements of the Quorum Hypothesis

I start by outlining the essential elements of the Quorum Hypothesis. Although I have attempted to make the hypothesis plausible in the Introduction, and discussed its physiological significance, I think it helpful, in discussing my observations, to start by listing its salient features. This will provide a context for the discussion.

According to the Quorum Hypothesis, a minimum number of antigen-specific CD4+ T cells is required for the antigen-dependent activation of a naïve CD4+ T cell to produce cytokine-producing cells, whereas a single antigen-specific CD4+ T cell can be inactivated on antigen encounter. Secondly, the critical collaboration between the CD4+ T cells required for CD4+ T cell activation is postulated to be mediated by an antigen-specific B cell. This proposed mechanism ensures that the critical CD4+ T cell cooperation involves the "recognition of linked epitopes", i.e. the pertinent peptides recognized in the context of MHC by the cooperating CD4+ T cells are derived from the same physical entity that has been endocytosed by the B cell via the BCRs. Thirdly, the model proposes a role for APC other than B cells in a first step in the activation of CD4+ T cells. Antigen stimulates the CD4+ T cells to multiply in this first step. This step was proposed on both observational grounds and because it partly overcame the "Scarcity Problem", as outlined in the introduction. As also outlined in the introduction, this model explains how peripheral CD4+ T cell tolerance is achieved in a manner consistent with The Historical Postulate.

This minimal description raises two questions. Although the experiments I carried out did not directly attempt to answer these questions, they were ever present in our considerations, and affected our experimental design. The first problem is referred to as the priming problem. The model is envisaged to apply to the activation of a naïve CD4+ T cell in an immunocompetent person or animal. There are populations of partially/fully activated B and T cells in such persons/animals and so, so long as some of these are specific for the antigen, there is no problem in visualising how the model applies. However, consider the situation in a fetus or neonate before

any immune responses have taken place. In this case, one would anticipate there are no activated lymphocytes. We need activated CD4+ T cells for antigen to optimally activate B cells, and the model proposes that B cells must be activated to in turn activate a naïve CD4+ T cell, as envisaged in step 2. Where, in this case, does the first activated lymphocyte come from? This is referred to as the "Priming Problem". Although there are potential solutions to how the priming problem can be "solved", our awareness of the problem and related issues led us to be very conscious of how our observations might depend on whether the lymphocytes we dealt with were in a truly ground state. The second question can for convenience be referred to as the "Numbers Problem". The model states that a minimum number of lymphocytes, in terms of CD4+ T cells (and B cells) are required to activate CD4+ T cells. At the same time, it is proposed that single CD4+ T cells can be given signals to multiply before step 2, in which the CD4+ T cell cooperation takes place. It could be that a single CD4+ T cell multiplies sufficiently well so that step 2 can be realised from the progeny of a single CD4+ T cell. In this case, the explanation of peripheral tolerance would be undermined, and so the major rationale for the model would be lost. "The numbers problem" is how can the situation just outlined be avoided? A general solution, without a detailed proposal for how this solution could be realised in cellular/molecular terms, is that the number of CD4+ T cells at the time of antigen impact is critical in the fate determination of the effector CD4+ T cells generated rather than the number of CD4+ T cells present a few days after antigen impact. In fact, our studies revealed unexpectedly such a situation, as we shall discuss.

9.2 Modifications to an experimental system already in use in our laboratory in my attempt to achieve my research goals

To test the Quorum Hypothesis, I first sought to develop an in vitro system where CD4+ T cell cooperation can be observed to affect the fate of the CD4+ T cells. In other words, I aimed to culture CD4+ T cells in limiting numbers so that the number of cytokine-producing cells per input CD4+ T cell increases as the number of input CD4+ T cells is increased.

I opted to use TCR transgenic CD4+ T cells so the number of CD4+ T cells/well at the initiation of culture could be readily varied. I employed a system previously developed in the lab using

