AGGREGATED Ig ELICITS CD4+ T CELL ACTIVATION AND IgG ANTIBODY RESPONSES

by

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Aggregated Species of Ig Elicit CD4 Activation and IgG Humoral Responses

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ABSTRACT

Immunoglobulin variable region diversity presents a unique challenge to the CD4 T cell repertoire to discriminate between self and nonself. Previous studies have suggested that deaggregated, monomeric IgG may be able to tolerize the CD4+ repertoire to its antigenic components, while aggregated species remain immunogenic even without adjuvant. I hypothesized that monomeric IgG might induce T cells to adopt a regulatory phenotype, and I had unique reagents to examine the primary and memory response at the level of a specific T cell clone. My data from in vivo studies utilizing the adoptive transfer of cells from a CD4+ T cell receptor (TCR) transgenic (Tg) line of mice show this distinction may be a function of aggregate stimulation of a T follicular helper (Tfh) response. I challenged mice with monomeric Ig and two aggregate populations: heat aggregated Ig and immune complexes. Both aggregate populations induced an IgG humoral response to an antigenic peptide within the Ig, which never occurred in mice receiving monomeric Ig. Aggregate populations induced an increased percentage of the TCR Tg cells to take on a Tfh phenotype. The aggregates drove higher percentages of these cells to further proliferative divisions in the early proliferative response, compared to monomeric Ig, which drove cells to fewer divisions. This was an intriguing indicator of the humoral immune response, and particularly intriguing given that the aggregates
were created from the monomeric Ig itself. Monomeric Ig did not induce cells to differentiate to become CD25+FoxP3+ regulatory T cells when compared to aggregate populations. I also tested these aggregate species capacity for driving TCR Tg cell division in mice depleted with anti-CD20 Ig. In B cell depleted mice, the heat aggregated Ig lost its stimulatory capacity, while immune complexes did not. This finding raised questions about the trafficking of antigenic species of the two aggregate populations. As a whole, this data suggests that immune complexes generated in vivo during monoclonal antibody therapy may be a significant source of immunogenicity that could lead to humoral rejection in patients.

The form and content of this abstract are approved. I recommend its publication.

Approved: Lawrence J. Wysocki
I dedicate this work to Mim, who has to be the most patient person on the planet,
even if she doesn’t know it.
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# TABLE OF CONTENTS

## CHAPTER

### I. INTRODUCTION

I.1 Prologue

I.2 Immunoglobulin as tolerogenic antigen

I.3 Immunoglobulin as immunogenic antigen

I.4 CD4+ T cells and the perception of immunoglobulin as antigen: model systems

I.5 Conclusion and the scope of the thesis

### II. MATERIALS AND METHODS

II.1 Production and characterization of reagents

II.1.1 T Cell medium (TCM)

II.1.2 Hank’s Buffered Salt Solution (HBSS)

II.1.3 Antibody production in mice

II.1.4 Handling and dialysis of ascitic fluid for anion exchange chromatography

II.1.5 Anion exchange chromatography

II.1.6 Low aggregation pharmaceutical buffer and Ig storage

II.1.7 Arsanilation of mouse serum albumin

II.1.8 mAb 36-71 Fab and F(ab’)_2 generation

II.1.9 Heat aggregation of Ig

II.1.10 Generation of immune complexes with Ars-MSA

II.1.11 Ultracentrifugation of Ig

II.1.12 Size-exclusion chromatography

II.1.13 Microflow imaging
II.1.14 Particle tracking analysis

II.1.14 Estimate of Ig mass in protein particles

II.1.15 Limulus amebocyte lysate endotoxin quantification

II.2 In vitro and in vivo analysis of CD4+ T cell behavior

II.2.1 Mice

II.2.2 Tissue harvesting and cell preparation

II.2.3 CFSE labeling

II.2.4 In vitro T cell activation assays

II.2.5 Adoptive transfers

II.2.6 Immunization protocols

II.2.7 Flow cytometry

II.2.8 Quantification of IgG α-Vκ36-71 antibody in sera

II.2.9 Anti-Vκ36-71 ELISPOT

II.3 Statistics

III. CHARACTERIZATION OF THE AGGREGATE CONCENTRATION, ADJUVANTICITY, AND IN VITRO STIMULATORY CAPACITY OF MONOCLONAL ANTIBODY 36-71

III.1 Introduction

III.2 Results

III.2.1 Production of mAb 36-71, 36-71 F(ab’)2 and Fab

III.2.2 Creation of heat aggregates and immune complexes

III.2.3 Ultracentrifuged mAb 3671 is monomeric when compared to heat aggregates and immune complexes

III.2.4 Immune complexes have an increased mass of particles with diameters in the nanometer range

III.2.5 Heat aggregates and immune complexes have an increased mass of particles with diameters in the micron range
III.2.6 Summary of mass distribution of Ig species ........................................... 54

III.2.7 Mass distribution of the alternative “large” heat aggregates and “large” immune complexes .......................................................... 54

III.2.8 Limulus amebocyte lysate assay does not rule out the possibility of LPS contamination ................................................................. 59

III.2.9 mAb 36-71 does not act as a non-specific adjuvant in an in vivo model ......................................................................................... 60

III.2.10 Heat aggregated mAb 36-71 is a more potent antigen for in vitro CA30 stimulation than monomeric Ig .............................................. 61

III.3 Discussion ........................................................................................................ 66

IV. IG AGGREGATION LEADS TO PRODUCTIVE, CD4-MEDIATED HUMORAL IMMUNITY WHILE MONOMERIC IG STIMULATES AN EXISTENT, BUT NON-PRODUCTIVE, CD4+ RESPONSE .......................................................... 71

IV.1 Introduction ..................................................................................................... 71

IV.2 Results ............................................................................................................. 73

IV.2.1 Identification of CA30 T cells by using congenic markers in the B6AF1 system ............................................................................. 73

IV.2.2 Monomeric Ig induces primary CA30 T cell proliferation, but heat aggregated Ig generates a weak humoral immune response .......... 73

IV.2.3 Heat aggregated, but not monomeric, Ig primes for a memory humoral response ........................................................................... 75

IV.2.4 Heat aggregated Ig drives CA30 T cells through more division cycles than monomeric Ig in the spleen ........................................ 81

IV.2.5 Heat aggregates induce an increase in CA30 T follicular helper (T\textsubscript{fh}) cells by day 14 ......................................................... 89

IV.2.6 Immune complexes generate a similar humoral immune response to heat aggregated Ig in the CA30 adoptive transfer model ......... 96

IV.2.7 Alternative “large” heat aggregates and immune complexes induce divergent proliferative profiles at day 5 ............................... 104

IV.2.8 The lack of a functional Fc diminishes presentation of a peptide in the F\textsubscript{ab} region ............................................................................. 107
IV.2.9 B cells are an important source of presentation for heat aggregated Ig antigen to CA30, but not critical for presentation of immune complex or monomeric Ig .............................................. 110

IV.3 Discussion........................................................................................................... 119

V. THE EFFECT OF IG FORM ON THE EXPANSION OF MEMORY CA30 T CELLS ............................................................................................................. 125

V.1 Introduction ........................................................................................................... 125

V.2 Results .................................................................................................................... 127

V.2.1 CA30 T cells exposed to monomeric Ig can expand in a memory response, but this expansion is similar to that of CD4+ T cells exposed to a tolerogen................................................................. 127

V.2.2 The primary expansion of residual CA30 T cells in negative control mice is larger than that of mice treated with various mAb 36-71 species................................................................. 131

V.2.3 Monomeric Ig is a poor inducer of memory expansion of CA30 T cells.............................. 133

V.2.4 Memory expansion of CA30 T cells primed with aggregated Ig species does not appear to be dependent upon rechallenge with the same antigen................................................................. 137

V.3 Discussion.............................................................................................................. 141

VI. DISCUSSION .......................................................................................................... 147

VI.1 Context and Discussion of the Findings.................................................................... 147

VI.1.1 Chapter III: Are particle mass and LPS contamination as important as immune complexes with regard to immunogenicity?........ 147

VI.1.2 Chapter IV: Monomeric Ig versus immune complexes: differential inducers of Th1 and Th2 development, or tolerogenic versus immunogenic stimuli?................................................................. 152

VI.1.3 Chapter V: Evidence of Th1 memory or a critique of my experimental protocol? ................................................................. 162

VI.2 Limitations ............................................................................................................ 164

VI.2.1 The use of the CA30 CD4+ TCR Tg T cell............................................................. 165
VI.2.2 The use of a single isotype.......................................................... 166

VI.3  Recommendation for future work .................................................. 168

VI.3.1 Building and transitioning to the B6.3kλ Tg mouse as a source of Ig .......................................................... 168

VI.3.2 The immunogenicity of immune complexes in monoclonal antibody therapy.................................................. 170

VI.3.3 The tendency of immune complexes to generate Th2 responses ...... 171

REFERENCES.........................................................................................174

APPENDIX

A. PERCENTAGES OF CD45.1+ CELLS IN EACH CFSE DIVISION FOR PROLIFERATION EXPERIMENTS..................200
LIST OF TABLES

Table

2.1 Reagents used for flow cytometry. ................................................................. 39

3.1 Mass distribution of experimental Ig species compared to ideal and recovered mass.......................................................... 55

3.2 Mass distribution of large heat aggregate Ig and large immune complex species compared to ideal and recovered mass......................... 56

4.1 Heat aggregated Ig generates a weak humoral response in some recipients of the CA30 T cell. ................................................................. 75

4.2 Heat aggregated Ig, but not monomeric Ig, primes for a memory humoral response.......................................................................................... 81

4.3 Immune complexes prime for a memory humoral response ...................... 99

4.4 Large heat aggregates and large immune complexes do not prime for a memory humoral response............................................................... 106

5.1 CA30 T cells exposed to monomeric Ig expand in a memory response, but this expansion is similar to that of CD4+ T cells exposed to a tolerogen................................................................................................. 131

5.2 The primary expansion of residual CA30 T cells in negative control mice is larger than that of mice treated with various mAb 36-71 species................................................. 136

5.3 Monomeric Ig is a poor inducer of memory expansion of CA30 T cells .......................................................................................................................... 140

5.4 Memory expansion of CA30 T cells primed with aggregated species does not appear to be dependent upon rechallenge with the same antigen .............................................................................. 143

A1 Mean number of divisions: monomeric and heat aggregated Ig ............... 200

A2 Mean number of divisions: monomeric and immune complex................... 200

A3 Mean number of divisions: large head aggregated Ig and large immune complex................................................................................................. 200

A4 Mean number of divisions: F(ab')2, Fab, and F(ab')2 complex..................... 201
A5 Mean number of divisions: Isotype control and α-CD20 m1g2A5D2

.......................................................... 201
## LIST OF FIGURES

**Figure**

3.1 Precipitation of mAb 36-71 with arsanilated mouse serum albumin ................. 47  
3.2 SEC chromatograms of experimental Ig species ............................................ 48  
3.3 Particle size distributions and integrated mass for nano-sized populations in experimental Ig species ................................................................. 50  
3.4 Particle size distributions, integrated mass and representative images of micron-sized particles in experimental Ig species ........................................... 52  
3.5 SEC, PTA, and MFI analyses of the large heat aggregated Ig and large immune complex samples ....................................................................................... 57  
3.6 mAb 36-71 does not act as a non-specific adjuvant in an in vivo model ............ 62  
3.7 Heat aggregated Ig is a more potent antigen than monomeric Ig for in vitro CA30 cell stimulation .................................................................................. 65  
4.1 Primary proliferative time course of the CA30 T cell in response to monomeric Ig and heat aggregated Ig ................................................................. 76  
4.2 Heat aggregated Ig, but not monomeric Ig, primes for a memory humoral response .............................................................................................................. 80  
4.3 Evidence of humoral memory response to a unique heat aggregated Ig species .................................................................................................................. 82  
4.4 Heat aggregated Ig drives CA30 T cells through more division cycles than monomeric Ig in the spleen, but not lymph nodes ......................................... 86  
4.5 Heat aggregated Ig induces an increase in CA30 T follicular helper (Tfh) cells by day 14 .............................................................................................. 93  
4.6 Immune complexes generate a similar primary expansion and humoral response to heat aggregates ................................................................................ 100  
4.7 Large heat aggregates and large immune complexes drive fewer divisions than expected ............................................................................................... 108  
4.8 The lack of a functional Fc diminishes presentation of a peptide in the Fab region but an F(ab')2 complex can rescue presentation ............................... 111
4.9 α-CD20 therapy diminishes proliferation of CA30 T cells in response to heat aggregated Ig ................................................................. 115

5.1 CA30 T cells exposed to monomeric Ig expand in a memory response, but this expansion is similar to that of CD4+ T cells exposed to a tolerogen............................................................................................................................... 129

5.2 The primary expansion of residual CA30 T cells in negative control mice is larger than that of mice treated with various mAb 36-71 species ........................................................................................................................................... 134

5.3 Monomeric Ig is a poor inducer of memory expansion of CA30 T cells .................................................................................................................. 138

5.4 Memory expansion of CA30 T cells primed with aggregated species does not appear to be dependent upon rechallenge with the same antigen ........................................................................................................................................ 142
CHAPTER I
INTRODUCTION

I.1 Prologue

When Emil von Behring and Shibasaburo Kitasato first identified a component of serum that could transfer passive protection against tetanus or diphtheria toxin between immunized and naïve animals, they arrived at the simple, yet powerful, conclusion that this discovery would spark a revolution in therapeutics. To say that their hunch augured well vastly underestimates the pharmaceutical-industrial complex that has emerged around the production of these antibodies, the name coined by Paul Ehrlich, for the use as therapy in an increasing number of disease states. It has been projected that in 2014, the year that this thesis was written, monoclonal antibodies will account for 166 billion dollars in sales for pharmaceutical companies, which will be approximately one third of the total prescription drug market [1].

Towards the end of their seminal publication, Kitasato and Behring sought to relay an optimistic message of therapeutic potential by quoting von Goethe’s Faust Part I, “Blood is a very special juice.” In the spirit of Mephistopheles, the speaker goading Faust into signing a contract for his eternal soul, this benefit came with a price [2]. The early history of commercial antibody production, initiated by Behring himself, would also presage more complicated aspects of our current understanding of the immunoglobulin he co-discovered. In a subsequent monograph, Behring stated a goal of mass production of antitoxin for the treatment of diphtheria in humans and set forth the experimental procedures by which he would ascertain an appropriate therapeutic protocol [3]. Starting in 1892, he worked with Farbwerke Hoechst to produce sheep anti-
diphtheria toxin to be used in humans. This antitoxin was a clinical success, but there was a proviso: it was evident from the earliest usages that patients often developed skin rashes in response to multiple injections and that some patients developed systemic symptoms (fever, arthralgias) that Clemens von Pirquet and Bela Shick later described as “serum sickness” [4, 5]. Behring agreed with the hypothesis of Pirquet and Shick: there was something foreign about transferring antitoxin from animals into humans that led to rejection. Behring spent years devising purification techniques to rid the antitoxin of this consequence.

While the modern techniques to produce therapeutic mAbs have sought to eliminate any traces of non-human structure, there are nevertheless immune responses to virtually all of these agents in some fraction of patients. This is a therapeutic sequela of a paradox of Ig ontogeny that has long fascinated the immunological community: if the immune system recognizes non-self, amino acid sequence diversity in pathogens and antibodies generate amino acid sequence diversity to bind pathogens, how does the immune system avoid responding to the antibodies mediating its own action? To answer this question definitively in the context of every aspect of the immune system during a graduate thesis would border on megalomania. Instead, the aims of this thesis will focus on the capacity for CD4+ T cells to perceive and respond to an antigenic sequence within an Ig by initiating a productive humoral immune response. The ability, or lack thereof, for a CD4+ T cell to initiate this response has significant ramifications for the success of therapeutics in some fraction of the patient population for virtually every mAb, as well as implications for the more general biological question posed above: how do we maintain antibody diversity without rejecting antibody diversity?
Since the history of research surrounding Ig spans over one hundred years, it is unsurprising that there is a wealth of published experimental data that must be considered when addressing questions in this vein. To confound the analysis further, much of this research falls into one of two diametrically opposed categories: *Ig as tolerogenic antigen* or *Ig as immunogenic antigen*. For the purposes of the introduction to this thesis, I hope to present a survey of the literature detailing these competing views. This summary of an established division in the literature will be followed by a discussion of Ig immunogenicity and CD4+ T cell response with a particular emphasis on the CA30 Tg T cell model system that was used in the experiments detailed in the later chapters. With this background established, I will present the hypotheses explored in the data chapters of the thesis and continue to the presentation of the body of the work.

### 1.2 Immunoglobulin as tolerogenic antigen

The 1940s to early 1960s was an era in which convergent lines of research laid a foundation for the study of immunological tolerance. In the 1930s, researchers had come to the conclusion that young animals made poor immune responses when challenged with commonly studied antigens, which was considered to be a function of some deficiency in Ig production [6-12]. In the 1940s, this work was extended through the study of serum gamma globulin to assay for changes in protein character during animal development; it became obvious that young animals tended to have a delay between birth and the capacity to produce specific, high titer Ig responses upon challenge [12-18]. Around the same time, Ray Owen identified fetal “graft” tolerance through his studies of the circulations of fraternal twin calves; twin calves that gestated with a common placental circulation, but had divergent blood types, could contain red blood cells that displayed red cell antigens
that came from either of the twins. Many of the twins assayed were adults, which implied a transfer of a regenerating progenitor cell that could continuously produce red blood cells in the recipient twin. Owen commented on the potential for immunological implications of this finding [19]. Frank Macfarlane Burnet and Frank Fenner cited both of these phenomena, the variation in Ig production during youth and the fetal tolerance in fraternal twin calves, in the second edition of “The Production of Antibodies” [12] Burnet would eventually win the Nobel Prize for his analysis of Owen’s experiment, hypothesizing that the transfer of foreign cells into an embryo could yield indefinite immunological tolerance to the cells, which was later borne out in the lab of co-Prize winner Peter Medawar. While it was not stated explicitly that these phenomena were the impetus for his conclusion, Burnet went on to state that it was likely that antibody production was affected by both the age of the animal as well as “the nature and frequency of the antigenic stimulus.” Considering this conclusion with his hypothesis regarding fetal tolerance merely a chapter before, it is reasonable to consider Burnet to be an intellectual forefather of thought on how tolerance to serum proteins is achieved and maintained [12, 20, 21].

A year before the reissue of “The Production of Antibodies”, Lloyd D. Felton delivered the presidential address to the American Association of Immunologists and spoke about a harmful manifestation of tolerance, “immune paralysis.” By injecting 500 µg of pneumococcus polysaccharide into mice, his group was able to eliminate the capacity for these mice to respond to an immunizing dose of polysaccharide [22]. During the 1950s, this “immune paralysis” intrigued a number of researchers who attempted to recapitulate these results by injecting large quantities of heterologous protein such as
albumin or gamma globulin, rather than polysaccharide, into rabbits or outbred mice. However, they could not repeat the paralysis until they followed the strategy implied by the work of Burnet and Medwar, by injecting the tolerizing dose of protein into neonatal animals. This protein-specific tolerization persisted for extended periods of time after its induction and could be maintained even longer by administering booster immunizations of the experimental antigen [20, 21, 23-42].

When David Dresser began his experiments injecting bovine gamma globulin (BGG) into CBA mice, he was extending knowledge of this tolerization phenomenon in an inbred strain of mouse, using a published method to detect antigen-elimination, and investigating kinetic variables. At first his results mirrored those of previous groups: the tolerization phenomenon occurred only in neonatal mice or, inconsistently, when BGG was injected intravenously. However, while studying the role of lipids as adjuvants, he made a notable and divergent finding: if he removed particulate matter by ultracentrifugation, the BGG tolerized adult animals via any route of administration. This finding was subsequently recapitulated in guinea pigs. While previous tolerance regimens had required large doses (hundreds of milligrams) of antigen and been inconsistent, ultracentrifuged BGG tolerized all adult mice at relatively low doses (hundreds of micrograms). Dresser had identified heterologous, deaggregated gamma globulin as a potent tolerogen that could be used in studies ranging from the testing of adjuvants to understanding the kinetic of immunological tolerance [40, 42-52].

William Weigle recognized the novelty of this tolerization by gamma globulin immediately and directed his research group to dissect the cellular mechanisms, a research question that he would follow throughout the rest of his career. Within two
years of Dresser’s discovery of the unique function of BGG, Weigle’s group demonstrated that the same phenomenon occurred with human gamma globulin (HGG) and that the splenocytes of these mice remained tolerant even if adoptively transferred into an irradiated, secondary host. This “adoptive tolerance” intrigued the researchers, as it could be mediated by either neonatally or adult tolerized splenocytes, and indicated to them that the mechanism of tolerance might be similar in both groups. Continuing their studies with HGG, the group challenged the tolerization phenomenon with intricate time courses and injections of adjuvant in the context of different mouse strains, describing subtleties regarding the onset and abatement of tolerance that will be discussed in the data chapters of this thesis [53-66].

The original description of “adoptive tolerance” to HGG utilized splenocytes, a bulk population that contained both T and B cells. In 1966, Henry Claman and his colleagues described the necessity for synergism between thymic- and bone marrow-derived cells for Ig production [67]. Jacques Chiller, Gail Habicht, and Weigle made the logical leap and initiated experiments to test which cell population, the thymic or the bone marrow, was responsible for the tolerance to HGG. The simple, surprising answer: both populations of cells were tolerized by the deaggregated HGG. The kinetics of the phenomena were different, bone marrow cells were tolerized for about two months while the thymocytes were tolerized for over four months, but both populations of cells had to be functional to initiate an antibody response to immunogenic HGG and it was possible to show that tolerized cells from either location maintained tolerance in a transfer experiment [68-77]. This differentiation between tolerance in the B and T cell compartment was paradigm-creating; in the year before this thesis was written this work
was republished in the *Journal of Immunology* “Pillars of Immunology” series with a commentary about its historical significance to the field of self-tolerance [78]. From the perspective of Ig, it raised the questions: how does heterologous Ig tolerizing the two types of cells, and was there a reason that Ig would induce this response rather than another serum protein such as albumin [54]?

To continue this progression of thought, it is valuable to consider a vein of Ig research that occurred in the years after Chiller, Habicht, and Weigle, but that ultimately drifted from the mainstream of immunology research. Concurrent with the development of this suppression literature, other groups were building a compelling dataset to verify the Clonal Selection hypothesis, conceived in parts by Burnet, Talmage, and Jerne [79-82]. As an important component of clonal selection involves the unique specificity of Ig receptors on lymphocytes, immunologists became intrigued by extensive diversity evident within the lymphocyte repertoire: how many specificities could a mouse have? Could you transfer specificities from mouse to mouse? What was the cellular basis of Ig allotype suppression [83-85]? Researchers injected Ig from myelomas into mice and observed tolerance or, if aggregated, immunogenicity [86]. The immune system became an infinite hall of mirrors: if antibodies could elicit antibodies against themselves and those antibodies could have antibodies against themselves as well, was this suppression in immune response to something like HGG just an extension of battling antibody specificities? Although perhaps an inelegant description, this was the nexus of the “network theory of the immune system” of Jerne that became a significant topic of research from 1974 to roughly 1982 [84]. An important component of the theory as it developed was the existence of T cells that suppressed antibody responses due to
idiotypic-anti-idiotypic interactions, which was thought to explain the tolerized thymocytes of Chiller et al; these T cells were known as “suppressors” and contained a gene that mapped a region of the Major Histocompatibility Complex called “I-J”. The existence of these suppressor T cells was debated within the literature with skeptics (including the chair of this thesis committee) and proponents based upon experimental data, until ultimately RNA hybridization revealed that the putative “I-J” gene did not exist at the site where others claimed to map it [83, 87-105]. To a great extent, network theory was relegated to the scrap heap of immunological thought. However, this does not mean that every experimental finding from the period is completely irrelevant. For example, regulatory T cells (T_{reg}) function in some of the ways that the literature predicted as mechanisms for the suppressor T cell. The capacity to suppress antibody responses with anti-idiotypic antibodies is still an intriguing finding and one must consider why this is, or is not, the norm in natural immune responses.

After the exodus from the realm of network theory, a few labs persisted in their efforts to define a mechanism for the tolerogenic effect of gamma globulin. The HGG tolerization was robust and highly reproducible. Many of the experimental observations were reminiscent of data that would be described in later, more controlled models testing central and peripheral tolerance, however, researchers struggled to find a definitive, unifying theories to explain the phenomenon. Ideas ranged from antibody-dependent cell cytoxicity to a lack of appropriate costimulation in both the B and T cell compartments, a suppressive effect mediated by macrophages to an interaction with B cells that specifically targeted IL-4 secreting Th_{2} T cells [106-128]. The striking binary nature of the tolerance data compelled researchers forward: tolerized T cell populations showed
virtually no proliferation or cytokine production in response to a secondary challenge \emph{in vitro}.

In recent years, focus on the tolerogenic effect of Ig has often shifted to a consideration of issues related to therapeutics. Multiple groups have identified the inclusion of peptides into the structure of IgG as a potential tolerogenic delivery mechanism that can decrease experimental immune responses or even temper pathology in a mouse model of autoimmune disease [129-148]. Findings such as these, in conjunction with computational methods of tolerogenic peptide analysis, have led to an intriguing new hypothesis: some IgG may contain naturally tolerogenic peptides that generate the production of T\textsubscript{reg} cells that can suppress ongoing response to other peptides within the structure [149-157]. Other hypotheses surrounding IgG suppression of the immune response derive from knowledge about the function of inhibitory receptors for the constant region of IgG (Fc\gamma) or sugar moieties (sialic acid) that are attached to the IgG during trafficking in the endoplasmic reticulum. A notable example of the former is the work of Solveig Reitan and Kristian Hannestad, who examined the adjuvanticity of Ig containing an immunogenic idiotope but paired with differing isotypes. They found that IgG and dimeric IgA were tolerogenic, while IgE, native, pentameric IgM, and F(ab’\textsubscript{2}) were immunogenic [158-160]. While they were unable to completely eliminate this dichotomy (tolerogenicity/immunogenicity) in various Fc receptor knockout mice, they concluded that it was likely that multiple Fc mediated mechanisms were involved in the process, including an inhibitory signal from the Fc\gammaRIIB on the surface of B cells and ADCC induced by stimulatory Fc\gamma on the surface of NK cells. In the case of sugar moieties, the laboratory of Jeffery Ravetch has identified a receptor for sialic acid (SIGN-
R1 in mice, DC-SIGN in humans) as the critical component for the suppression of immune function by high doses of intravenous administration of Ig (IVIg) [161-164]. As opposed to much of the tolerogenic phenomenology discussed thus far, IVIg mediates a generalized down regulation of immune function rather than a targeted tolerization, and has been used to alleviate acute episodes of autoimmune disease. From their data, the group has surmised that SIGN-R1 is initiating this downregulation in function, however the direct mechanism of this systemic downmodulation is still in question. Ravetch and his colleagues propose that engagement of SIGN-R1 is causing the secretion of a soluble factor that leads to upregulation of the inhibitory FcγRIIB on effector macrophage populations that suppress ongoing inflammatory responses.

To summarize this historical progression of work in regards to the tolerogenicity of Ig: the administration of deaggregated gamma globulin, whether heterologous or isologous, does not yield a productive immune response as defined by the development of anti-gamma globulin antibody. Furthermore, animals treated this way are tolerized in so far as they do not make a secondary or primary response upon challenge with an immunogenic form of the Ig and adoptive transfer of lymphocytes has demonstrated that they cannot participate in a productive response even when removed from the tolerizing environment. There is mixed evidence for this tolerance being active (i.e. regulatory cells) vs. deletion of potential effectors, and both mechanisms may be able to play some role within both the B and T cell compartment. More recent research has suggested roles for either cellular regulation or deletion, although much of the regulatory literature has either been predominantly putative or phenomenological at the level of suppression of autoimmune function. One deficit in a great deal of this literature has been its reliance on
assumed behavior of bulk populations rather than on cells with identifiable or defined antigenic specificity, and as such, it has been difficult to dissect the potential for regulation versus deletion. The physiological results obtained are of great interest, and their relevance should not be discounted, but one of the compelling reasons for the pursuit of this thesis is the capacity of our lab to track T cells of a defined specificity, as will be described in more detail.

1.3 Immunoglobulin as immunogenic antigen

In 1905, Pirquet and Schick described “Die Serumkrankeit” (The Serum Sickness), the systemic symptoms associated with an adaptive immune response to an administered heterologous horse serum in humans [5]. They recognized that the kinetics of the reaction, and the presence of a precipitin in some patients, indicated that the horse antibodies were acting as an antigen for ongoing production of an anti-horse Ig response. This recognition that a heterologous serum protein could induce a productive immune response was well established by the time that Dresser and others were injecting animals with various species of albumin or gamma globulin. In fact, it was the work of Dresser with BGG that was unusual, if adult animals were injected with purposefully aggregated or not ultracentrifuged Ig, they made a productive humoral immune response that was in contrast to the tolerance that he observed. These results complemented findings from groups investigating the capacity of antibody-antigen immune complexes to initiate cutaneous skin reactions, recapitulating the effect originally described by Nicolas Arthus in 1903 [165, 166]. In the case of the work of Dresser, a component of gamma globulin that could be eliminated through centrifugation (i.e. an aggregated species) was responsible for inducing the immune response in a dominant fashion when injected with
its tolerizing counterpart; in the Arthus recapitulations, immune complexes formed in moderately high antigen excess along the precipitation curve, a situation where the majority of the Ig could be expected to be in a complexed form, were highly efficient at inducing cutaneous inflammation or systemic anaphylactic response [167-180]. The discovery of the existence and function of the Fc receptors would give these observations a plausible mechanism [181, 182]. Furthermore, while B cells could utilize antigen-specific, receptor-mediated intake to increase antigen presentation as a mechanism for increasing their capacity to stimulate T cells, Ig and Fc receptors presented an avenue through which other antigen presenting cells (APC) could bind antigen in a specific and receptor-mediated fashion (immune complexes) to augment presentation. While these observations did not speak specifically to the nature of the antigenicity of Ig per se, in retrospect, they did demonstrate that immune complexes have a natural adjuvanticity that can increase the presentation of antigenic peptides from within the complex to T cells. It is important to mention that FcγRIIB has been shown to deliver an inhibitory signal upon the binding of aggregated IgG, and that this inhibition is believed to regulate antibody production during an immune response [183]. While this caveat certainly must be taken into account when considering the totality of the effect of immune complexes on the progression of the immune response, the observations commented upon above suggests that this inhibition is not completely eliminating immune function when tested experimentally. Furthermore, FcγRIIB deficient mice can be made tolerant in Ig tolerization experiments, and the receptor has always been cited as one component in a milieu of regulatory factors [120, 160].
An alternative method for evaluating the immunogenicity of Ig is to examine humoral responses against Ig-derived epitopes in either the constant or the variable region. Rheumatoid factor (RF) is the classical example of an Ig that targets the Fc of IgG in the system. It was discovered by two research groups independently in the years bookending World War II, the fog of war surrounding whether the original report was disseminated widely [184, 185]. In both cases, it was identified as a factor, found in the sera of some patients with rheumatoid arthritis (RA), which could agglutinate sheep red blood cells (SRBC) if an anti-SRBC antibody was also present in the reaction. RF was most often described as being an IgM anti-Fcγ, although it has been shown that RF can be any isotype, not only IgM. The validity of testing for RF as an assay for RA, and the role of RF in the pathophysiology of RA have both been questioned as knowledge about the disease has become more sophisticated [186]. However, for the purposes of evaluating the capacity for the Fcγ to act as an immunogenic antigen for a productive immune response, the existence of RF is confirmation. Rheumatoid factor B cells are a common constituent of the natural repertoire and have been shown to expand during adaptive immune responses to unrelated protein antigens [187-194]. Recent work with transgenic mice expressing Ig chains from an RF has shown that RF B cells can exist quiescently within a normal mouse without becoming tolerized or deleted, but that these cells may secrete RF when bred to an autoimmune background [195, 196].

While RF targets the Fc component of the IgG, there is also ample experimental evidence to indicate that the variable regions of an Ig can also induce a productive humoral response [197-204]. This is particularly interesting given the knowledge that the variable region contains somatic diversity from several mechanisms that occur both
during development of the B cell receptor and the affinity maturation that occurs in the germinal center during a humoral response. Isologous anti-idiotypic antibodies are a manifestation of the immunogenicity of this somatic antibody diversity. Again, the literature supporting the network theory relied upon the generation of these anti-idiotypic antibodies to create the eponymous network, and results of associated studies must be considered despite the consensus movement away from the theory since the early 1980s. More recently, the work of Reitan and Hannestad examining anti-idiotypic generation demonstrated that isotypes that are typically found in polymeric form in the body (IgM, IgA) could elicit anti-idiotypic responses in the absence of adjuvant [158-160].

Amidst the years of controlled experimental investigation in animals, the clinical community has been carrying out a larger, less controlled, and higher stakes experiment into the immunogenicity of Ig: the usage of monoclonal antibodies in patients. Since the first published mAb treatment in a cancer patient in 1980, and particularly since the FDA approval of Muromanab-CD3 for allograft rejection in 1984, physicians, academic scientists, and pharmaceutical companies have sought ways to bring these mAb to the clinic [205, 206]. There are currently over 30 mAbs approved for therapy by the FDA and reports of over one hundred in ongoing clinical trials. In 2009, the FDA published a guide suggesting to pharmaceutical companies that assaying for immunogenicity should be an important component of drug development with mAbs and other biologics [207]. The scientific community had already sounded the alarm about immunogenicity of mAbs years earlier. As of the early 1990s, researchers had observed that chimeric mouse antibodies used in therapy regularly generated an anti-Ig response, which they dubbed a HAMA (human anti-mouse antibody) or HACA (human anti-chimera antibody) and are
still a concern to this day [208-224]. They concluded that it was likely an immune
response to murine components of the chimeric Ig, but some also conceded that it was
likely that the humanization of therapeutic Igs, or even fully human Igs, would probably
still lead to anti-idiotypic immune response (later to be called a HAHA – human anti-
human antibody) [207, 219, 221, 225]. While it was difficult to draw a direct correlation
between the development or quantity of the HAMA and a specific clinical outcome, it
was clear to these groups that this immune response had potential to end therapeutic
efficacy and create pathology for patients. Pharmaceutical companies responded by
attempting to develop reagents that incorporated increasing amounts of human protein
structure or, in recent years, were derived completely from human Ig genes. Although it
is rarely an emphasized point in papers about clinical trials, evidence of an immune
response against a given mAb is generally a component of the adverse outcomes reported
by researchers. Sometimes they will report an anti-Ig response; sometimes they report
outcomes associated with anti-Ig antibodies like infusion anaphylaxis or delayed-type
hypersensitivity reactions at the injection site. Most mAbs, if not all, generate evidence
of an anti-Ig response whether they are chimeric or completely derived from human
genesis. The rates of reaction or measured anti-Ig development range based upon the
specific Ig, the disease being treated, and the schedule of administration; the anti-CD20
antibody rituximab yielded an anti-Ig response in less than 10% of lymphoma patients,
but in as high as 60% in one study of patients being treated for systemic lupus
erythematosus [217, 218]. This knowledge has led physicians to design treatments with
the co-administration of a mAb and chemotherapeutic agents such as methotrexate or
azathioprine in the hopes that these agents will ablate any immune response [226].
However, the correlation between anti-Ig development and the success of treatment is still murky, at best, for most agents and the evidence for this co-administration of chemotherapeutic is generally anecdotal in part due to an insufficient study population. One notable exception to this paucity in data is in the usage of anti-TNF-α mAbs, particularly for rheumatoid arthritis. In the last year, researchers have begun to make a quantitatively justified case that the development of anti-Ig responses has a significantly negative effect on hypersensitivity reactions, therapeutic response and soluble drug levels over time [227]. In concordance with the theoretical underpinnings, administration of a chemotherapeutic with the mAb could eliminate some of the negative outcomes associated with developing an anti-Ig response. While more substantive data will be needed to make clinical judgments about treatment regimens, the fact remains that the therapeutic use of Ig has demonstrated that it can be immunogenic. The rates of anti-Ig antibody development may vary based upon a number of factors, but some percentage of patients is able to make a productive immune response against virtually every mAb and ongoing data collection is finally beginning to justify the concern that has existed for over two decades.

The release of the guide on immunogenicity testing of biologics by the FDA in 2009 hinted at another complicated aspect of this line of research: immunologists and clinicians are no longer the only scientists hoping to dissect the capacity for mAbs to induce or evade the immune response. Pharmaceutical scientists, those that examine the physicochemical attributes of manufactured pharmaceuticals, have begun to question what controllable aspects of the proteins they make can be modified to decrease rates of rejection. The variables they test can range from modifying the glycosylation patterns of...
the Fc to optimizing amino acids in the variable domains to decrease the immunogenicity [228-230]. The influence of antigen size on Ig immunogenicity has been a subject of investigation for some time; the original tolerance protocol of Dresser relied upon the ultracentrifugation of BGG to remove polymeric complexes, and larger accumulations of antigen such as heat aggregates and immune complexes have been shown to induce humoral and DTH responses. Pharmacologists recognize that aggregated Ig could be immunogenic and have begun to study Ig species that have been manipulated aggressively to simulate mishandling during production, storage, or administration [231-233]. While there are definitely cultural differences between the immunologists and the pharmacologists in terms of their techniques and the extent to which data between the two realms can be compared, it is valuable to consider this type of experimentation into aggregate size as a complementary element of immunogenicity research.