DO11.10 CD4+ T cells, as described Chapter 1. The previous studies had the aim of examining how different conditions of culture affect the differential generation of Th1 and Th2 cells and employed a minimum number of 1000 transgenic CD4+ T cells per culture well. We were unable to observe cooperation when titrating CD4+ T cells between 6 and 100 per well, as the number of IFN-γ-producing cells per input CD4+ T cell did not rise when increasing the number of input CD4+ T cells. DO11.10 CD4+ T cells, by virtue of their being RAG-sufficient, do not suppress the endogenous recombination of the alpha chain of the TCR, resulting in the generation of bispecific CD4+ T cells. These bispecific CD4+ T cells can potentially be activated by environmental antigens. We initially speculated that the use of these partially activated, bispecific CD4+ T cells contributed to our inability of observe cooperation. The existence of activated, or partially activated B and T cells might circumvent, or minimize the need for, CD4+ T cell cooperation in the generation of IFN-γ-producing Th cells. I attempted to overcome this possibility by using a CD4+ T cell isolation kit that depletes "activated" CD25+ CD44+ cells. I did not formally quantify, by flow cytometry, the ability of this kit to remove CD25+ CD44+ cells, but it decreased the number of isolated CD4+ T cells by 60-70%, a surprisingly large number. Nevertheless, we again did not observe cooperation when we cultured CD25- CD44- DO11.10 CD4+ T cells similar numbers of CD4+ T cells per well. It remained possible that the CD25- CD44- DO11.10 CD4+ T cells did not represent a naïve population. In other words, it is possible that population of CD25- CD44- DO11.10 CD4+ T cells contained cells had been activated or partially activated. We naturally examined the consequences of culturing even lower numbers of CD4+ T cells/well, namely an average of 1 and 3 cells per well. We also assessed the generation of IL-2-producing cells. As reported in Chapter 4, our observations showed that single CD4+ T cells generated IL-2-producing cells, whereas an average of 3 input CD4+ T cells led to the generation of both IL-2- and IFN-γ-producing cells. This is a striking result in two respects. Firstly, as many cytokine-producing cells were recovered at day 5 of culture, typically about 100 when an average of 1 CD4+ T cell was plated per well, it is clear that the single CD4+ T cell multiplied extensively. Yet the fate of the CD4+ T cells, when plated at an average of 1 or 3 cells per well, as judged by the effector CD4+ T cells produced, was dramatically different. This observation demonstrates that the number of CD4+ T cells at the initiation of culture, rather than after a few days, is important in "fate determination". This observation might therefore provide a model

system for examining how the "Number Problem" might be resolved, as listed under "Future Directions".

Secondly, the observation contradicts the prediction of the Quorum Hypothesis that single CD4+ T cells should be inactivated by antigen. There are at least four non-mutually exclusive possibilities that can account for this observation:

1) The population of CD25- CD44- DO11.10 CD4+ T cells contained a large number of CD4+ T cells that were in a non-naïve state, whereby their induction by antigen to produce IL-2 is cooperation independent.
2) The source of APC might contain sufficiently activated B cells (or other APC) that single CD4+ T cells can be activated to generate IL-2-proding CD4+ T cells.
3) The level of LPS in the cultures created a non-physiological circumstance such that single CD4+ T cells can be induced by antigen to produce cytokines
4) The Quorum Hypothesis is incorrect

The third possibility seems particularly plausible. LPS binds to TLR-4 and induces the expression of the costimulatory molecules CD80 and CD86 (B7.1 and B7.2, respectively) and stabilizes peptide-MHC II complexes on the surface of B cells and DCs (104, 172-174). Furthermore, LPS binds to TLR-4 on the surface of murine CD4+ T cells and influences their activation (167, 168). Therefore, it seemed likely that the presence of LPS throughout the five days of culture might have created a non-physiological environment where CD4+ T cell inactivation is an unlikely outcome and that single CD4+ T cells could be induced to generate IL-2-producing cells.

We had originally started our studies employing CD4+ T cells from DO11.10 transgenic mice that were in a RAG -/- background, to minimize environmental priming of bi-specific CD4+ T cells.

We anticipated their use would provide a more uniform source of CD4+ T cells in a naïve state. These mice became unavailable from the supplier. We therefore decided to explore whether we could find another source of TCR transgenic mice in a RAG-/- background to provide a relatively homogeneous population of naive CD4+ T cells. Dr Colin Anderson offered to give us breeding pairs of Marilyn transgenic mice, whose features have been described in Chapter 5. I decided this system would allow us, at least partially, to overcome the environmental priming of our CD4+ T cells. High on my priority was to test whether we could get consistent activation of CD4+ T cells without adding LPS to our cultures.

9.3 The Marilyn system: the demonstration, and the partial elucidation of the mechanism, of CD4+ T cell cooperation in the generation of IL-2 and IFN-γ-producing CD4+ T cells and the raising of a new question

I have described in Chapter 5 that Marilyn CD4+ T cells, cultured at low densities without LPS, failed to generate cytokine-producing cells, in contrast to those cultured at higher densities. One might argue that low density cultures, by virtue of their containing low numbers of input CD4+ T cells, result in the generation of too few cytokine-producing cells to be detected by our modified ELISPOT assay. This is highly unlikely. Higher density cultures generate about 10 cytokine-producing cells per input CD4+ T cells by day 5 of culture. If the response was density independent, we would expect the number of cytokine-producing cells generated from cultures containing 30 input CD4+ T cells to be 300 per culture, which would be readily detectable by ELISPOT. We also explored the possibility that low density cultures could not readily generate cytokine-producing cells due to a deficit in their proliferative capacity. Our observations proved otherwise. The demonstrated lack of a difference in proliferation despite a stark difference in production of cytokine-producing cells again reflects a mechanism exists that has solved the "Numbers Problem"! This finding is also a critical step, according to our model, towards establishing an in vitro system for analyzing the antigen mediated inactivation of CD4+ T cells. Marilyn CD4+ T cells cultured at a low density differentiated into cytokine-producing cells when cultured with 1000 OT-II CD4+ T cells. Importantly, OT-II CD4+ T cells only efficiently helped Marilyn CD4+ T cells if their respective peptides were on the same APC, suggesting that CD4+ T

cell cooperation occurs by a linked mechanism, consistent with the mechanism envisaged in the Quorum Hypothesis.

Our studies shed light on recent observations, published by Polonsky et al, supporting the existence of a quorum-dependent mechanism in the generation of central memory T cells (175). In their *in vitro* system, they demonstrated that the upregulation of expression of IL-2 receptor alpha, CD69, and CD44 was quorum independent. However, the generation of central memory T cells, as assessed by their expression of CD62L and gene expression profile, was indeed so. Importantly, and similar to our findings, this quorum mechanism was independent of the proliferation capacity of the cultured T cells but was dependent on short-range signalling, leading to the suggestion that quorum sensing occurs by a linked mechanism.

Two other features of our experimental system call for comment. We cultured CD4+ T cells in the presence of $3x10^4$ APCs and we use peptides as antigen rather than intact protein. This choice is again one of history, given that we started with an in vitro system in which peptide was used to activate CD4+ T cells. It would be more physiological for several reasons to use a protein antigen. Nevertheless, given the system employed and the observations made, and recognizing there are many more APCs presenting the peptide than the number of CD4+ T cells at the initiation of culture, a question arises: how can cooperation occur by a linked mechanism? It is difficult to envisage how CD4+ T cells interact with the same APCs to cooperate, as there is a gross excess of presenting APCs at the initiation of culture, a situation that, at face value, makes this process statistically highly unlikely. The problem becomes even more remarkable when considering the response of cultures containing an average of 1 and 3 CD4+ T cells per well, especially when considering that LPS induces B cell proliferation (176), further exacerbating the APC excess. We discussed in Chapter 1, above, how scarce B and T cells can interact in vivo through lymphocyte recirculation and lymph node organization (97-100). Here, we feel bound to consider how relatively few CD4+ T cells are able to cooperate *in vitro* in the presence of a vast excess of APCs. We propose that the interaction between CD4+ T cells and APCs is dynamic and cooperative, whereby a productive interaction (i.e., TCR binding to cognate peptide-MHC II complexes)

between a CD4+ T cell and an APC leads to stronger future interactions between CD4+ T cells and that particular APC. Therefore, CD4+ T cells initially start scanning the APCs in culture, leading to the formation of interaction clusters, whereby a few APCs accumulate more interactions with CD4+ T cells compared to other APCs, enabling them to mediate further cooperation. It is also likely that once an APC accumulates sufficient interactions, it becomes more efficient at activating CD4+ T cells. The possibility of multiphase dynamic interactions is not unusual and is the subject of intense investigation (177-179). For example, it has been shown that CD4+ T cells stimulated with anti-CD3 antibodies were able to induce the expression of B7 on B cells, leading in turn to more robust CD4+ T cell activation by B cells (180, 181). With that being said, it remains possible that cooperation between CD4+ T cells in the generation of IFN-γ-producing cells observed when culturing an average of 3 CD25- CD44- DO11.10 CD4+ T cells per well could have occurred by an unlinked mechanism. Further investigation is required to determine the mechanism of cooperation using peptide pulsed APCs. It is worthwhile noting that the APCs used in our system were isolated from wild-type animals that were at least 8 weeks old and housed in specific pathogen free conditions. Therefore, it is likely that some of the APCs isolated have been previously activated by environmental antigens. Such activated APCs may interact more efficiently with CD4+ T cells compared to other APCs and any serve as "nuclei" for CD4+ T cells cooperation.