I.4 CD4+ T cells and the perception of immunoglobulin as antigen: model systems

While there is a substantial history of literature examining the extent to which Ig activates a productive immune response, the vast experimental data have a deficiency in regards to the role of individual CD4+ cells in tolerization or activation. Certainly, there have been observations about CD4+ cells and indications about how they might perceive immunoglobulin. A tolerized thymic compartment could not provide sufficient help to a naïve bone marrow compartment to yield a productive anti-HGG response [68, 69]. HGG treatment seemed to decrease both IL-2 and IL-4 production in T cell populations leading to the interpretation that the tolerogenic Ig could suppress both Th1 and Th2 CD4+ cells, with an increased emphasis on suppression of Th1 cells [116, 234].
Sequencing data have implied that the Fc may contain tolerogenic peptides that induce the proliferation of suppressive T\textsubscript{reg} cells [153-155, 157]. However, the production of high affinity IgG anti-mAb responses in patients against therapeutic Ig implies that there must be CD4+ cells participate in productive immune responses to the mAb. Precursor percentages of some anti-mAb CD4+ cells have been defined in a naïve human repertoire [235, 236].

The biggest deficiency in all of this data is the reliance on experimental results that imply, rather than directly show, the effect of Ig on single T cells. Prior to the era of transgenic mice, it was difficult, if not impossible, to track a monoclonal population of T cells that were activated by an Ig-derived peptide. There are currently two mouse models with transgenic CD4+ T cells that recognize specific peptides generated via somatic mutation in isologous Ig V regions. Both have been used to study central CD4+ tolerance to Ig-derived peptides as well as the consequences of failures of tolerance. Both will be described, in particular the CA30 – V\textsubscript{κ}\textsuperscript{36-71} model which is the basis for the work in this thesis.

The first model derived from the observation that injections of an isologous IgA antibody derived from a myeloma could induce anti-idiotypic antibodies [201]. This anti-idiotypic response was T cell-dependent, and the antigenic light chain epitope was determined via antigen presentation with T cell clones derived from sensitized mice [200, 237-245]. The researchers created two transgenic mice: one expressing the lambda light chain with the antigenic somatically mutated residues (\(\lambda_{2315}\)) and the other expressing an \(\alpha/\beta\) T cell receptor which recognized the antigenic peptide in the context of the class II MHC I-E\textsuperscript{d}. If the two mouse strains were crossed, there was a profound deletion in
CD4+/CD8+ thymocytes indicating that central tolerance mechanisms were influenced by the presence of the λ2315 light chain in the mouse [246]. Interestingly, large injections of IgG bearing the λ2315 could also delete the TCR-Tg cells in the T cell-only transgenic animal. If a mother expressed the λ2315 and the TCR-Tg but the offspring expressed only the TCR-Tg, the transferred maternal IgG was insufficient to delete the TCR-Tg in the neonatal animal due to its low concentration. This finding indicated that high concentrations of IgG could lead to Ig-derived antigen presentation to CD4+ cells, particularly in the thymus. Further work with these mice focused more on the consequences of T cell/B cell cooperation and the illicit licensing of B cells expressing the λ2315 to secrete autoreactive antibodies due to CD4+ recognition of the light chain epitope [247-250].

The second model, used in this thesis, was developed in our lab to investigate the development of tolerance to Ig-diversity. The λ2315 system had begun as an investigation into the capacity for large amounts of isologous IgG to induce an anti-idiotypic response with the assumption that idiotypic antigen were probably immunogenic, just expressed at low enough levels within the system to evade tolerance induction. In 1995, our lab asked a more fundamental question: if T cells perceive peptides derived from Ig V regions can all Ig V regions induce CD4+ regulated humoral responses or is there a requirement for non-germline, somatic diversity in the Ig to direct a CD4+ mediated response [251]? To investigate this question, it was important to inject a panel of Ig that utilized the same germline genes, but some of which utilized somatically mutated receptors that would hypothetically contain an antigenic peptide. There was such a panel of Ig available that bound the hapten Ars (p-azophenylarsonate) and had been characterized extensively for
Ig genes and somatic mutation [252-254]. The experiment yielded T cell hybridomas that revealed the answer: animals were tolerant to isologous germline Ig sequences, but could generate a fuseable CD4+ response to Ig with somatically generated diversity. These results could be replicated by utilizing a different strain, C58 as opposed to A/J, that possessed the I-A\(^k\) MHC II, but did not possess the germline Ig V gene to which the A/J mouse had shown tolerance. By generating and challenging C58 hybridomas, the lab could demonstrate that A/J APC were able to present peptides from the germline Ig, but unable to make a CD4+ T cell response against it and thus tolerant. To study the mechanisms behind this tolerance, our lab generated two transgenic mice: the V\(\kappa^{36-71}\) mouse, which expressed the somatically mutated \(\kappa\) light chain of the Ars-reactive mAb 36-71, and the CA30 Tg, which expressed an \(\alpha\beta\) T cell receptor specific for an epitope in the V\(\kappa^{36-71}\) framework-1 region [255]. Crossbreeding between the two transgenic strains led to deletion of the CA30 Tg T cells during development, while transfer of large numbers of CA30 Tg T cells into mice expressing only the V\(\kappa^{36-71}\) caused development of a lupus-like autoimmune disease. Both of these experiments relied upon interactions between large numbers of transgenic T cells and B cells, and so the lab shifted to model more physiological circumstances to investigate the role of tolerance in the system. A series of bone marrow chimeras demonstrated multiple levels of CD4+ tolerance, both central and peripheral, that could serve as the regulatory mechanisms observed in the previous experiments [256]. Similarly, a dual transfer model in which V\(\kappa^{36-71}\) B cells and CA30 T cells were transferred into a secondary animal to simulate the introduction of a novel B cell clone with a somatically mutated receptor containing antigenic peptides showed a mechanism of peripheral regulation, in which there was a disruption of memory
development by Tg B cells in an ongoing immune response and rapid differentiation of Tg B cells into short lived plasmablasts [257]. In both the bone marrow chimeras and dual transfer model, there were preliminary data indicating that the secretion of antigenic Ig may have played a role in some of the observed mechanisms of tolerance. In particular, in the dual transfer model, there was early evidence that a transfer of sera from a V\(\kappa^{36-71}\) Tg animal could induce CA30 T cell proliferation when both were injected into a \(\kappa^{-/-}\) recipient. However, sera was used rather than a monoclonal reagent, so it was possible that this result was a function of T cell stimulation by any isotype. Regardless, this model presented an excellent opportunity for investigating the questions expressed over the course of this introduction, namely the extent to which Ig, and particularly IgG, could tolerize or activate CD4+ cells to make a productive humoral response to novel epitopes within the IgG itself.

I.5 Conclusion and the scope of the thesis

There has been a debate within the immunological literature since at least 1961 as to the nature of perception of isologous Ig by the immune system. Depending on preconceived bias, one can point to a long progression of papers to support a hypothesis either that Ig is intrinsically tolerogenic or immunogenic. While the history of mAb therapeutics and the development of HAMA and HACA made an increasingly compelling argument for Ig being at least partially immunogenic, pharmaceutical scientists and their belief that aggregated, damaged species of Ig are responsible for inducing anti-Ig immune responses was sufficiently compelling to suspend disbelief at the outset of our work. Given that our lab has a unique and powerful system to transfer and track transgenic, Ig-specific CD4+ cells, we started the project with a goal: to
characterize the CD4+ response to an IgG1 containing an antigenic sequence within its light chain. Our initial hypothesis was an extension of the landmark work by Chiller et al: we believed that the CA30 T cells would not make a primary CD4+ response to a monomeric, deaggregated species of IgG1 containing an antigenic sequence.

In Chapter III, I will describe the production and purification of antibody, initial in vitro testing of presentation of antibody species, and a panel of tests to characterize particle concentration within the samples that would be used in in vivo transfer experiments. I characterized my monoclonal reagent as a predominantly monomeric protein with very small populations of potentially immunogenic particles and little evidence of adjuvant activity in vivo or in vitro. Conversely, I characterized heat aggregated Ig and immune complexes as reagents with large percentages of soluble and insoluble complexes that displayed early in vitro evidence of strong immunostimulatory capacity. These data describe the first particle concentration analysis of immune complexes, which are naturally occurring structures.

In Chapter IV, I will describe a series of novel in vivo adoptive transfers that explored the primary response of a monoclonal CD4+ TCR Tg cell to species of immunoglobulin without adjuvant. While others have reported the capacity for TCR Tg cells to proliferate after in vivo exposure to antigenic Ig, this is the first presentation of the full contraction and expansion of the primary response to monomeric Ig as well as two species hypothesized to be immunogenic: heat aggregated Ig and immune complexes. I demonstrated that both heat aggregated Ig and immune complexes, but not monomeric Ig, can prime mice to make a consistent secondary humoral response, and that this difference is not due to stimulation of regulatory T cells by monomeric Ig, but
instead appears to be a function of T follicular helper differentiation in response to heat aggregated Ig and immune complexes, which has never been reported. Furthermore, I will describe a differential pattern of early proliferation in T cells exposed to monomeric Ig vs the immunogenic species that is a unique phenomenon in which T cells exposed to the immunogenic species have higher percentages of cells residing at later stages of division than those exposed to monomeric Ig, which has not been described without the use of adjuvant. I identified an important role for the Fc in the early presentation of Ig-derived peptides, in contrast to monomeric Ig, which was readily presented \textit{in vivo}.

Finally, I will present an early exploration of the role of B cells in the early presentation of Ig-derived peptides and will show evidence that exogenously administered heat aggregated Ig may be dependent on B cells for presentation in the spleen, whereas immune complexes are not, which has important ramifications for mAb therapy.

In Chapter V, I will describe experiments examining the dynamics of CA30 cells after a secondary challenge. I will present data that suggests that monomeric Ig generates a poor memory population in comparison to peptide, and that this poor memory response is not augmented extensively by LPS or immune complexes. Furthermore, I will show experiments suggesting that even with a strong adjuvant, the memory response to Ig is weaker than peptide and that monomeric Ig is a poor stimulator of secondary response. This is the first report of the memory behavior of a TCR Tg cell exposed to Ig species.
CHAPTER II
MATERIALS AND METHODS

II.1 Production and Characterization of Reagents

II.1.1 T Cell medium (TCM)

For *in vitro* and adoptive transfer experiments, cells were washed and cultured in T cell medium (TCM): RPMI 1640 supplemented with 2-mercaptoethanol (50 µM), penicillin G (10^5 U/L), streptomycin sulfate (100 mg/L), sodium bicarbonate (2g/L) (Sigma Aldrich, St. Louis, MO), HEPES (7.5 mM) (Fisher Scientific, ), L-glutamine (2 mM), 1x MEM essential amino acids, 1x non-essential amino acids, 1x sodium pyruvate (Gibco-BRL, Grand Island, NY), and 10% fetal calf serum.

II.1.2 Hank’s Buffered Salt Solution (HBSS)

For cell harvesting for flow cytometry, tissues were processed in Hank’s Buffered Salt Solution (HBSS): 0.137 M NaCl, 0.25 mM Na$_2$HPO$_4$, 4.2 mM NaHCO$_3$, 5.4 mM KCl, 0.44 mM KH$_2$PO$_4$, 1.3 mM CaCl$_2$, 1.0 mM MgSO$_4$, and 0.1 g glucose. (Sigma-Aldrich, St. Louis, MO).

II.1.3 Antibody production in mice

C$_{129}$S4-Rag$^{2^{tm1.1Flv}}$ Il2rg$^{tm1.1Flv/J}$ (Rag2$^{-/-}$cyt$^{-/-}$) mice were injected i.p. with 0.2 ml incomplete Freund’s adjuvant (IFA) (Sigma Aldrich, St. Louis, MO) 5 days before the i.p. injection of 5 x 10^6 hybridoma cells in phosphate buffered saline (PBS). Ascitic fluid began to accumulate 5 to 7 days later and was collected via peritoneal lavage after mice were sacrificed in accordance with guidelines of the National Jewish Health Institutional Animal Care and Use Committee (IACUC).
II.1.4 Handling and dialysis of ascitic fluid for anion exchange chromatography

Ascitic fluid was placed at 37°C for 20 minutes and then incubated on ice for 1 hour. After induction of clots, the fluid was centrifuged at 20,000 x g for 30 minutes at 4°C, removed from the clot and visible lipid fraction with a Pasteur pipette, and passed through a 0.22 µm filter (Millipore, Billerica, MA) under standard sterile cell culture precautions. Filtered ascites fluid was then immediately precipitated or stored at 4°C for less than 24 hours. Ascitic fluid was precipitated while stirring on ice; saturated (NH₄)₂SO₄ solution was added to the ascitic fluid at a rate of 1 ml/min until reaching 45% (v/v). This solution was incubated on ice with stirring for 1 hour to maximize precipitation. After precipitation, the solution was centrifuged for 1 hour at 20,000 x g at 4°C and the supernatant removed and tested for residual antibody. The precipitate was dissolved in a similar volume of phosphate buffered saline with 0.01% NaN₃ (PBS-A) to the original precipitated solution volume, as this minimized protein precipitation during dialysis, and stored at 4°C or immediately dialyzed for anion exchange chromatography. For dialysis, the dissolved precipitate was placed in dialysis tubing with a molecular weight cutoff of 35,000 kilodaltons (kD) and dialyzed against ≥20 volumes of 10mM NaPO₄ pH 7.9. The protein was dialyzed over 48 hours which included 4 to 5 changes of 10mM NaPO₄ buffer, the first two changes typically containing 0.01% NaN₃ to prevent bacterial growth and the final 2 to 3 changes eliminating its inclusion. After dialysis, protein was immediately subjected to anion exchange chromatography and never stored for >12 hours at 4°C.
II.1.5 Anion exchange chromatography

Diethylaminoethyl (DEAE) anion exchange cellulose DE52 (Whatman, GE-Healthcare, Pittsburgh, PA) was used in the purification of IgG. DE52 beads were swollen in distilled H₂O for 1 hour. The beads were then incubated with 5 equilibrations of 100 mM NaPO₄ pH 7.9 buffer, followed by 10 equilibrations of 10 mM NaPO₄ ph7.9. After this equilibration, the beads were loaded into a 50 mL syringe with a small plug of glass wool in the base, with a target volume 0.15 ml packed resin for every 1 mg of protein to be loaded onto the column. After packing the column, the dialyzed protein in 10mM NaPO₄ pH 7.9 was passed through the column and loaded onto the anion exchange resin. The column was washed 3 times, each wash containing 1 bed volume of the column of NaPO₄ pH 7.9. Protein was obtained by progressive column elutions in which the column was subjected to 2 times the bed volume washes with 10 mM NaPO₄ pH 7.9 with salt concentrations starting at 0.01 M NaCl and increasing 0.01 M with each subsequent wash. The fraction obtained during each wash cycle was concentrated in an Amicon Ultra spin filter (Millipore, Billerica, MA) with a molecular weight cutoff of 50 kD and then assayed for protein content using the absorbance at 280 nm measured by spectrophotometer. Once the elution point had been defined for an antibody, in the case of this work typically 0.03 M NaCl, this concentration was used for subsequent anion exchange purifications.

II.1.6 Low aggregation pharmaceutical buffer and Ig storage

Protein was buffer exchanged from 10mM NaPO₄ ph7.9 into a low aggregation pharmaceutical buffer that has been previously described (20 mM histidine, 222 mM trehalose dihydrate pH 5.5) using ≥4 spins in an Amicon Ultra spin filter, MW cutoff
Ig concentration was brought to 4 mg/ml in the pharmaceutical buffer, and then the entire volume was passed through a 0.22 µm filter. Polysorbate 80 (PS80) (Sigma Aldrich, St Louis, MO) was added to 0.02% (v/v). Ig was then distributed into 2 mg aliquots in sterile 1.5 ml microcentrifuge tubes and stored at -20°C. Individual tubes of this Ig were subjected to a single freeze-thaw cycle prior to injection into animals.

II.1.7 Arsanilation of mouse serum albumin

Mouse serum albumin was dissolved in a solution of boric acid and sodium chloride (0.16 M H₃BO₃, 0.16 M NaCl) to a concentration of 10 mg/ml. Two solutions were prepared and incubated on ice: arsanilic acid was dissolved in 1 ml of 1 N HCl to create a 0.15 M arsanilic acid solution and NaNO₂ was dissolved in cold water to create a 0.2 M solution. While stirring the arsanilic acid solution, the 0.2 M NaNO₂ was titrated into the arsanilic acid in 50 µL drops to create a diazonium salt. To assess this titration after the addition of each drop, 5 µl of the resultant solution was tested using starch iodide strips to assess for the presence of HNO₂. When the strip showed a blue hue immediately upon testing with the solution, the diazonium salt was prepared for coupling. This diazonium salt solution was titrated into the mouse serum albumin solution in 50 µL drops while stirring on ice. The pH of the resulting solution was maintained between at pH 9.5 by adjusting with 0.5 N NaOH. As the titration was occurring, the coupling of the hapten to the protein was assessed by diluting the solution 1/50 in 0.2 N NaOH and reading the absorbance at 477nm. Coupling could be monitored by using the extinction coefficient of $8.85 \times 10^3$ to calculate the number of moles of Ars in the solution and then dividing by the moles of MSA in the reaction. Once a coupling of 12 – 15 Ars per MSA ($\text{Ars}_{12-15}-\text{MSA}$) was achieved, the protein was diluted with 1 ml of 10x PBS, placed in
dialysis tubing with a MW cutoff of 35,000 kD, and against ≥20 volumes of PBS. The protein was dialyzed over 48 hours which included 4 to 5 changes of PBS, the first two changes typically containing 0.01% NaN₃ to prevent bacterial growth and the final 2 to 3 changes eliminating its inclusion. After dialysis, the protein concentration was calculated based upon the end dialysis volume, the protein was passed through a 0.22 µm filter and divided in small volumes into 1.5 ml microcentrifuge tubes and stored at -20°C.

II.1.8 mAb 36-71 Fab and F(ab’)₂ generation

As mAb 36-71 is an IgG₁, the sulfhydryl protease ficin could be used to generate both Fab and F(ab’)₂ fragments. For both procedures, mAb 36-71 was buffer exchanged from the low aggregation pharmaceutical buffer to a final concentration of 10mg/ml in a 0.1 M citrate buffer pH6.0 using an Amicon Ultra spin column with MW cutoff of 50kD. Immobilized ficin (Thermo Fisher Scientific, Waltham, MA) was equilibrated with 10 times resin slurry volume (2mL) of Fab digestion buffer (0.1M citrate, 5mM EDTA, 25mM cysteine) or F(ab’)₂ digestion buffer (0.1M citrate, 5mM EDTA, 4mM cysteine). Equilibrated ficin was incubated with 1 ml of mAb 36-71 in Fab or F(ab’)₂ digestion buffer for 40 hours (F(ab’)₂) or 5 hours (Fab) while being rotated in a 37°C incubator. The digested material was buffer exchanged into PBS-A and concentrated using an Amicon Ultra spin column with MW cutoff of 30kD. Both species were size excluded independently via fast protein liquid chromatography (kindly completed by Fran Crawford in the Kappler/Marrack lab, NJH) and the fractions containing the two species pooled independently. Both the Fab and F(ab’)₂ were buffer exchanged into the low aggregation pharmaceutical buffer, passed through a 0.22 µm filter, and divided into sterile 1.5 ml microcentrifuge tubes and stored at -20°C.
II.1.9 Heat aggregation of Ig

mAb 36-71 was buffer exchanged from low aggregation pharmaceutical buffer into PBS at a concentration of 7 mg/ml using an Amicon Ultra spin column with MW cutoff of 50 kD. Individual 1 ml aliquots were incubated in a water bath at 63°C for 20 minutes and then immediately placed on ice for 1 hour, by which time large insoluble aggregates were visible within the solution. Insoluble aggregates were spun down by centrifuging at 10,000 x g for 5 minutes, and the supernatant was measured for absorbance at 280 nm using a spectrophotometer, allowing for an estimation of the mass of the insoluble fraction. The soluble fraction was removed, pooled, and frozen at -20°C.

The precipitated aggregates were washed 3 times with low aggregation pharmaceutical buffer and then divided into sterile 1.5 ml microcentrifuge tubes and stored at -20°C.

II.1.10 Generation of Immune Complexes with Ars-MSA

Due to the specificity of mAb 36-71, it was possible to generate immune complexes with the Ars-MSA reagent. Due to some variability between batches of arsanilated albumin, it was necessary to determine an equivalence point for each batch prior to the generation of complexes for in vivo or in vitro experiments. 100 µg of mAb 36-71 were mixed with varying amounts of Ars-MSA and diluted to 100 µl with PBS. These mixtures were incubated at 37°C with rotation for 3 hours and then centrifuged at 13,000 x g for 5 minutes. After centrifugation, the supernatants were measured for absorbance at 280 nm using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA) to establish a precipitation curve. Once an equivalence point had been established, experimental immune complexes were generated using similar conditions and either Ars-MSA at the equivalence point (referred to later as “large”
immune complexes due to the insoluble fraction) or at an amount of Ars-MSA 4 times larger than the mass at the equivalence point (referred to as immune complexes). Similar amounts of Ars-MSA created in antigen excess were also incubated with Fab and F(ab’)\textsubscript{2} fragments, although the amounts of Fab (66 µg) and F(ab’)\textsubscript{2} (73 µg) were varied to normalize the amount of antigenic 36-71 epitope in the sample. Immune complexes were never frozen or stored prior to injection into animals or usage in \textit{in vitro} culture and were used within 2 hours of generation.

\textbf{II.1.11 Ultracentrifugation of Ig}

To obtain monomeric Ig, multiples of 100 µg of mAb were diluted in sterile PBS to a concentration of 1 mg/ml and centrifuged at 165,000 x g in a fixed angle TLA-120.1 rotor (Beckman Coulter, Brea, CA) for 3 hours. The top 2/3 of the supernatant was removed for injection to avoid disturbing the pellet, and the sample was stored in a sterile 1.5 ml microcentrifuge tube until used for experimentation within less than 6 hours.

\textbf{II.1.12 Size-exclusion chromatography}

Analytical size-exclusion chromatography was performed using an Agilent 1100 chromatography system (Agilent Technologies, Santa Clara, CA) as has been described previously[233]. Prepared stocks of ultracentrifuged Ig, heat aggregated Ig, immune complexes, or diluent PBS was spun at 13,000 x g and the supernatant removed carefully to eliminate large insoluble particles. Protein was loaded onto a Tosoh G3000 SWXL 7.8 x 30 cm column (Tosoh Bioscience, Tokyo, Japan) and eluted with a mobile phase of PBS pH 7.4 at a flow rate of 1 mL/min. The eluate was monitored at 280 nm and 215 nm to assess for protein recovery and percentages of monomer and dimer. Triplicate samples were analyzed for each Ig preparation. Monomer and dimer percentages were calculated
based upon areas under the respective peaks of absorbance at 215 nm. Total protein recovery was calculated based upon the absorbance at 280 nm with the following equation: \( \frac{x \times (y/60)}{e \times p} = \text{total protein} \) where \( x = \text{total area integrated under the curve} \), \( y = \text{flow rate} \), \( e = 1.4 \text{ cm}^2/\text{mg} \), and \( p = \text{path length (1 cm)} \).

II.1.13 Microflow imaging

A Brightwell (Ottawa, ON, Canada) 4100 instrument was used for microflow imaging (MFI) to assess particle size and particle counts as has been described previously[233]. Prepared stocks of ultracentrifuged Ig, heat aggregated Ig, immune complexes, or diluent PBS were incubated at RT for 1 hour to allow settling of large insoluble particles. 550 µL of Ig sample was loaded onto the machine to allow total volume analysis of 500 µL; this was performed in triplicate for each Ig preparation. The instrument was configured to allow for 1-50 µm particle detection by using “set point 3” mode and low magnification.

II.1.14 Particle tracking analysis

A NanoSight LM20 (NanoSight Ltd., Amesbury, UK) instrument with a 405 nm laser was used to perform particle tracking analysis (PTA) as has been described previously[258]. Prepared stocks of ultracentrifuged Ig, heat aggregated Ig, immune complexes, or diluent PBS were incubated at RT for 1 hour to allow settling of large insoluble particles. 500 µL of sample was loaded into the flow chamber before data acquisition. Video was captured for 60 seconds using NTA 2.3 software at a setting recommended for low polydispersity samples, size detection limit automatically determined by the software (1 – 600 nm), and with manually defined shutter and gain settings. Triplicate readings were obtained for each Ig preparation.
II.1.14 Estimate of Ig mass in protein particles

Both MFI and PTA provided calculated concentrations of particles/mL for “bins” of particles defined by their circular diameter. Given this concentration and the reported diameter, an estimate of protein mass for a given bin of particles could be calculated as reported previously[233]. This equation relied upon a number of assumptions: first, the volume of a particle sphere could be calculated using the reported diameter. This volume was assumed to be 75% protein and 25% water. An average density of more than 30 proteins was calculated to be 1.43 g/mL, and it was assumed that this value could be used to calculate the mass of protein as the mass of the particle would be bounded by that of protein (1.43 g/mL) and water (1.0 g/mL). Thus an estimated mass could be calculated using the following equation: $0.75*v*1.43*n = \text{estimated mass per bin}$, where $v =$ calculated volume of particles in the bin based upon the diameter and $n =$ number of particles in the bin, which could be calculated from the concentration of particles/mL and a known volume of solution.

II.1.15 Limulus amebocyte lysate endotoxin quantification

Bacterial endotoxin was quantified in Ig samples, Ars-MSA, and sterile PBS using a chromogenic microplate assay (Thermo Fisher Scientific, Waltham MA) based upon the classic *Limulus polyphemus* amebocyte lysate test. Briefly, sample replicates were incubated with *Limulus polyphemus* amebocyte lysate at 37°C for 10 minutes. A chromogenic substrate was added to the sample and incubated for 10 minutes, after which 25% acetic acid was added to stop the potential conversion of the substrate by *Limulus* proteases. Sample absorbance at 405 nm was detected in a Victor² 1420 multilabel
counter (Wallac, Turku, Finland) and compared to a standard curve to calculate concentration.

II.2  

In vitro and In vivo analysis of CD4+ T cell behavior

II.2.1 Mice

A/J, C57BL/6 (B6), C;129S4-Rag2<sup>tm1.1Flv</sup> Il2rg<sup>tm1.1Flv</sup>/J (Rag2<sup>−/−</sup> Il2rg<sup>−/−</sup>), B6.Cg-Thy1Tg(TcraTcrb<sup>425Cbn/J</sup> (B6.OTII.pl), and B6xA/J F1 (B6AF1) mice were bred in-house. A/J CA30 Tg mice (CA30) have been described previously and were maintained on an A/J κ<sup>−/−</sup> background in-house[255]. B6.PL-Thy1<sup>a</sup>/CyJ (B6.pl) and B6.SJL-<i>Ptprc<sup>a</sup></i> <i>Pepcd</i> /BoyJ (B6.SJL) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in the Biological Resource Center at National Jewish Health (Denver, CO). CA30 mice were bred to B6.pl (CA30.Thy1.1) and B6.SJL (CA30.CD45.1) to create congenically-marked CA30 cells suitable to be transferred into B6AF1. Mice used for experiments were generally 8-14 weeks old and included both sexes. All mice were handled and bred with Institutional Animal Care and Use Committee (IACUC) approval in accordance with institutional guidelines.

II.2.2 Tissue harvesting and cell preparation

Mice were euthanized with CO<sub>2</sub> in accordance with institutional guidelines, and spleen and peripheral lymph nodes (typically inguinal, brachial, axillary, and cervical) were extracted and stored in TCM (for adoptive transfer or in vitro experiments) or HBSS (for flow cytometric analysis). Single cell suspensions were prepared by passing cells through a 40 µm cell strainer (BD Bioscience, New Bedford, MA) and 2 cycles of centrifugation at 300 x g for 5 minutes in a Multifuge 3 S-R centrifuge (Heraeus, Germany) and washed with TCM or HBSS. Erythrocytes were depleted from samples of
spleenocytes, but not lymph nodes, by incubating cells in lysis solution (8.3 g/L NH₄Cl, 10 mM Tris-HCL pH 7.5) for 3 minutes followed by 2 washes. Cells were enumerated with a Coulter AcT diff 2 Hematology Analyzer (Beckman Coulter, Brea, CA) and resuspended in TCM (in vitro experiments), HBSS (flow cytometric analysis) or sterile PBS (adoptive transfer).

**II.2.3 CFSE labeling**

For adoptive transfers and in vitro experiments to assess T cell proliferation, cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) using a protocol modified from the manufacturer’s recommendations (Thermo Fisher Scientific, Waltham, MA). Briefly, lymph node cells were washed and resuspended in PBS, 0.1% BSA to a concentration of 1 - 3 x 10⁷ cells/mL. CFSE was added from a stock solution of 5 mM CFSE diluted in DMSO to the cells for a final working concentration of 10 µM CFSE. Cells were incubated at 37°C for 5 minutes and then washed twice in TCM to sequester residual CFSE. Cells were enumerated and resuspended in sterile PBS (for adoptive transfer) or TCM (for in vitro experiments).

**II.2.4 In vitro T cell activation assays**

CD4+ T cells were purified from the lymph nodes of transgenic animals (OTII or CA30.SJL F1) using the EasySep magnetic cell enrichment protocol (StemSep, Vancouver, BC, Canada) and enumerated with a Coulter AcT diff 2 Hematology analyzer and flow cytometric analysis. In the case of CA30 activation assays, T cells were labeled with CFSE and enumerated. For both assays, 2 x 10⁵ purified T cells were cultured with 2 x 10⁵ irradiated (1100 rads) splenocytes from B6 (OTII) or B6AF1 (CA30) mice in TCM. Antigens were added at varying concentrations to a total volume of 200 mL;
antigens included ultracentrifuged Ig, heat aggregated Ig, immune complexed Ig, the antigenic \( \text{Vk}^{36-71} \) peptide (DIQMTIQSSLSA), or the antigenic OVA\(_{323-339}\) peptide (ISQAVHAAHAINEAGR). Cultures were incubated at 37°C in 5% CO\(_2\) for 24 hours (OTII) or 5 – 6 days (CA30) and then analyzed for CD69 upregulation (OTII) or CFSE dilution (CA30).

**II.2.5 Adoptive transfers**

After harvesting and initial enumeration, a fraction of lymph node cells was stained and assayed on a flow cytometer to identify transgenic T cell percentages of CA30 (CD4+V\(\beta\)8+) or OTII (CD4+V\(\alpha\)2+). Based upon this percentage and the Coulter enumeration, T cells were diluted with sterile PBS to obtain a concentration of \(5.0 \times 10^5\) T cells/mL (all but proliferation experiments) or \(5.0 \times 10^5 – 2.5 \times 10^7\) T cells/mL (CFSE proliferation experiments). Recipient mice received an i.v. transfer of 100µL of the specific cell suspension and allowed to rest for 24 hours prior to receiving a primary immunization. The day of transfer was considered to be “day -1” in immunization protocols.

**II.2.6 Immunization protocols**

As many of the experiments in this thesis were based upon variations on base immunization protocols, exact protocols will be discussed in figure captions. Mice received primary antigen injections 24 hours after the transfer of T cells on “day 0” of immunization protocols. Primary injections were delivered i.p. unless otherwise specified. In most cases, injections of antigen contained 100 µg of Ig species in 100 µl of sterile PBS without adjuvant or 10 µg – 300 µg of peptide. In some cases, antigen was co-emulsified in complete Freund’s adjuvant (CFA) or precipitated in alum. To
precipitate in alum, antigen was mixed 1:1 (v/v) with 0.2 AlKSO₄ and precipitated with 1M NaCO₃, the precipitate was sedimented, washed 3x in sterile PBS, and resuspended in sterile PBS for injection. All immunizations were staggered amongst multiple cages of animals. Immunization protocols can generally be summarized in a few categories:

*Early proliferation CFSE:* 5 x 10⁴ – 2.5 x 10⁶ CFSE-labeled T cells were i.v. transferred on day -1, antigen was i.p. injected on d0, mice were sacrificed on d3 – d5. Splenocytes and, in some experiments, lymph node cells were collected, stained, and analyzed via flow cytometry. Blood was collected to assay for the presence of transferred Ig species in the sera.

*Primary response time course:* 5 x 10⁴ T cells were i.v. transferred on day -1, antigen was i.p. injected on d0, mice were sacrificed at multiple time points between d0 – d30. Splenocytes were collected, stained, and analyzed via flow cytometry. Blood was collected to assay for the presence of transferred Ig species in the sera and the development of an anti-Ig response by the recipient.

*T follicular helper phenotyping:* 5 x 10⁴ T cells were i.v. transferred on day -1, antigen was i.p. injected on d0, mice were sacrificed on d14. Splenocytes were collected, stained, and analyzed via flow cytometry. Blood was collected to assay for the development of an anti-Ig response by the recipient.

*Anti-Ig antibody development:* 5 x 10⁴ T cells were i.v. transferred on day -1, antigen was i.p. injected on d0, and received booster injections of 100µg of an Ig species in sterile PBS on d21 and, in some cases, d42. Animals were bled prior to boost and 21 days after boost to assess for anti-Ig antibody development.
**T cell boost expansion:** 5 x 10^4 T cells were i.v. transferred on day -1, antigen was i.p. injected on d0, and mice received a booster injection of either 10µg of peptide and 10µg of bacterial LPS (Invivogen, San Diego, CA) or 100µg of an Ig species in sterile PBS on day 30 or day 21. In all cases, mice were sacrificed 5 days after boost. Splenocytes were collected, stained, and analyzed via flow cytometry. Blood was collected to assay for the development of an anti-Ig response by the recipient.

**α-CD20 B cell depletion experiment:** Mice were injected i.p. with 500 µg of mlgG2a5D2, an α-mouse CD20 IgG2aκ reagent donated generously by Genentech (San Francisco, CA) or monoclonal IgG2aκ from the myeloma UPC 10 (Sigma-Aldrich, St. Louis, MO) in sterile low aggregation pharmaceutical buffer 24 hours prior to adoptive transfer (day -2). 24 hours later, 5 x 10^4 CFSE-labeled T cells were i.v. transferred, and antigen was injected 24 hours after cell transfer. Mice received 100 µg monomeric mAb 36-71, heat aggregated mAb 36-71, immune complexes, or negative control Ig. Mice were sacrificed after 5 days and splenocytes were collected, stained, and analyzed via flow cytometry.

### II.2.7 Flow cytometry

Reagents used for FACS were purchased from Biolegend (San Diego, CA), eBioscience (San Diego, CA), Thermo Fisher Scientific (St. Louis, MO), Tonbo Biosciences (San Diego, CA) or generated in house (Refer to Table 2.1 for list of reagents used for flow cytometry). Cells were resuspended in staining buffer (PBS, 2% FCS, 0.1% NaN₃). Cells were surface stained with conjugated antibodies diluted in staining buffer on ice for 30 minutes in the presence of anti-CD16/32 (mAb 93) to block Fc receptors. Following surface staining, cells were washed 2x with staining buffer and
then immediately analyzed with a flow cytometer, or fixed by incubating in 1.6% paraformaldehyde in staining buffer in the dark for 15 minutes at RT. If cells were fixed and not immediately analyzed, they were stored in the dark at 4°C for less than 24 hours prior to analysis. In some cases (FoxP3), cells were stained for intracellular antigen after surface phenotyping using a protocol from the Ebioscience FoxP3 transcription factor staining kit. Briefly, cells were fixed and permeabilized, incubated with conjugated antibody diluted in permeabilization buffer for 1 hour on ice, and then washed 2x with permeabilization buffer and 2x with staining buffer before being analyzed immediately with a flow cytometer. For experiments involving the identification of congenic CD4+ T cells, cells were gated for forward and side scatter and then gated on CD4+, MHC II-, CD19-, F4/80-, CD8α- cells. At times, a data filter was implemented to exclude 99% of events falling outside of the CD4+, MHCII-, CD19-, F4/80-, CD8α- gate for the purposes of enriching for CD4+ event collection. Flow cytometry data was acquired on a FACScan, LSRII, or CyAn ADP flow cytometer and analyzed using FlowJo 9.7.1 (Tree Star, Ashland, OR).