9.4 How the analysis of the mechanism of CD4+ T cell cooperation mediated by APC might be taken further

We have previously titrated the APC number per culture well and determined that 3x104 is optimal for supporting the antigen-dependent activation of CD4+ T cells with T cell-depleted splenocytes as the source of APCs. Adding too few or too many APCs had detrimental consequences on the number of cytokine-producing cells generated per input T cell. We speculate that this could be due to factors beyond antigen presentation, such as trophic influences. The effect of APC number on the ability of CD4+ T cells to cooperate could be further explored in the future by using different ratios of pulsed and non-pulsed APCs, up to a total of $3x10^4$, to determine how this ratio of pulsed to non-pulsed APCs affects the generation of cytokine-producing cells. Such an approach can also be used to determine in this system whether cooperation is only achieved if B cells are double-

pulsed. We speculate that in such studies, the number of cytokine-producing cells per input would be higher if a lower number of pulsed APCs is used. It would be particularly interesting in the context of the Marilyn system to examine whether in the relatively LPS-free cultures the number of T cells required to support the generation of cytokine-producing cells could be considerably reduced, and the efficiency of generation of cytokine-producing cells per input CD4+ T cell increased.

9.5 Different roles for B cells and DCs in context of the Quorum Hypothesis

Our studies demonstrated that a mixture of B cells and DCs, presumably serving as a source of APC, reliably supported the generation of cytokine-producing cells only from high density cultures of Marilyn CD4+ T cells when LPS was not added. However, in the presence of LPS, only B cells supported the generation of cytokine-producing cells from low density cultures. The Quorum Hypothesis postulates that the B cells are the critical APCs for mediating CD4+ T cell cooperation. I shall attempt, below, to consider the pertinence of these findings in the context of the Quorum Hypothesis.

Under steady state conditions and according to the Quorum Hypothesis, CD4+ T cells specific for self-antigens are kept at low numbers, as explained in Chapter 1. In addition, due to absence of an inflammatory environment, the quorum threshold is high, which precludes the activation of self-specific CD4+ T cells. Suppose an infectious entity impinges upon the immune system. DCs, which also present a variety of self-antigens, will take up the pathogen and will mature. However, due to the high quorum threshold, the CD4+ T cells interacting with these DCs will not differentiate into cytokine-producing cells. It is worthwhile noting that some of the CD4+ T cells interacting with the DCs will be specific for self-antigens. After their interaction with DCs, we suppose, for reasons already outlined, the CD4+ T cells will migrate to the B cell zone in the lymph nodes. As mentioned above, B cells only efficiently present epitopes from antigens for which their B cell receptor is specific (94, 182). Furthermore, B cells specific for self-antigens are also kept at low numbers, as discussed previously. Therefore, pathogen-specific B cells will take up the pathogen, present pathogen-derived epitopes, and mediate cooperation between CD4+ T cells

specific for the pathogen. Due to the presence of inflammatory signals, the quorum threshold may be somewhat lower and B cells will efficiently support the generation of cytokine-producing CD4+ T cells specific for the pathogen. Self-specific B cells are typically removed, and if present, will be less likely to be exposed to the inflammatory signals, as they will not interact with the pathogen (as long as the pathogen does not crossreact with self-antigens or produce PAMPs that act over long distances). Therefore, the quorum threshold for activation of self-specific CD4+ T cells will remain relatively high. As such, self-specific CD4+ T cells will fail to generate cytokine-producing cells. This model explains how the different ability of DCs and B cells to mediate quorum sensing allows the immune system to discriminate between self and foreign antigens.

We recognize that some of the statements we have made above will make some readers uneasy. DCs are considered by many to be the professional APCs responsible for activation and differentiation of CD4+ T cells (84, 104, 105, 109, 111, 112, 172, 183-186). We would like to note that many systems use artificially differentiated DCs, artificially high number of CD4+ T cells, and/or B cell knockout murine models where lymphopenic expansion could result in an abnormally high CD4+ T cell count (187). We do recognize that no in vitro or in vivo system is perfect. However, the big picture must be considered when inferences are made. We have previously put forward physiological considerations that we believe need to be taken into account by models of self-nonself discrimination and argued that overall the evidence is not inconsistent, and on the whole supports, an obligatory role of B cells in the activation of naïve CD4+ T cells under physiological conditions (7, 90, 188).