II.2.8 Quantification of IgG α-Vκ36-71 antibody in sera

Microlon ELISA plates (Greiner Bio-One, Monroe, NC) were coated with 2 mg/ml IgM antibody ArsA11.1 in ascites fluid diluted in PBS O/N at 4°C and then incubated in a blocking buffer (2% BSA, 1% gelatin, 0.05% Tween 20 in PBS) for 2 hours at 37°C. Mouse serum was diluted between 1:100 and 1:25 in first well and titrated in serial 2-fold dilutions down the plate. An α-Vκ36-71 IgG2b antibody that has been previously described (mAb 17-63) was used as a standard starting at 200 ng/ml and serially diluted by half dilutions down the first two columns of the microlon plate.
Table 2.1 Reagents used for flow cytometry.

<table>
<thead>
<tr>
<th>Antibody clone</th>
<th>Reactivity/description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>15A7</td>
<td>Fas (CD95)</td>
<td>eBioscience</td>
</tr>
<tr>
<td>53-6.7</td>
<td>CD8α</td>
<td>eBioscience</td>
</tr>
<tr>
<td>A20</td>
<td>CD45.1</td>
<td>eBioscience or Tonbo Biosciences</td>
</tr>
<tr>
<td>B20.1</td>
<td>Vα2</td>
<td>eBioscience</td>
</tr>
<tr>
<td>BM8</td>
<td>F4/80</td>
<td>eBioscience</td>
</tr>
<tr>
<td>Clone 90</td>
<td>CD38</td>
<td>Biolegend</td>
</tr>
<tr>
<td>Clone 93</td>
<td>CD16/32 (Fc block)</td>
<td>Biolegend or made in house</td>
</tr>
<tr>
<td>eBio1D3</td>
<td>CD19</td>
<td>eBioscience</td>
</tr>
<tr>
<td>FJK-16S</td>
<td>FoxP3</td>
<td>eBioscience</td>
</tr>
<tr>
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</tr>
<tr>
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<td>CD69</td>
<td>Biolegend</td>
</tr>
<tr>
<td>IM7</td>
<td>CD44</td>
<td>Biolegend</td>
</tr>
<tr>
<td>KJ16-133</td>
<td>Vβ8.1/Vβ8.2</td>
<td>eBioscience</td>
</tr>
<tr>
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<td>MHC II (I-A/I-E)</td>
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</tr>
<tr>
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<td>CD90.1 (Thy1.1)</td>
<td>Biolegend</td>
</tr>
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<td>B220/CD45R</td>
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<td>PD-1 (CD279)</td>
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<td>CXCR5</td>
<td>eBioscience</td>
</tr>
<tr>
<td>Streptavidin</td>
<td>biotin conjugated antibodies</td>
<td>Thermo Fisher Scientific</td>
</tr>
</tbody>
</table>

Following a 3 hour incubation at 37°C, plates were washed 3 times with PBS and then incubated with 50 ng/well of a biotinylated polyclonal goat α-mouse IgG (Southern Biotech, Birmingham, AL) diluted in blocking buffer. Plates were incubated for 1 hour at 37°C and then washed 3 times with PBS, at which point 10 ng/well of a DELFIA SA-Europium reagent (Perkin Elmer, Waltham, MA) were added to the samples diluted in blocking buffer. Following a 30-minute incubation at 4°C, plates were washed with PBS and were developed in europium enhancement solution (100 mM Sodium acetate, 1 mM TTA, 750 mM TOPO, pH 3.2) (made in house). Europium counts were read in a Wallac
Victor® 1420 multilabel counter at 590 nm for 1 second/well. Regression analysis was performed using Prism graphing software (GraphPad Software, La Jolla, CA).

II.2.9 Anti-Vκ^{36-71} ELISPOT

Microlon ELISA plates were coated with 1 mg/ml of Vκ^{36-71} containing IgM ArsA11.1, which was stored in ascites fluid and coated on plates diluted in PBS overnight at 4°C. Plates were incubated with RPMI 10% FCS for 2 hours at 37°C. Splenocytes (5.0x10^5) were added to the first well of a row and titrated in serial 2-fold dilutions down the plate in TCM. After 7 hours, plates were washed 3 times with PBS, 0.05% Triton X-100. Polyclonal goat α-mouse IgG was applied at 0.5 ng/ml in blocking buffer and allowed to incubate overnight at 4°C. Plates were washed in PBS, and SA-AKP (Biolegend, San Diego, CA) was applied at 1:2000 in blocking buffer. After washing, plates were developed in 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 10 mM MgCl₂ with 1 mg/ml BCIP (Pierce, Rockford, IL) and developed for 2 hours at 37°C. Plates were scanned into TIFF images for blinded counting.

II.3 Statistics

Statistical analyses were performed using PRISM 5.0 (GraphPad, La Jolla, CA). Pairwise statistical analyses were made between samples using the Student’s t-Test as specified in the figure captions.
CHAPTER III

CHARACTERIZATION OF THE AGGREGATE CONCENTRATION, ADJUVANTICITY, AND *IN VITRO* STIMULATORY CAPACITY OF MONOCLONAL ANTIBODY 36-71

III.1 Introduction

As the primary aim of this dissertation was to ascertain the tolerogenicity or immunogenicity of a peptide delivered in an Ig for a specific CD4+ T cell, and the Ig species were produced in house, it was important to examine quantifiable differences between the reagents that would be used in *in vivo* experiments. In some capacity, this characterization served to build a framework to assess the literature of tolerogenicity, which was often based upon functional capacity of a reagent rather than specific and quantifiable physical attributes, in the context of advanced techniques that are being used by pharmaceutical researchers in the assessment of therapeutic proteins used in the clinic.

When Dresser first discovered the tolerogenic capacity of bovine gamma globulin in adult CBA mice, the innovation was that he ultracentrifuged his preps of BGG at 20,000 – 30,000 g for 30 minutes [43]. He described this step as important for the removal of particulate matter and later commented that the pellets of this ultracentrifugation had been examined with an electron microscope and had shown no signs of bacterial contamination. The inflammatory nature of immune complexes had already been identified in the context of studies of the Arthus phenomenon, so it logically followed that aggregated forms of Ig might exhibit similar behavior due to complement fixation. William Weigle had studied this immune complex phenomenon and had also published on immunogenicity of a different heterologous serum protein, BSA, in rabbits.
He had found that native BSA was immunogenic in most, but not all, rabbits, but that heat aggregated BSA was highly immunogenic. As his lab replicated Dresser’s phenomenon with HGG, they began to use heat aggregated HGG as a positive control for immunogenicity and as the boosting antigen to assess for a secondary response [61, 62]. The parallels between an aggregated, particulate protein and the pellet of the ultracentrifuged B/HGG were obviously apparent as well; in 1969, Golub, a member of the group, showed that the immunogenic portion of native HGG could be removed by injecting it into a mouse in large quantities and then taking the serum from that mouse and injecting it into a secondary mouse [66]. This “biological filter” likely utilized the phagocytic apparatus responsible for inducing an immune response to remove the particulate matter that would have been removed by ultracentrifugation. It was even able to tolerize a strain that was previously untolerizable with ultracentrifuged Ig: the Balb/c, quite the immunological parlor trick. However, the researchers at the time were satisfied with the concept that aggregated Ig could induce an immune response and were much more interested in the cellular mechanisms of the tolerance that they had witnessed with the ultracentrifuged Ig, and so the functional, rather than physical, characteristics of their aggregates took precedence.

In the modern realm of therapeutics, the antigenicity of manufactured protein is a critical conundrum in a billion dollar business that is increasingly coming under the scrutiny of regulatory infrastructure. These scientists would concur with the observations of the earlier HGG researchers that the aggregated protein created by the heat or in the visible pellet after ultracentrifugation could be immunogenic. However, the sophistication of their perception of aggregate species and their concern over the creation
of these aggregates has increased asymptotically since the days of Dresser. At almost
every step of manufacturing, storage, and administration, there is the potential for protein
aggregation, and the aggregates in question can range from dimerization to particles with
diameters in micron range [231, 233, 258, 260, 261]. Size exclusion chromatography has
been a useful technique to identify loss of mass in a therapeutic protein population, but
the limit on the mass that can be detected (<1000 kDa) has disallowed the
characterization of larger aggregate populations that do not fall beneath this mass limit.
More recently, protein tracking analysis (PTA) and microflow imaging (MFI) have
allowed the identification of subvisible particles with diameters in the nanometer and
micron range. Given that the FDA has recently expressed concern that particles in the 1-
10μm diameter range may be immunogenic, and studies have shown that even smaller
particles may be phagocytosed readily by APC, these analytical tools will probably
become de rigeur for analysis of therapeutic proteins during development, from
manufactured lot to manufactured lot, and in further studies of drug immunogenicity in
mouse and man [262].

Another consideration in regards to immunogenicity of the Ig to be used is the
content of soluble toll-like receptor (TLR) agonists such as lipopolysaccharide (LPS). In
1963, Henry Claman identified LPS as a nonspecific agent that could preclude tolerance
by ultracentrifuged BGG if it was administered on the same day [263]. Subsequent
characterization enlarged the window of nonspecific activity for LPS tolerance ablation
to three days before or after the dose of ultracentrifuged BGG. LPS is well known for its
role in inducing inflammation and has been implicated in both B and T cell activation.
For this reason, the FDA has set limits for the LPS content of pharmaceutical grade
reagents, which generally range from 500 pg/kg drug (5 endotoxin units (EU)) to 50 pg/ml (0.5 EU/ml) to be considered sterile water. The classic test for LPS is Limulus amebocyte lysate (LAL) assay, based upon the original observation that bacterial endotoxin causes clotting of the amebocytes of the horseshoe crab, *Limulus polyphemus* [264, 265].

The objective of the work in this first chapter was to investigate physical and chemical characteristics of Ig samples that would be used in *in vivo* experiments with the CA30 T cell. This included production and purification of monoclonal mAb 36-71, the production and purification of a Fab and F(ub’)_2 from 36-71, and aggregation of mAb 36-71 through heat-induced unfolding/aggregation and the creation of immune complexes with hapten (*p*-azophenylarsonate) conjugated mouse serum albumin. The monomeric Ig was compared to these aggregated species using techniques to quantify soluble aggregates and subvisible particles in the nanometer and micron range. All species were subjected to endotoxin testing via the LAL assay, and based upon the results, monomeric Ig was subjected to an *in vivo* test of adjuvanticity. Finally, the monomeric Ig and the aggregated species were tested in an *in vitro* presentation assay as an early assessment for how they might drive T cell proliferation *in vivo*.

### III.2 Results

#### III.2.1 Production of mAb 36-71, 36-71 F(ub’)_2 and Fab

The CA30 Tg CD4+ T cell recognizes an antigenic peptide located in the framework 1 (FR1) region of the κ light chain of mAb 36-71, an IgG₁ with somatic mutations increasing its affinity for the hapten *p*-azophenylarsonate by ~200 fold over the germline antecedent [255, 266]. To generate sufficient mAb 36-71 for experimentation,
the Ig was grown as an ascites in Rag2\(^{−/−}\) c\(\gamma\)\(^{−/−}\) mice and purified via DE52 anion exchange cellulose. This is in contrast to some pharmaceutical techniques utilizing antibody production in culture supernatants and purification via isotypic determinants. Ascites was chosen for its efficiency in antibody production as well as theoretical considerations based upon previous literature indicating glycosylation variation using cell culture production methods [267-269]. Anion exchange was chosen as a technique to obtain monomeric mAb based upon historical precedent as well as concern that acidic elutions typically associated with isotypic purification might lead to protein aggregation during elution. Fab and F(ab’\(^{\prime}\))\(_2\) were created using ficin, a sulfhydryl protease from fig latex with increased efficiency in cleavage for IgG\(_1\). After size exclusion, these reagents were stored in a low aggregation pharmaceutical buffer that has been described previously and aliquoted for storage at -20°C. All Ig products used were subjected to a single freeze-thaw cycle to limit aggregation. To ensure monomeric protein and remove aggregates from Ig preps prior to injection, I diluted Ig species in PBS to 1 mg/ml and ultracentrifuged at 165,000 x g in a TLA-120.1 fixed angle rotor. After ultracentrifugation, I removed the top 2/3 of the supernatant and used this product within two hours of production. I will use the term “monomeric Ig” to refer to ultracentrifuged mAb 36-71, but Fab and F(ab’\(^{\prime}\))\(_2\) species were also ultracentrifuged prior to injection.

**III.2.2 Creation of heat aggregates and immune complexes**

Heat aggregates were created in a manner consistent with previous literature, centrifuged, and the supernatant removed and assayed at OD\(_{280}\) to estimate the amount of mass remaining in the insoluble fraction that would be used for experiments. At the concentration, time, and temperature used, heat aggregation rendered roughly 66% of the
original mass into insoluble aggregates in all aliquots. Washed and stored aggregates from these preps were used for most experiments. A case where the aggregation percentage was higher (~100%) due to an increase in temperature (~65°C) will be discussed sporadically as “large heat aggregates” due to the increased incorporation of Ig.

Immune complexes were formed by incubating 100 µg of mAb 36-71 with varying amounts of Ars-MSA in 100 µl of total volume. Complexing was measured by centrifuging preps and assaying at OD$_{280}$ for evidence of protein loss to insoluble complexes. Figure 3.1 demonstrates the pattern of precipitation seen with two different preps of Ars-MSA, with the equivalence point at approximately 8 µg (Ars$_{14}$-MSA) and 16 µg (Ars$_{12}$-MSA). The term “immune complex” will be used to refer to complexes made with quantities of Ars-MSA approximately 4 times that required to reach the equivalence point(s) demonstrated in Figure 3.1, a practice based upon historical precedent[176-179]. There was no discernible difference between mice treated with immune complexes created with either Ars-MSA prep. In a few cases, “large immune complexes” will refer to complexes made at the equivalence point(s) due to the increased incorporation of Ig.

**III.2.3 Ultracentrifuged mAb 3671 is monomeric when compared to heat aggregates and immune complexes**

Size exclusion chromatography was used to assess for monomeric protein and soluble aggregates in each of the various preps. Samples were centrifuged at 13,000 x g and then loaded onto the column. When assessed at absorbance at 280 nm, the void volume can been seen around 5.8 minutes, the monomeric peak for monomeric protein
Figure 3.1 Precipitation of mAb 36-71 with arsanilated mouse serum albumin

Increasing amounts of Ars$_{12}$-MSA (closed square) or Ars$_{14}$-MSA (open triangle) antigen were added to 100 µg of mAb 36-71 in a total volume of 100 µl of PBS. Samples were incubated for 3 hours while rotating at 37°C and then centrifuged at 13,000 x g for 5 minutes. Supernatants were measured for OD$_{280}$. Values were calculated for soluble mass remaining in supernatant based upon ratio of calculated mass from absorbance/expected mass. Values reflect means of three samples complexed independently and error bars specify standard deviation.

can be seen around 8.1 minutes and a peak of the buffers (PBS with diluted histidine-trehalose) can be seen at 11.1 minutes (Figure 3.2A). The monomeric sample provided only a monomeric protein peak and a buffer peak with an integrated mass indicating 82% recovery of the ideal injected mass (74 µg/90 µg). The heat aggregated samples provided only a buffer peak, showing no monomeric protein or protein in the void volume. The immune complexes provided a peak in the void volume, a monomeric peak, and a buffer peak; the recovery was 44% of the ideal injected mass for the void volume (65 µg/146 µg) and 7.5% for the monomeric protein (11 µg/146 µg). To assess for dimers, absorbance at 215 nm was also measured to increase the sensitivity of detection (Figure 3.2B). The heat aggregated samples continued to show no signal for soluble Ig at 215
Figure 3.2 SEC chromatograms of experimental Ig species
SEC chromatograms of injections measured at both (A) 280 nm and (B) 215 nm for soluble protein analysis of the monomeric Ig (top), heat aggregated Ig (middle), and immune complex (bottom). The void volume appears at 5.8 minutes, the monomeric Ig peak occurs at 8.1 minutes, and the diluted buffer peak appears at 11.1 minutes. In (B), the gray box denotes region magnified in the inset, which demonstrates a dimer peak that appears at 7.1 minutes. Chromatograms are representative of triplicate analyses.

nm. By examining the absorbance in the time between the monomeric peak and the void volume in the Figure 3.2B inlay, dimeric populations of the Ig become more readily apparent; while it was difficult to differentiate mass definitively between dimeric and trimeric species in the immune complex population, the monomeric sample showed a dimeric population that was about 1.2% of the injected mass (1.1 µg/90 µg).
III.2.4 Immune complexes have an increased mass of particles with diameters in the nanometer range

Particle tracking analysis was used to assess the concentration of particles with diameters in the nanometer range in each sample (Figure 3.3A). Both buffer (PBS and diluted histidine-trehalose) and monomeric protein contained nanoparticles; the nanoparticle concentration in the monomeric sample peaked in the 60 – 80 nm range, ultimately reaching a concentration roughly 3 times that of the PBS particles, which peaked in the 50 – 70 nm range. Similar to the size exclusion data, the heat aggregates contained low concentrations of nanoparticles, with the highest concentration falling in the 50 – 70 nm range. Immune complexes showed the highest concentrations of nanoparticles, reaching concentrations 1.6 times the highest concentration of monomeric nanoparticles or 5 times that of the buffer, with the highest concentrations falling in the 210 – 230 nm range.

These particle tracking data could be used to estimate total protein mass per mL of protein solution, which is shown as a function of increasing nanoparticle size (Figure 3.3B). There was a base level of 100 ng/ml of nanoparticles in the monomeric (100 ng/ml) and 200 ng/ml in the heat aggregated samples. With the increased concentration of nanoparticles, particularly those with a larger diameter, the immune complex contained by far the most mass with an estimate of 7 µg/ml, roughly 35 – 70 times that of the other Ig samples.
Figure 3.3 Particle size distributions and integrated mass for nano-sized populations in experimental Ig species
(A) Particle size distributions were collected for nano-sized populations via particle tracking analysis of PBS, monomeric Ig, heat aggregated Ig, and immune complex. Monomeric samples (black line) are provided for comparison on each graph with other species represented in gray. Size of bins of 1 nm were used for representation of concentrations and error bars shown are standard deviation of triplicate samples. (B) Integrated mass for the monomeric Ig (black), heat aggregated Ig (red), and immune complex (blue) using an estimation of protein mass per size bin as discussed in the methods. Error bars shown are standard deviation of estimated mass of the triplicate samples.
III.2.5 Heat aggregates and immune complexes have an increased mass of particles with diameters in the micron range

It has been shown previously that freeze-thaw cycles significantly increase the number of 1 – 5 micron diameter subvisible particles, and also increase the total mass of aggregated protein, much of which occurs in particles larger than 5 micron\[233\]. Microflow imaging (MFI) was used to assess the concentrations of particles with diameters in the micron range in each of the samples (Figure 3.4A). Both buffer and monomeric Ig contained micron sized particles in the range of 1 – 5 microns, with concentrations roughly equivalent for all subvisible particles in this size range $\left(2.4 \times 10^3\right.$ particles/ml). The heat aggregate and immune complex samples contained significantly higher concentrations in the 1 – 5 micron range. The heat aggregated Ig samples peaked at $1.33 \times 10^5$ particles/ml at a size of 3 microns, a concentration 53-fold higher than the largest concentration for the buffer or monomeric protein (occurring at 1 micron). The immune complex samples peaked at $8.6 \times 10^4$ particles/ml in the 1 micron range, a concentration 34-fold higher than the buffer or monomeric protein at the same size.