Lastly, we also recognize that we only used LPS as an example of a PAMP. A variety of PAMPs and DAMPs exist that bind to a variety of PRRs resulting in activation of different signalling pathways and leading to different outcomes in CD4+ T cell activation and DC maturation (108, 109, 189-191). Furthermore, the characterization of the different DC subsets is the subject of intense investigation (192-195). Therefore, our findings on the difference between B cells and CD11c+ DCs should be interpreted with caution. Nevertheless, LPS is a potent pro-inflammatory PAMP and CD11c+ is expressed on a wide variety of DCs, including plasmacytoid DCs (pDCs),

CD8+ classical DCs (CD8+ cDCs), CD11b+ cDCs, Langerhans cells and other DCs (reviewed in (196), Table 1).

9.6 Other important considerations pertaining to our in vitro system

We have established a powerful in vitro system that enabled us to demonstrate the ability of CD4+ T cells to cooperate, by a linked mechanism, for their activation and the generation of cytokine-producing cells. However, some concerns, in addition to the ones addressed above, may be raised that we would like to discuss here. Firstly, we acknowledge that our experimental system for analysing the respective role of B cell and DCs is not ideal. Antigen-specific CD4+ T cells were cultured with many more antigen-presenting B cells than there would be antigen-specific B cells in vivo. We did not investigate the effect of such an environment. A mixture of pulsed and non-pulsed B cells could be used to determine the effect of changing the number of pulsed B cells on the induction of cytokine-producing cells, as already discussed. Secondly, about 1% of T-depleted splenocytes are typically CD11c+. Therefore, culturing CD4+ T cells with $3x10^4$ CD11c+ DCs/well, as we did, is not physiological. Decreasing the number of DCs in culture had a detrimental effect on CD4+ T cell survival. Nevertheless, in vitro systems in the literature study the role of DCs by culturing CD4+ T cells exclusively with DCs.

Another major consideration to address is our definition of cytokine-producing cells. We claimed that culturing Marilyn CD4+ T cells at low densities failed to generate cytokine-producing cells. However, we did not investigate whether these CD4+ T cells made other cytokines such as IL-17, IL-10 or TGF-β. Further work is required to attempt to fully characterize the CD4+ T cells cultured at different densities.

Lastly, we did not address the role of macrophages, present in the T-depleted splenocytes population, in CD4+ T cell activation. Given that the macrophages are not antigen-specific APCs, we hypothesize that they would have a similar role as DCs. We have not yet conducted an analysis of the role of macrophages in our in vitro system.

9.7 Difficulties encountered when attempting to develop an in vitro model of CD4+ T cell inactivation

We commenced our work on the antigen-dependent inactivation of CD4+ T cells when we had found that Marilyn CD4+ T cells cultured at low densities proliferated without differentiating into IL-2 or IFN-γ-producing cells. We describe our unsuccessful attempts to develop such a system, and the difficulties encountered. The first difficulty was that it took two years to establish a Marilyn breeding colony here that produced a steady supply of experimental mice, despite considerable advice from Dr Anderson, who had given us the breeding mice from his colony.

To determine whether CD4+ T cells can be inactivated by antigen, a small number (N) of Marilyn CD4+ T cells would be cultured with antigen for some time (t). Afterwards, the CD4+ T cells would be restimulated for five days under conditions known to support the generation of cytokine-producing cells, when a similar number of naïve T cells are cultured. A lack of response may indicate antigen-dependent inactivation. Both proliferation and cytokine production would be measured. A control population of N Marilyn CD4+ T cells would be cultured without antigen to confirm that any potential lack in generation of cytokine-producing cells was due to antigen and not due to cells failing to generate cytokine-producing cells due to some unfavourable in vitro conditions. Such experimental setup requires the following conditions to be met:

1) The stimulating condition used after t days post culture must be optimal enough to allow generation of cytokine-producing cells regardless of their densities. Otherwise, on day t, there will be too few CD4+ T cells to generate detectable cytokine-producing cells on day t+5, especially if no antigen was added on day 0.

2) The response detected on day t+5 must be generated only by the responding N CD4+ T cells cultured on day 0.

3) The time t must be sufficient for inactivation to take place and not too long such that CD4+ T cells die, in the absence of antigen, due to overcrowding, media depletion or other confounding factors.