Similar to the nanoparticle analysis, the particle counts from the MFI could be used to estimate the total mass of particles in the measured range, 1 – 50 micron, which is shown as a function of increasing subvisible particle size (Figure 3.4B). There was a base concentration 433 ng/ml in the monomeric samples, which was modest in comparison with the heat aggregate and the immune complex samples. The heat aggregate and immune complex contained 215 µg/ml and 693 µg/ml respectively, which were over 500-fold and 1600 fold greater than the concentration in the monomeric samples.
Figure 3.4 Particle size distributions, integrated mass and representative images of micron-sized particles in experimental Ig species
Figure 3.4 Particle size distributions, integrated mass and representative images of micron-sized particles in experimental Ig species

(A) Particle size distributions were collected for micron-sized populations via microflow imaging of PBS, monomeric Ig, heat aggregated Ig, and immune complex. Size of bins of 0.25 micron were used for representation of concentrations and error bars shown are standard deviation of triplicate samples. PBS (gray) and monomeric Ig (white) are shown in different graph than heat aggregated Ig (gray) and immune complex (white) due to large disparity in particle concentration. (B) Integrated mass for the monomeric Ig (black), heat aggregated Ig (red), and immune complex (blue) using an estimation of protein mass per size bin as discussed in the methods. Error bars shown are standard deviation of estimated mass of the triplicate samples. (C) Representative images of subvisible particles from heat aggregated Ig (top) and immune complex (bottom) of various size distributions.
An interesting, albeit qualitative, observation from the MFI analysis was the difference in the appearance of aggregate species of the same approximate size diameter from heat aggregate and immune complex samples (Figure 3.4C). The representative examples in the 10 – 15 micron, 15 – 25 micron, and 25 – 40 micron ranges demonstrate the typical differences between the two species. The heat aggregate subvisible particles appeared as dense, dark deposits with compact, globular formation as compared to the immune complex particles which appeared less dense, longer, and translucent.

**III.2.6 Summary of mass distribution of Ig species**

Table 3.1 presents a summary of the mass distribution of the Ig species based upon the SEC, PTA, and MFI that compares the recovered mass of each species to the ideal mass and the total mass that was actually recovered. Although there was some loss of ideal mass, the recovered mass for the monomeric Ig was virtually all monomer (98.49%) with a small fraction of dimer (1.44%). Heat aggregated Ig was missing the predominance of the ideal mass (78.48%) with the remaining mass being almost completely comprised of micron particles (99.91%). The immune complex had a wide distribution of mass, with the majority of the ideal and actual mass falling in the soluble complex (46.74%) and micron particle (44.83%) range.

**III.2.7 Mass distribution of the alternative “large” heat aggregates and “large” immune complexes**

As mentioned previously, there were two species of heat aggregates and immune complexes that were created that incorporated more Ig into the aggregates or complexes due to increased temperature of heat aggregation or incubation with Ars-MSA closer to
Table 3.2 Mass distribution of experimental Ig species compared to ideal and recovered mass

<table>
<thead>
<tr>
<th>Ig Species</th>
<th>Soluble Monomer</th>
<th>Soluble Complex</th>
<th>Nanometer Particles</th>
<th>Micron Particles</th>
<th>Missing Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of Ideal</td>
<td>% of Actual</td>
<td>% of Ideal</td>
<td>% of Actual</td>
<td>% of Ideal</td>
</tr>
<tr>
<td>Monomeric</td>
<td>82</td>
<td>98.49</td>
<td>1.20</td>
<td>1.44</td>
<td>0.01</td>
</tr>
<tr>
<td>Heat Aggregated</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.02</td>
</tr>
<tr>
<td>Immune Complex</td>
<td>7.50</td>
<td>7.97</td>
<td>44.0</td>
<td>46.7</td>
<td>0.43</td>
</tr>
</tbody>
</table>

*Visible, insoluble fraction

the equivalence point. These species were also analyzed using SEC, PTA, and MFI with the data and summary presented in Figure 3.5. SEC showed that the large heat aggregates (LHA) had both monomeric protein (7.9%) and soluble aggregates that were difficult to differentiate between dimer and void volume populations (2.5%), while large immune complexes (LIC) had no soluble protein (Figure 3.5A). PTA showed nanoparticles in both the LHA and LIC, with the LHA nanoparticles peaking in the 50 – 70 nm size range and accounting for 300 ng/ml, and the LIC nanoparticles peaking in the 135 – 170 nm size range and accounting for 7 µg/ml (Figure 3.5B). MFI showed micron sized particles in both groups, with both the LHA and the LIC peaking in the 1 micron diameter range (2.8 x 10^5 and 1 x 10^5 particles/ml respectively), although the LHA also had a peak at 2.5 microns (2.46 x 10^5 particles/ml), similar to the previously analyzed heat aggregates. These particles accounted for 944 µg/ml in the LHA and 23.9 µg/ml in the LIC (Figure 3.5C). The qualitative appearance of the LHA and LIC micron sized particles followed a similar pattern to the previously analyzed heat aggregates and
immune complexes (Figure 3.5D). Interestingly, each of the mass distributions was more similar to the opposite species in the previously analyzed aggregates and complexes (Table 3.2). Analysis of the LHA actually overestimated the ideal mass, predominantly due to variability in the MFI readings; of the mass that was recovered, it was distributed amongst all groups with contributions from monomer (0.75%), soluble aggregate (0.24%), nanometer particles (0.03%), and a majority of micron sized particles (99%). In contrast, very little of the LIC ideal mass could be accounted for with only nanometer particles (0.6%) and micron particles (2%). Given that the antibody-antigen complexes were formed closer to the equivalence point and could be expected to be larger and more likely to precipitate, it may not be surprising that 97.4% of the ideal mass was unaccounted for in these analyses.

**Table 3.2 Mass distribution of large heat aggregate Ig and large immune complex species compared to ideal and recovered mass**

<table>
<thead>
<tr>
<th>Ig Species</th>
<th>Soluble Monomer</th>
<th>Soluble Complex</th>
<th>Nanometer Particles</th>
<th>Micron Particles</th>
<th>Missing Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of Ideal</td>
<td>% of Actual</td>
<td>% of Ideal</td>
<td>% of Actual</td>
<td>% of Ideal</td>
</tr>
<tr>
<td>Large Heat Aggregated</td>
<td>7.90</td>
<td>7.25</td>
<td>2.50</td>
<td>2.39</td>
<td>0.30</td>
</tr>
<tr>
<td>Large Immune Complex</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.60</td>
</tr>
</tbody>
</table>

*Visible, insoluble fraction*
Figure 3.5 SEC, PTA, and MFI analyses of the large heat aggregated Ig and large immune complex samples
Figure 3.5 SEC, PTA, and MFI analyses of the large heat aggregated Ig and large immune complex samples

(A) SEC chromatograms of injections measured 280 nm for soluble protein analysis of the large heat aggregated Ig (left) and large immune complex samples (right). The void volume appears at 5.8 minutes, the monomeric Ig peak occurs at 8.1 minutes, and the diluted buffer peak appears at 11.1 minutes. All chromatograms are representative of triplicate samples. (B) Particle size distributions (left) were collected for nano-sized populations via particle tracking analysis of large heat aggregated Ig (black line) and large immune complex (gray line). Size of bins of 1 nm were used for representation of concentrations and error bars shown are standard deviation of triplicate samples. Integrated mass (right) was calculated for the samples using an estimation of protein mass per size bin as discussed in the methods. Error bars shown are standard deviation of estimated mass of the triplicate samples. (C) Particle size distributions (top) were collected for micron-sized populations via microflow imaging of large heat aggregated Ig (gray stripes) and large immune complex (white). Size of bins of 0.25 micron were used for representation of concentrations and error bars shown are standard deviation of triplicate samples. Integrated mass (bottom) for the large heat aggregated Ig (black), and large immune complex (gray) using an estimation of protein mass per size bin as discussed in the methods. Error bars shown are standard deviation of estimated mass of the triplicate samples. (D) Representative images of subvisible particles from large heat aggregated Ig (top) and large immune complex (bottom) of various size distributions.
III.2.8 *Limulus* amebocyte lysate assay does not rule out the possibility of LPS contamination

A commercially available colorimetric LAL assay was obtained to test for LPS in the reagents to be used in *in vivo* experiments. Reagents were tested in PBS and at concentrations prepared for injection into mice. Due to the recommendation of the protocol for the assay, the number of reagents tested, and the limited amebocyte lysate provided, data was collected as duplicate measurements at a single point and, as such, will not be shown graphically. However, a curve based upon an LPS standard was generated with each test for quantitation purposes and to ensure functional reagents, and this curve displayed linearity and range commensurate with recommended tolerances. The LAL test was performed three times using independent kits: the first assay was performed on monomeric Ig and a prep of heat aggregate, which indicated that the monomeric Ig prep (0.355 EU/ml) would be considered sterile water (0.5 EU/ml) and the heat aggregate prep would not (0.592 EU/ml). Subsequent assays were performed on monomeric Ig (same prep as first assay), heat aggregates (different prep, but made from same prep of monomeric Ig), immune complexes, and Ars-MSA approximately six months later. The results from these second and third assays were perplexing, as they showed all reagents except the heat aggregate (monomeric Ig, immune complex, Ars-MSA) had LPS concentrations well above sterile water (>1.0 EU/ml). Conversely, the heat aggregate had LPS concentrations below the threshold for sterile water (0.139 EU/ml). Based upon these results, it was not possible to conclude that the Ig preps had LPS concentrations similar to that of sterile water. However, the inconsistency in the results, such as heat aggregate made from aliquots of positive-testing monomeric Ig
testing as a very low negative, call this assay into question. There are published reports of false activation of the LAL assay by sugar moieties both from Ig and products that are purified using cellulose resins, and both the Ig and the MSA used were purified using cellulose products [270-272].

**III.2.9 mAb 36-71 does not act as a non-specific adjuvant in an in vivo model**

To assess the adjuvanticity of the monomeric mAb 36-71 in vivo, it was tested as a non-specific inflammatory mediator in a model CD4+ immune response to ovalbumin, similar to a previously published study. The experiment is briefly outlined in Figure 3.7A: in short, 5 x 10^4 transgenic CD4+ T cells (OT-II) that recognize a peptide from chicken ovalbumin (OVA_{323-339}) were adoptively transferred into mice and the next day mice were injected i.p. with no antigen, 300 µg OVA_{323-339}, 300 µg OVA_{323-339} emulsified in CFA, or 300 µg OVA_{323-339} and 100 µg of monomeric mAb 36-71 (Figure 3.6A). Based upon the previous work, I expected the OT-II cells to expand and increase in number by day 3 and then contract some by day 5 unless there was adjuvant, in which case the numbers would continue to expand. Mice were sacrificed on day 3 and day 5 and the T cell populations identified using a gating strategy relying on the expression of the congenic marker Thy1.1 on the OT-II cells (Figure 3.6B). When cells were enumerated based upon cell counts and cell percentages, only the peptide emulsified in CFA caused an increase in OT-II cell numbers between day 3 and day 5, while the OVA_{323-339} or the OVA_{323-339} and mAb 36-71 caused a cell expansion by day 3, but a decrease by day 5 (Figure 3.6C). In fact, the OT-II numbers were decreased in mice that received mAb 36-71 compared to mice that received OVA_{323-339} alone. To further ensure that this apparent lack of adjuvanticity was not a function of some epitope within
mAb 36-71 activating or impeding the OT-II and competing with OVA\textsubscript{323-339}, an \textit{in vitro} antigen presentation assay was set up to assess for CD69 upregulation on transgenic OT-II cells in response to antigen. OVA\textsubscript{323-339} was able to induce CD69 upregulation in OT-II cells alone, in the presence of mAb 36-71, and in the presence of the antigenic 36-71 peptide; the mAb 36-71 and 36-71 peptide could not stimulate CD69 upregulation (Figure 3.6D). In total, these experiments indicated that 100 µg of mAb 36-71, the amount that would be used for future experiments, did not have sufficient non-specific adjuvanticity to induce a productive transgenic T cell response to a well studied model antigen. This lack of adjuvanticity was unlikely to be some specific cross-reaction between the mAb 36-71 antigens and the OT-II T cell itself, giving even more credence to the interpretation that the mAb 36-71 was not teeming with non-specific adjuvant activity.

\textbf{III.2.10 Heat aggregated mAb 36-71 is a more potent antigen for \textit{in vitro} CA30 stimulation than monomeric Ig}

As a final experiment before beginning \textit{in vivo} characterization of the CA30 response to the Ig species, I used an \textit{in vitro} antigen presentation assay to ensure that the monomeric Ig and heat aggregates I had produced could stimulate these transgenic cells. Purified CA30 cells were CFSE labeled and cultured with irradiated splenocytes and antigen for 6 days, at which time they were harvested and assessed for the percentage of CA30 cells that diluted the CFSE as evidence of proliferation. The antigenic peptide of mAb 36-71 was used as a positive control, as this would not require processing for presentation and had been documented previously to be a potent stimulator of CA30
Figure 3.6 mAb 36-71 does not act as a non-specific adjuvant in an in vivo model
Figure 3.6 mAb 36-71 does not act as a non-specific adjuvant in an in vivo model

Mice were treated in an experimental protocol presented in (A). Briefly, C57BL6 mice received adoptive transfer of $5 \times 10^4$ congeneric OTII.Thy1.1 followed 1 day later by i.p. injection of antigen. Mice were sacrificed at day 3 or day 5 and splenocytes were assayed for Thy1.1+ cells. (B) Representative FACS plots expressing the percentages of Thy1.1+ cells in the CD4+, MHC II-, CD19-, CD8α-, F4/80- gate for each treatment group. (C) Scatter plot showing the percentage of Thy1.1+ of total CD4+ in the mice at day 3 (gray circle) and day 5 (black square) for each mouse in the treatment group (n=4 per group per day). (D) Scatter plot representing an in vitro assay to assess for activation of OTII.Thy1.1 cells by the mAb 36-71 or Vκ<sup>36-71</sup>. Briefly, 2.5 x $10^5$ OT-II.Thy1.1+ purified T cells were cultured with 2.5 x $10^5$ lightly irradiated (1100 rad) C57BL6 splenocytes. Cells were stimulated with 1 µM OVA<sub>323-339</sub> peptide or 1 µM Vκ<sup>36-71</sup> peptide or 100 nM mAb 36-71 or combinations thereof. Cells were incubated for 24 hours and then assessed via FACS for CD4+,Thy1.1+, MHC II- events to identify CD69 upregulation.
cells. Because of the possibility that mAb 36-71 might not induce proliferation at all, monomeric Ig with 1 µg/ml LPS was also included as an experimental sample. The 6 day time course was dictated by observing cell proliferation in the monomeric Ig cultures, as the heat aggregated and 36-71 peptide samples proliferated rapidly, determined by the observation of visible proliferative foci in these wells. This led to a stark difference in the CFSE profiles by day 6, with heat aggregated and peptide CD4+ cells having diluted the CFSE robustly and monomeric samples having a relatively small percentage of cells in the middle of a proliferative process (Figure 3.7A). The heat aggregated Ig induced a higher percentage of cells to proliferate at a concentration over 1000-fold lower than that of monomeric Ig (Figure 3.7B). Unexpectedly, the addition of the LPS to the monomeric Ig did not lead to increases in CA30 proliferation. It has been previously documented that aggregated antigen is superior at inducing CD4+ cell proliferation *in vitro*, and this held true for the Ig species. Most importantly, these results showed that my Ig was capable of inducing CA30 proliferation and, as such, *in vivo* experimentation would be feasible. I have preliminary data indicating that mAb 36-71 immune complexes generate a unique phenotype in culture; they do induce some proliferation, but they do not induce the same proliferative foci seen in heat aggregated or peptide samples. Instead, immune complexes induce large syncytial structures within the wells that resemble pieces of tissue (large, somewhat translucent, containing tens to hundreds of cells). We are curious whether this may be related to a report of immune complexes inducing neutrophil extracellular trap release, but have no further evidence to support this [273].
Figure 3.7 Heat aggregated Ig is a more potent antigen than monomeric Ig for in vitro CA30 cell stimulation

CA30.Thy1.1 cells were purified and CFSE labeled. 2.5 x 10^5 CA30 cells were cultured with 2.5 x 10^5 lightly irradiated (1100 rad) B6AF1 splenocytes. Cells received titrated amounts of V_{k}^{36-71} peptide, mAb 36-71, heat aggregated Ig, or mAb 36-71 with 0.2 µg LPS per well. Cultures were allowed to proceed for 6 days, at which point cells were stained for CD4+, Thy1.1+, MHC II- events to assess for CFSE proliferation. (A) CFSE profiles from representative samples for each treatment. A negative control is presented in black and the experimental condition is notated with the checked gray line. (B) Percentage of CFSE diluted cells a compared to negative control at different points in the titration of reagents. Results are mean of triplicate samples with bars denoting standard deviation. Data are representative of three independent experiments.
III.3 Discussion

The primary objective of the work in this chapter was to manufacture and characterize the Ig reagents to be used in *in vivo* experiments with the CA30 T cell, and to do so utilizing modern methods of pharmaceutical investigation that could provide a quantitative basis upon which to explain potential differences in the phenomenology related to them. Although ultracentrifuged, monomeric Ig and heat aggregated Ig are the operationally defined reagents of Dresser and Weigle, they can be further assessed in the context of a level of physical scrutiny that is unparalleled and the basis for a rapidly growing pharmaceutical literature.

The production of mAb 36-71 and its variants was relatively straightforward, with a few nuances. The choice to produce mAb 36-71 in ascites in part for concern about glycosylation variation was not based upon specific evidence comparing the ascites with a culture supernatant, but instead on reports from the literature. Recent work on glycosylation of antibodies and their function would indicate that changes in these post-translational modifications can increase the inflammatory capacity, decrease binding to FcRs, or perhaps suppress immune function globally, meaning that this decision to use ascites could have significant consequences on my *in vivo* results. Similarly, this potential difference in glycosylation could be a source of the confusion with my results in the LAL assay, although I think it is more likely that the DE52 cellulose purification is responsible for this finding. One advantage of the DE52 should be that the majority of the LPS would stick to the anion exchange column and should elute in a later fraction than Ig due to its negative charge. The result of the OT-II *in vivo* assay, as well as the fact that I did not see LPS induced proliferation in splenocytes that have not been
irradiated (data not shown) with any of my Ig products leads me to believe that the sources of sugar residues are more likely the cause of the elevated values seen in the *Limulus* assay. If the Ig was contaminated with some TLR agonist, I believe that I would have seen a more substantial response in the *in vivo* assay, but in retrospect, I should have used LPS or another soluble TLR agonist instead of CFA as a positive control, as this would have made the experiment more tightly controlled.

Discussing the creation of the heat aggregate and the immune complexes may seem rote, however it raises two points that affect interpretations of *in vivo* experiments. The first is that due to the amount of protein and volume used to create the aggregates, it was impractical to create a titration curve varying concentrations, time, and heat. Instead, I relied on an established protocol and read the OD$_{280}$ of the resultant product [274]. The variant heat aggregate, aggregated at approximately 2 degrees °C aggregated a higher percentage of protein. Based upon a given batch of Ars-MSA, the formation of immune complexes followed an orderly and replicable pattern, as would be predicted by theory and techniques such as the Ouchterlony double immunodiffusion test. Second, the assays used to generate and characterize the heat aggregate do not reveal anything about the state of the Fc, which complicates interpretations of *in vivo* data. Immune complexes are assumed to have intact Fc given the lack of any treatment that would cause perturbation/misfolding in the protein structure itself. While the previous literature would suggest that misfolding of the Fc does not occur until a higher temperature (71°C) than the variable region (61°C), the fact that the aggregation procedure relies upon misfolding of protein means that the actual forms of the aggregate that are formed may contain vastly different available Fc arrays for binding[275]. While heat aggregate may
be a good positive control based upon precedent in these immunogenicity assays, the immune complex would seem to be the more consistently replicable reagent using a specific monoclonal antibody and antigen system. The qualitative observation that the two species cause differential effects in splenocyte proliferation and behavior in vitro implies that it may be worthwhile to characterize differences in the Fc functionality between the two species in the future.

In regards to the SEC, PTA, and MFI data, the first thing to observe is that these techniques produced an enormous amount of data that can be analyzed from multiple different perspectives. The data were analyzed based upon previously published methods that placed emphasis on particles of specific groups of sizing (1 – 5 micron, for example) and accounting for mass of the sample. The SEC data was the most straightforward, identifying the soluble monomer and soluble aggregate populations in each sample, while the PTA and MFI could be used to define mass distributions based upon any category within the particles that were measured. This type of resolution may eventually be useful in terms of identifying particle populations that are particularly immunogenic or tolerogenic, but in the current study the most useful aspect was to identify global distribution of the Ig mass. The ultracentrifuged, monomeric Ig was predominantly monomeric, with a small amount of dimeric Ig and even smaller amounts of nanometer and micron sized particles. There was a noticeable loss of mass compared to the ideal protein yield, however this could be explained by machine error or evaporation in the ultracentrifuge; in the past I have identified the Ig concentration of samples post-centrifugation and have found virtually no loss, however I did not evaluate the mass in the samples after ultracentrifugation due to time constraints with the pharmaceutical
analyses. There was no visible precipitate in the sample or the ultracentrifugation tube, implying that little of the Ig was lost to an insoluble fraction. The heat aggregate mass distribution was weighted to micron sized particles and an insoluble fraction that was not analyzed in the pharmaceutical assays, but clearly visible in these samples. In contrast, the immune complexes had a wide distribution of the mass including all types of size species, with the majority falling in the micron and soluble aggregate categories. Interestingly, the “larger” species of heat aggregate and immune complex appeared to be similar to the opposite species (i.e. LHA to immune complex) in terms of mass distribution. A prediction that arose from the mass distribution in the heat aggregate and immune complex samples was that these species would have different patterns of immunogenicity in vivo, with immune complexes perhaps having tempered immunogenicity due to an increased monomer population in the sample.

Finally, the preliminary in vitro assay was useful for hypothesis generation for the in vivo studies as well as confirmation that the monomeric Ig and heat aggregated Ig could both stimulate CA30 proliferation. The fact that heat aggregate was over 1000-fold more potent in stimulating proliferation indicated that there was probably a facilitated uptake of these particles as compared with monomeric Ig which was probably taken up through macropinocytosis. This finding was not surprising based upon previous work examining increased presentation of particulate, aggregated OVA as compared to deaggregated OVA protein in T cell stimulation assays, however I had predicted that monomeric Ig might be readily taken up through some Fcγ related mechanism and, as such, had believed that the disparity between the monomeric Ig and heat aggregate might be decreased. From the perspective of the in vivo work, this result, along with the
historical literature, supported a hypothesis that monomeric Ig would not be immunogenic for the CA30 T cell when injected into mice.
CHAPTER IV

IG AGGREGATION LEADS TO PRODUCTIVE, CD4-MEDIATED HUMORAL IMMUNITY WHILE MONOMERIC IG STIMULATES AN EXISTENT, BUT NON-PRODUCTIVE, CD4+ RESPONSE

IV.1 Introduction

The rejection of monoclonal antibody therapeutics is a clinical quandary that recalls a long debate over the immunogenicity of Ig. Seminal work in this field implied that deaggregated Ig could tolerize both the bone marrow (B cell) and thymus (T cell) compartments, and the quest to discover the mechanism(s) through which this tolerance in T cells is mediated has spanned nearly half a century. Three of the consistent deficiencies in this literature has been the usage of bulk population of T cells, inferred data from pathological phenomenology, or in silico hypothetical analysis, to explore these mechanisms.

Our lab possessed a unique reagent, the CA30 transgenic T cell, which recognizes an antigenic peptide found in the somatically generated κ-light chain of an antibody generated in a canonical response of A/J mice to the hapten p-azophenylarsonate[255]. This Ig, mAb 36-71, was an IgG1 that could be readily produced and purified and could be heat aggregated or complexed with a haptenated protein[252, 253]. Using an adoptive transfer model, congenic CA30 T cells could be transferred into recipient mice that subsequently received different preparations of the mAb 36-71, and the responses of these specific T cells could be tracked with a resolution heretofore unreported in the literature. These specific T cell responses could be compared to humoral responses to
mAb 36-71 determinants generated in a similar fashion to determine whether aspects of the CA30 activation correlated with productive anti-Ig responses.

Based upon the historical literature as well as experience with a pilot study in an in vitro model, I hypothesized that monomeric 36-71 would not be perceived by transferred CA30 cells and this lack of perception would prevent the rise of a productive humoral response. If any response to the monomeric Ig existed, I predicted that it would elicit CA30 conversion to regulatory T cells (T_{reg}). Conversely, I hypothesized that heat aggregated and immune complexed mAb 36-71 would lead to robust CA30 activation and strong humoral responses. I undertook adoptive transfers with these unique reagents to test these hypotheses. My results support a more complicated, nuanced picture of the in vivo immune response to an antigenic Ig: that monomeric Ig can readily elicit a CD4+ response, but one that is abortive and non-productive in generating a humoral response, but does not lead to the production of conventional T_{reg}. Conversely, heat aggregated and immune complexed Ig without significant adjuvant elicit a CD4+ response that appears modest in terms of cell numbers, but induces a population of cells to differentiate into T follicular helper (T_{fh}) cells that facilitate a productive humoral response. While both induce this humoral response, the mechanisms by which they initiate the response may require different cell populations, and B cells may be playing a more important role in activating and suppressing CD4+ responses to these antigen than we had previously appreciated.
IV.2 Results

IV.2.1 Identification of CA30 T cells by using congenic markers in the B6AF1 system

The CA30 T cell recognizes a $\text{V}_\kappa^{36-71}$ FR-1 peptide in the context of the MHC II I-A$^k$, which is why the CA30 transgenes are maintained on an A/J genetic background. A class II tetramer bearing the antigenic peptide that could label CA30 cells was previously constructed and described, however the low yield of production and a rapid rate of loss of function at 4°C led me to adopt a congenic marker for identifying transferred CA30 T cells. The CA30 mouse was bred to B6 mice containing either the Thy1.1 or CD45.1 congenic markers that were used to identify the T cells when transferred into mice of background Thy1.2 or CD45.2. Initially, work was carried out using the Thy1.1 marker, but this strategy was altered when plans were laid to use the Thy1.1 marker to identify an alternate transgenic T cell (OT-II) in B6AF1 recipient mice while CD45.1 was used to identify CA30 T cells. While the OT-II B6AF1 turned out to be a functional failure due to a superantigen-mediated deletion of V$\beta$5-expressing OT-II cells, the breeding of CA30.CD45.1 mice was already well underway, at which point this congenic marker was used for all future analyses.

IV.2.2 Monomeric Ig induces primary CA30 T cell proliferation, but heat aggregated Ig generates a weak humoral immune response

At the outset of in vivo experiments, my hypothesis was that ultracentrifuged, monomeric mAb 36-71 would not be perceived by adoptively transferred CA30 T cells, while heat aggregates would lead to robust CA30 proliferation and activation. To test this, I adoptively transferred $5 \times 10^4$ CA30 cells into B6AF1 recipients and injected these
animals i.p. 24 hours later with 100 µg of negative control monomeric IgG1 (mAb 36-65), monomeric mAb 36-71, mAb 36-71 precipitated in alum, or mAb 36-71 heat aggregates, and then sacrificed the mice and assayed splenocytes at time points during a 35 day primary response. Representative FACS from different treatments and timepoints are presented in Figure 4.1A. The results of this time course demonstrated a few notable characteristics (Figure 4.1B). First, contrary to expectation, the CA30 cells readily perceived, expanded, and retracted in response to the monomeric mAb 36-71, making a primary response similar to other transgenic models, in particular models of peptide-induced tolerogenicity with rapid expansion and contraction indicating a non-productive response[276, 277]. The alum precipitated mAb 36-71 generated a primary response that mirrored other transgenic models using adjuvant to induce a productive response; the population of CA30 T cells on day 7 was 53 fold higher than the population elicited by the monomeric Ig. The response to the heat aggregates was also surprising and appeared counter to the initial hypothesis; there was no statistical difference between the CD45.1+ cell numbers or percentage CD45.1+ of total CD4 compared to the response to the monomeric Ig. I had expected robust proliferation more similar to the large, plateaued response to the mAb 36-71 precipitated in alum. In retrospect, I believe that this initial experiment may have been underpowered for the subtlety of the CA30 response to heat aggregates, as a subsequent analysis of cell numbers at day 14 in the primary response with more mice showed a statistically significant increase in CA30 cells between heat aggregated and monomeric Ig treated groups (Figure 4.1C).

To assess whether these injections in the initial time course were leading to a humoral response, sera were obtained from mice at day 30 post-immunization and tested
for IgG reactivity to the $\nu_{36-71}$ light chain in a DELFIA assay utilizing a plate-bound IgM containing this light chain (ArsA11.1) as a target antigen. The alum-precipitated mAb 36-71 elicited an IgG anti-$\nu_{36-71}$ response in all mice that were treated (8/8), the monomeric Ig did not elicit such a response in any of the mice treated (0/8), and the heat aggregate treatment elicited this response in 3/8 mice that received it as a primary antigen (Table 4.1). The alum-generated response produced a mean IgG $\alpha$-$\nu_{36-71}$ concentration of 13 µg/ml, while the three mice in the aggregate treated group that made antibody produced a mean concentration of 142 ng/ml, a concentration 92 fold lower than that elicited by the alum. The fact that heat aggregated Ig elicited a small, detectable primary humoral response in a fraction of animals that received treatment contrasted with the monomeric Ig treatment, which never elicited a response.

**IV.2.3 Heat aggregated, but not monomeric, Ig primes for a memory humoral response**

From the previous experiment, it appeared that the heat aggregate was able to generate a weak humoral immune response to the somatically mutated $\nu_{36-71}$ light chain, but the unexpected expansion of the CA30 cells in mice treated with monomeric Ig was

<table>
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<tr>
<th>Treatment</th>
<th>Mice Producing $\alpha$-$\nu_{36-71}$ at day 30</th>
<th>Mean concentration $\alpha$-$\nu_{36-71}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Control</td>
<td>0/8</td>
<td>-</td>
</tr>
<tr>
<td>Monomeric Ig</td>
<td>0/8</td>
<td>-</td>
</tr>
<tr>
<td>Heat Aggregated Ig</td>
<td>3/8</td>
<td>0.142 µg/ml (SD 0.03)</td>
</tr>
<tr>
<td>Monomeric Ig in Alum</td>
<td>8/8</td>
<td>11.85 µg/ml (SD 10.83)</td>
</tr>
</tbody>
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Figure 4.2A  Primary proliferative time course of the CA30 T cell in response to monomeric Ig and heat aggregated Ig
(A) B6AF1 mice received adoptive transfer of 5 x 10^4 congenic CA30.45.1 followed 1 day later (day 0) by i.p. injection of 100µg antigen in sterile PBS or alum. Mice were sacrificed at different time points and splenocytes assayed for CD45.1+ cells. Presented are representative FACS plots from mice of different primary treatments (top) and days (side) expressing the percentages of CD45.1+ cells in the CD4+, MHC II-, CD19-, CD8α-, F4/80- gate.
Figure 4.1B  Primary proliferative time course of the CA30 T cell in response to monomeric Ig and heat aggregated Ig

(B) Mean CD45.1+ numbers in splenocytes (top) or CD45.1+ percentage among total CD4+ (bottom) of 4 mice per group per day in primary proliferative response of CA30 T cell to Ig species. Antigen injected i.p. on day 0 included 100 µg of negative control IgG1 (crosses, dotted), 100 µg of monomeric Ig (circles, solid), 100 µg of heat aggregated Ig (open circles, gray) or 100 µg of Ig precipitated in alum (squares, dashed). After identifying CD45.1+ population by FACS as in (A), cell numbers were calculated by multiplying CD45.1+ percentage among CD4+ by CD4+ percentage among live cell gate by total number of cells in the spleen. Bars denote standard deviation of values. Results are representative of 1 experiment.
Figure 4.1C  Primary proliferative time course of the CA30 in response to monomeric Ig and heat aggregated Ig

(C) Representative FACS plots (left) and number of CD4+ CD45.1+ cells (right) in splenocytes of mice at day 14 after injection of Ig species. B6AF1 mice received adoptive transfer as in (A) and (B) and i.p. injection of 100 µg of monomeric Ig of heat aggregate in sterile PBS one day later (day 0). Mice were sacrificed at day 14 and splenocytes were assessed by FACS. To increase the yield of CD45.1+ cells, events were pre-gated to exclude 99% of events outside a CD4+, MHC II-, CD19-, CD8α-, F4/80- gate. Symbols represent individual mice and mean of cell percentages is denoted by the bar in the scatter plot. Statistics represent a pairwise comparison calculated by a two-tailed Student’s t-test with p<0.05 (*). Data are representative of three independent experiments containing 5 or more mice per group.

curious. While there was a significant increase in the number and percentage of CA30 cells in mice that received heat aggregate compared to monomeric Ig, this increase was 2.5 fold as compared to over 50 fold for mice that received alum-precipitated Ig. Perhaps both the monomeric Ig and the heat aggregate were eliciting humoral immune responses, but the magnitude was sufficiently low that they were difficult to detect. To address this question, I sought to amplify the humoral response in mice to the Vk36-71 by boosting these mice with heat aggregated Ig and testing for an increase in α-light chain IgG. I adoptively transferred CA30 cells and injected mice with 100 µg of monomeric Ig, heat aggregated Ig, or the negative control IgG1. After 21 days, these mice were bled and
boosted and then allowed to rest for 21 days before being bled again (Figure 4.2A). The negative control IgG\textsubscript{1} was included to act as an indicator of primary response by surviving naïve CA30 cells at the time when the other mice were being boosted; based upon literature that indicated that monomeric Ig might be tolerogenic, it was possible that I could find evidence of this by detecting a primary anti-light chain response in the negative mice and no response to the boost in the monomeric Ig mice. Conversely, the monomeric Ig mice might boost an undetectable primary humoral response, suggesting that CA30 proliferation was productive. In this experiment, the mice given a primary injection of heat aggregated Ig were the only ones that made either a primary or secondary humoral response (Figure 4.2B, Table 4.2). In the primary response, 2/5 mice made an Ig\text{G anti-Vk}\text{36-71} response to the heat aggregate, and that number rose to 5/5 mice in the secondary response. In 4/5 mice, the amount of detectable α-V\text{k}\text{36-71} antibody was increased over that of the primary response, as would be predicted. Neither the monomeric Ig (0/5, 0/5) nor the negative IgG\textsubscript{1} (0/5, 0/5) produced a primary or secondary Ig\text{G anti-Vk}\text{36-71} response. While this may have been the result that was expected for the monomeric Ig, there was an interesting point about the negative IgG\textsubscript{1} and the boost: in the original literature about the HGG tolerance, mice with cells that were not tolerized, but also not primed, made a primary response to the heat aggregated HGG in the time period when the tolerized mice were “not responding” [68]. The lack of a detectable Ig\text{G response in any of the negative mice at day 21 post-boost (primary for that group) was interesting insofar as it was a deviation from the historical precedent.