Initially, we explored the possibility of using LPS to create the stimulatory environment in the second culture step employed to determine whether antigen had inactivated the Marilyn cells. However, such a setup posed a major challenge. We contemplated what N should be that would result reliably in failure to generate cytokine-producing cells and we decided to start with 10 Marilyn CD4+ T cells. However, as shown in figure 5-1, LPS is not reliable in supporting the generation of cytokine-producing cells from cultures containing 6 or 12 input Marilyn CD4+ T cells. Indeed, when 10 Marilyn CD4+ T cells were cultured with T-depleted splenocytes without peptide for 3, 4, 6, or 8 days and then stimulated with LPS and peptide, no cytokine-producing cells were detected. Therefore, we decided to abandon LPS and shift our attention to using irradiated effector Marilyn CD4+ T cells as a source of helper cells to be added on day t. This protocol is somewhat analogous to that originally developed and justified in our laboratory to show that the generation of CD4+ T cells mediating delayed-type hypersensitivity required CD4+ T ell collaboration. Briefly, 1000 Marilyn CD4+ T cells were stimulated by antigen for 5 days without LPS. On day 5, cells were harvested, purified, and irradiated with 900 rads. The irradiated cells were then added in different numbers as a potential source of effector CD4+ T cells.

Prior to setting up the inactivation experiments, we first sought to determine whether irradiated Marilyn CD4+ T cells were able to help non-irradiated Marilyn CD4+ T cells. However, when we titrated irradiated helpers with Marilyn CD4+ T cells, we failed to observe an enhancement in the number of cytokine-producing cells generated. We did not further explore why this approach failed. However, we knew that the activation of a limiting numbers of Marilyn CD4+ T cells could be helped by OT II cells, and decided to explore this approach. Unfortunately, by the time we established the in vitro system whereby OT-II CD4+ T cells provided help to Marilyn CD4+ T cells, the lab was closed due to the pandemic. We believe that this system would be powerful enough to enable us to study CD4+ T cell inactivation

9.8 Concluding remarks and future directions

We have provided evidence that the generation of IL-2 and IFN-γ-producing Marilyn CD4+ T cells requires CD4+ T cell cooperation. Using peptide pulsed APCs and OT-II CD4+ T cells as a source of helper cells, we demonstrated that CD4+ T cell cooperation mostly occurred by a linked mechanism. We also provided preliminary evidence potentially supporting different roles for B cells and DCs in quorum sensing that is consistent with the Quorum Hypothesis.

We believe that our studies and the in vitro system developed open avenues for answering two major questions: what different signals are required to cause CD4+ T cell multiplication or their multiplication and differentiation into cytokine-producing cells, and what genes are differently involved in these two fates; secondly, given that the interaction of antigen with different numbers of CD4+ T cells results in different fates of the CD4+ T cells, how does the immune system count the number of CD4+ T cells or, in other words, how is quorum detected? These suggestions are based upon my three main findings: 1) CD4+ T cells cultured at different densities responded differently to antigen in a repeatable manner, 2) OT-II CD4+ T cells constitute an efficient source of help for Marilyn CD4+ T cells and 3) our system is robust in that our modified ELISPOT assay allows us to detect cytokine-producing cells generated from a single input CD4+ T cell if this activation is efficient.

The first set of investigations pertains to the characterization of cells cultured at different densities. Using high throughput RNA-seq and flow cytometry, gene expression and surface marker expression could be corelated with situations that lead only to CD4+ T cell proliferation or such proliferation and also the generation of cytokine-producing cells. Such studies can shed light on the sequence of expression of genes and markers leading up to different fates.

The second major project involves the determination of how quorum is sensed. Our studies raise two related major questions. The first is at what point after antigen encounter do circumstances

determine the different fate of CD4+ T cells, these different fates involving either only T cell proliferation or proliferation and generation of cytokine-producing cells, or the generation of different spectrums of cytokine-producing cells? As already argued, these determinations must be set in place early after antigen encounter. Suppose that a culture of a number S (small) of CD4+ T cells results in F1 (fate 1) and of a number M (medium) results in F2 (fate 2). One could assess the potentiality of, say, CD4+ T cells or B cells, harvested at day 2 from M cultures, to affect various outcomes. Do day 2 CD4+ T cells from M cultures still express fate 2 when cultured at lower numbers for a further three days? Do day 2 B cells from M cultures affect the fate of CD4+ T cells when given at initiation of an S culture towards an F2 fate? Is fate determined by both cell populations? Once the role of different cell populations has been defined, the second question may be addressable. What determines or influences the quorum threshold at the cellular and molecular levels? Lastly, I hope my studies may contribute to the development of an in vitro system for discriminating the events leading to the inactivation and activation of CD4+ T cells by antigen.

CHAPTER 10 - REFERENCES

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