As an aside, the boost in anti-V\text{k}\text{36-71} Ig\text{G} in animals that were primed with heat aggregated Ig, but not in mice receiving monomeric Ig, concurred with an earlier pilot
Figure 4.2  Heat aggregated Ig, but not monomeric Ig, primes for a memory humoral response
Mice received experimental vaccination as in (A). Briefly, B6AF1 mice received adoptive transfer of 5 x 10⁴ CA30.CD45.1 cells and 1 day later were bled and i.p. injected with either 100 µg of either negative control Ig (open circle, gray), monomeric Ig (square, black), or heat aggregated Ig (triangle, gray) in sterile PBS. Mice were rested for 21 days, bled, and then received a booster injection of 100 µg of heat aggregated Ig in sterile PBS. Mice were bled at day 42. (B) IgG α-Vκ³⁶-⁷¹ concentration was quantified in sera using a DELFIA assay and extrapolated based upon a standard curve generated by a previously reported IgG₂b α-Vκ³⁶-⁷¹ (mAb 17-63). Individual mice are represented by continuous line connecting sera concentration at each time point. Each group contained 5 mice.
Table 4.2 Heat aggregated Ig, but not monomeric Ig, primes for a memory humoral response

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mice Producing α-Vκ&lt;sub&gt;36-71&lt;/sub&gt; in primary</th>
<th>Mean concentration IgG α-Vκ&lt;sub&gt;36-71&lt;/sub&gt;</th>
<th>Mice Producing α-Vκ&lt;sub&gt;36-71&lt;/sub&gt; in secondary</th>
<th>Mean concentration IgG α-Vκ&lt;sub&gt;36-71&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Control</td>
<td>0/5</td>
<td>-</td>
<td>0/5</td>
<td>-</td>
</tr>
<tr>
<td>Monomeric Ig</td>
<td>0/5</td>
<td>-</td>
<td>0/5</td>
<td>-</td>
</tr>
<tr>
<td>Heat Aggregated Ig</td>
<td>2/5</td>
<td>0.307 µg/ml</td>
<td>5/5</td>
<td>4.17 µg/ml (SD 6.33)</td>
</tr>
</tbody>
</table>

study that was not expanded (Figure 4.3A). A batch of heat aggregates that had been created accidentally, rather than in a controlled manner, were able to stimulate a primary response by day 30 (4/4) and a secondary response at day 35 (4/4), 5 days after a boost with the aggregates, while mice that received monomeric Ig developed neither a primary (0/4) nor secondary response (Figure 4.3B). Splenocytes of these mice were tested by ELISPOT analysis for α-Vκ<sub>36-71</sub> plasmablast formation, which showed a mean of 52 plasmablasts/10<sup>6</sup> splenocytes that received two treatments with heat aggregate and no detectable ASC in mice that received a monomeric primary and heat aggregated secondary (Figure 4.3C). The mice that received two doses of heat aggregate also showed an increased total ASC response on a control plate for total Igκ-bearing cells, so these mice were tested for the formation of a rheumatoid factor (IgM anti-IgG), which was negative (data not shown).

**IV.2.4 Heat aggregated Ig drives CA30 T cells through more division cycles than monomeric Ig in the spleen**

Monomeric Ig was able to induce a primary proliferative response of the CA30, but this proliferation did not lead to a primary or secondary humoral response. The heat
Figure 4.3 Evidence of humoral memory response to a unique heat aggregated Ig species
Figure 4.3 Evidence of humoral memory response to a unique heat aggregated Ig species

Mice received experimental vaccination as in (A). Briefly, B6AF1 mice received adoptive transfer of 5 x 10^4 CA30.CD45.1 cells and 1 day later were bled and i.p. injected with either 100 µg of monomeric Ig (circle, black), or a uniquely generated heat aggregated Ig (square, gray) in sterile PBS. Mice were rested for 28 days, bled, and then received a booster injection of 100 µg of the same heat aggregated Ig in sterile PBS. Mice were sacrificed at day 33 and blood and splenocytes were collected for DELFIA and ELISPOT analysis. (B) IgG α-Vκ^36-71 concentration was quantified in sera using a DELFIA assay and extrapolated based upon a standard curve generated by a previously reported IgG_2b α-Vκ^36-71 (mAb 17-63). Individual mice are represented by continuous line connecting sera concentration at each time point. (C) IgG α-Vκ^36-71 ELISPOT data from day 5 post boost (day 33). Images of ELISPOT (left) are representative of five mice per group. Frequency of IgG α-Vκ^36-71 splenic ASCs (right) was determined by ELISPOT. Symbols represent individual mice and line represents mean values.
aggregates induced primary and secondary humoral responses, but the difference in CA30 cell numbers and percentages was less than I had initially predicted. In approximately the last decade, the kinetics of cell division in a CD4+ primary response have been studied in two different models that provide conflicting views. The first was a study that contrasted CD4+ (OT-II) and CD8+ (OT-I) proliferation in response to infection with a Listeria monocytogenes strain that expressed OVA, which showed that CD4+ cells divided a limited number of times and most cells only divided a few times even if antigen dosage was increased, whereas CD8 cells expanded rapidly to any dose that activated the cells[278]. Conversely, experiments utilizing a doxycycline-induced MHC II-peptide Tg mouse showed that a CD4+ Tg cell would proliferate as long as antigen was being presented in a primary response, and that activating dendritic cells with α-CD40 could prolong this presentation and proliferation[279-281]. Although, it is important to note that the activation of dendritic cells and prolonged presentation was not seen when an Ig-coupled protein was used to load dendritic cells instead of the MHC II-peptide transgene. I favored the second model as an analog for my experiments as I believed that the inflammatory milieu induced by Listeria might influence CD4+ cells to behave differently than those in my system with little-to-no adjuvant. I hypothesized that the α-Vκ36-71 Ig that I saw in response to the heat aggregated Ig was a consequence of an increase in CA30 cell divisions compared to those treated with monomeric Ig. By examining a day 5 time point with an increased number of mice, I predicted that I would see an increase in CA30 cell cell divisions. To assess the number of divisions that occurred, I wanted to label the CA30 cells with CFSE, a fluorescent dye that stains proteins and is diluted by cellular mitosis, thus allowing for tracking of number of
divisions. I performed the experiment by adoptively transferring $5 \times 10^4$ CA30 cells and then injected mice with either the negative control Ig, the monomeric Ig, or the heat aggregated Ig. The CFSE proliferation profiles between the two groups were notably different; the cells that were treated with the monomeric Ig seemed to have the majority of cells in the 3rd or 4th division (Figure 4.4A). Conversely, the heat aggregated Ig treated cells seemed to have a higher percentage of cells that reached the 5th, 6th, and 7th division. I used the Flowjo cell proliferation algorithm to identify the pattern of division in each experiment and calculated the percentages of the CA30 cells that could be found at each stage of division; the mean CFSE divisions for various Ig species can be found in Table A1. As I had predicted, the heat aggregated Ig had a significantly higher percentage of cells remaining in the 7th division (Figure 4.4B). This agreed with my hypothesis, that a higher percentage of cells would be found that had divided more times in the heat aggregate group.

I thought that this increase in the percentage of cells found at a later division might lead to an increase in the total percentage of the CA30 cells in the spleen as compared to monomeric Ig, but this was also shown to be incorrect. To ensure that there was not an abundance of CA30 cells in the lymph nodes of mice that I had missed, I extracted individual lymph nodes (right and left inguinal, both brachial, both axillary) at day 5 from these mice and pooled the individual lymph nodes from each mouse within a treatment group and assessed for CFSE proliferation and CA30 T cell percentage (Figure 4.4D). I suspected that I would find evidence of enrichment of heavily divided CA30 T cells in the lymph nodes of the animals that were injected with heat aggregated Ig. This
Figure 4.4 A-C Heat aggregated Ig drives CA30 T cells through more division cycles than monomeric Ig in the spleen, but not lymph nodes
Figure 4.4 A-C Heat aggregated Ig drives CA30 T cells through more division cycles than monomeric Ig in the spleen, but not lymph nodes
Mice received adoptive transfer of $5 \times 10^4$ CFSE labeled CA30.CD45.1 cells and 1 day later (day 0) received 100 µg of negative control Ig, monomeric Ig, or heat aggregated Ig. Mice were sacrificed at day 5 and splenocytes were analyzed via FACS. (A) Representative FACS plots of CFSE and CD45.1 staining in the CD4+, MHC II-, CD19-, CD8α-, F4/80- gate (top) and histograms containing CFSE proliferation profile gates generated by FlowJo proliferation algorithm and applied to all samples (bottom). Histograms are gated on CD4+CD45.1+ cells. (B) Mean percentages of CD4+CD45.1+CFSE+ cells in the 7th division as calculated from the gates derived by the FlowJo proliferation algorithm. (C) Mean percentage of CD45.1+ cells among total CD4+ cells for mice depicted in other figures. Symbols represent individual mice and mean of cell percentages is denoted by the bar in the scatter plot. Statistics represent a pairwise comparison calculated by a two-tailed Student’s t-test with p<0.005 (**). Data are representative of two independent experiments containing 4 mice per group.
Figure 4.4 D–F Heat aggregated Ig drives CA30 T cells through more division cycles than monomeric Ig in the spleen, but not lymph nodes
Mice received adoptive transfer of 5 x 10⁴ CFSE labeled CA30.CD45.1 cells and 1 day later (day 0) received 100 µg of negative control Ig, monomeric Ig, or heat aggregated Ig. Mice were sacrificed at day 5 and pooled LN cells were analyzed via FACS. (D) Representative histograms containing CFSE proliferation profile gates generated by FlowJo proliferation algorithm and applied to all samples. Histograms are gated on CD4+CD45.1+ cells and depict pooled cells from axillary LN. (E) Mean percentage of CD45.1+ cells among total CD4+ cells for pooled axillary, brachial, right inguinal, and left inguinal lymph nodes. (F) Mean percentages of CD4+CD45.1+CFSE+ cells that had not divided as calculated from the gates derived by the FlowJo proliferation algorithm. Symbols represent pooled axillary, brachial, left inguinal, or right inguinal lymph nodes from 4 mice and mean of cell percentages is denoted by the bar in the scatter plot. Statistics represent a pairwise comparison calculated by a two-tailed Student’s t-test with p<0.05 (*). Data are representative of pooled lymph node cells from one experiment.
assumption was wrong; in fact, the percentages of CA30 cells among total CD4+ population were lower in the lymph nodes of the mice that received heat aggregates, and the CFSE profile of these lymph nodes showed an increased undivided population (Figure 4.4E,F). The percentages of this undivided population were significantly larger in mice that received heat aggregated Ig than in mice that received monomeric Ig. Based upon previous experience with a similar phenomenon in the spleen in CFSE experiments utilizing larger adoptive transfers, I suspect that this decreased proliferation is a function of either increased clearance of the heat aggregated Ig or sequestration by a splenic population such as the marginal zone B cell.

IV.2.5 Heat aggregates induce an increase in CA30 T follicular helper (Tfh) cells by day 14

The evidence thus far indicated that heat aggregates could lead to a small, but significant increase in CA30 cells at day 14 when compared to monomeric Ig, it induced IgG α- Vκ36-71 in some mice by day 21 and this response would expand to booster injection whereas animals that received monomeric Ig never produced antibody, and that there was a different proliferative pattern for heat aggregates that led to a larger percentage of the CD also undergoing more divisions (7) whereas monomeric Ig had most cells divide but a large percentage. This implied that there might be functional differences in CA30 T cells between mice receiving the different forms of Ig, which might affect the early proliferation (to day 5) and accumulation at the time of the germinal center (day 14), ultimately leading to a humoral response in one case but not the other. There were two phenotypes that we were curious to investigate: regulatory T cells (Treg) and T follicular helper (Tfh) cells. Based upon research into the “T-regitopes”
postulated to exist in the Fc of Ig as well as the historical precedent for long-lived
tolerance in treated thymocytes, we thought that the lack of antibody production and
accumulation of T cells at lower cell divisions might be a function of the CA30 cells in
animals receiving monomeric Ig differentiating into T<sub>regs</sub>[149, 151, 153-155, 157].
Conversely, while it was possible that the IgG α-<i>Vk</i><sub>36-71</sub> generated in response to heat
aggregated Ig was due to a T-independent process, the fact that a secondary
immunization could boost the response led me to think that a germinal center might be
involved in the generation of antibody, and the different proliferative profile of the CA30
let me to wonder whether these cells were differentiating into T<sub>fh</sub>. To test these
hypotheses, I set up an adoptive transfer with 5 x 10<sup>4</sup> CA30 T cells and injections of 100
µg of monomeric Ig and heat aggregated Ig and sacrificed the mice on day 7, 10, or 14 on
the assumption that these days should fall in the early development or close to the peak of
a potential germinal center response. I injected some mice with 100 µg of mAb 36-71
precipitated in alum to act as a positive control to aid in the identification of T<sub>fh</sub> and
germinial center development and other mice with the negative control IgG<sub>1</sub> to assess for
differences in the germinal center formation. Germinal center B cells were identified by
staining and assessing for a CD38<sup>lo</sup>Fas<sup>hi</sup> population. At day 14, this population was
readily seen in mice that had received alum precipitated Ig, but no increase in this
population was seen in mice receiving the monomeric Ig or the heat aggregated Ig
(Figure 4.5A). That the heat aggregated Ig did not induce a noticeable germinal center B
cell population was surprising given that previous work had demonstrated an α-<i>Vk</i><sub>36-71</sub>
boost in response to a secondary injection. However, the relatively low amount of α-
<i>Vk</i><sub>36-71</sub> that I had seen in response to a primary injection in most cases (hundreds of
nanograms/ml) made me suspicious that the precursor frequency of the B cell recognizing the heat aggregated Vκ36-71 epitope might be low and that the lack of adjuvant might be limiting the expansion of this response.

While there was not a difference between the monomeric Ig and the heat aggregated Ig in terms of the percentage of germinal center B cells, this did not mean that the CA30 cells were not phenotypically different. I stained cells with typical markers to identify T<sub>reg</sub> cells, the high affinity IL-2 receptor CD25 and the transcription factor forkhead box P3 (FoxP3), in the hopes that I might identify an expanded population in the mice that received monomeric Ig (Figure 4.5B). Given that I was most interested in the phenotype of the CA30 cells, I focused on the cells co-staining with these two markers in the monomeric Ig and heat aggregated groups. At day 7, day 10, and day 14, there was no statistical difference between the percentages of CA30 cells that were CD25<sup>+</sup>FoxP3<sup>+</sup> in the two groups (Figure 4.5C). There was a variation in the T<sub>reg</sub> percentage in both groups, where there seemed to be a decrease in the percentage from day 7 to day 10 and then an increase from day 10 to day 14, however the intergroup differences on all days were insignificant, and the difference in percentage ranged from roughly 2 – 5 percent. A naïve CA30 spleen examined during one of these experiments showed a T<sub>reg</sub> percentage of about 5. This was not the expansion of T<sub>reg</sub> cells that I had expected to see in the monomeric Ig population, so I wondered if the expansion in T<sub>reg</sub> cells might have occurred in the total CD4<sup>+</sup> population rather than the CA30 population specifically. If the mAb 36-71 possessed “T-regitopes” in the Fc, perhaps the regulation was occurring at the level of a non-CA30 T cell. However, an examination of the T<sub>reg</sub> percentage of the full CD4<sup>+</sup> repertoire of the mice receiving these treatments also showed
no difference between the two groups (Figure 4.5D). This does not fundamentally rule out the possibility that meaningful T\text{reg} cells were being formed in this repertoire or even within the CA30 subset, however it does imply that there was not a substantial expansion of T\text{reg} cells in either population.

In the hypothesis I posited, the counterbalance to the T\text{reg} cell would be the formation of the T\text{fh} cells in response to the heat aggregated Ig. The lack of a substantive germinal center B cell population in either of the groups, particularly the heat aggregated Ig, did not bode well for the appearance of this population, but I thought that the increased precursor population of CA30 cells might offer an opportunity to see the phenotype arise. T\text{fh} cells were identified by their expression of the C-X-C chemokine receptor type-5 (CXCR5) and the inhibitory receptor programmed cell death-1 (PD-1), which have been previously described as markers of these cells (CXCR5\text{hi}PD-1\text{hi}). To alleviate the problem of noise generated by low CA30 counts, I increased the number of adoptive transfer mice analyzed, as well as focused on collecting and storing a majority CD4+ events (99% to 1% falling outside of the CD4+, MHC II-, CD19- CD8α-, F4/80-gate), which allowed for manageable flow cytometry files as well as increased CA30 cells to allow for cleaner analyses. The result of expanding the population showed that there was a significant increase in the number of CA30 cells that were CXCR5\text{hi}PD-1\text{hi} in mice receiving treatment with heat aggregated Ig (Figure 4.5E). While the general germinal center B cell population had not increased, that was an analysis of polyclonal B cell response to an antigen without additional adjuvant, whereas the CA30 analysis focused on a monoclonal cell population and allowed for increased resolution of a
relatively modest immune response. This CA30 result suggested that the lack of the primary or secondary response to monomeric Ig was not due to suppression or to

Figure 4.5 A-D Heat aggregated Ig induces an increase in CA30 T follicular helper (Tfh) cells by day 14
Figure 4.5 A-D Heat aggregated Ig induces an increase in CA30 T follicular helper (Tfh) cells by day 14

Mice received adoptive transfer of $5 \times 10^4$ CFSE labeled CA30.CD45.1 cells and 1 day later (day 0) received 100 µg of negative control Ig, monomeric Ig, heat aggregated Ig, or Ig in alum. Mice were sacrificed at day 7, 10, and 14 and splenocytes were analyzed via FACS. (A) Representative flow plots of percentage of germinal center B cells in treated mice at day 14. Cells were gated as CD4-B220+ and analyzed for CD38loFashi cell percentages. Flow plots are representative of 4 mice per group (B) Representative flow plots of percentage of CD25+FoxP3+ Treg among CD45.1+ cells in treated mice at day 14. Cells were gated as CD45.1+ CD4+, MHC II-, CD19-, CD8α-, F4/80-. A naïve CA30.CD45.1 was substituted for negative control due to low cell numbers 7-14 days after adoptive transfer. Flow plots are representative of 4 mice per group, except for the naïve CA30 (C) Mean percentages of CD25+FoxP3+ cells among CD45.1+ cells at day 7, 10, and 14 in mice treated with monomeric Ig or heat aggregated Ig. Symbols represent individual mice, bars represent the mean, and error bars represent standard deviation. Statistics represent a pairwise comparison calculated by a two-tailed Student’s t-test with $p<0.05$ (*). (D) Representative flow plots of percentage of CD25+FoxP3+ Treg among CD45.1+ cells in treated mice at day 14. Cells were gated as CD4+, MHC II-, CD19-, CD8α-, F4/80-. Flow plots are representative of 4 mice per group.
Figure 4.5 E Heat aggregated Ig induces an increase in CA30 T follicular helper (T_{fh}) cells by day 14

Mice received adoptive transfer of 5 x 10^4 CFSE labeled CA30.CD45.1 cells and 1 day later (day 0) received 100 µg of monomeric Ig or heat aggregated Ig. Mice were sacrificed at day 14 and splenocytes were analyzed via FACS. To increase the yield of CD45.1+ cells, events were pre-gated to exclude 99% of events outside a CD4+, MHC II-, CD19-, CD8α-, F4/80- gate. (A) Representative flow plots of percentage of CXCR5^{hi}PD-1^{hi} cells among CA30.CD45.1 population. Cells were gated on CD4+CD45.1+ events. (B) Number of CD4+ CD45.1+ CXCR5^{hi}PD-1^{hi} cells per mouse. Symbols represent individual mice, bar represents the mean, and error bars represent the standard error. Statistics represent a pairwise comparison calculated by a two-tailed Student’s t-test with p<0.05 (*). Data are representative of three independent experiments containing at least 5 mice per group.
significant increase in the percentage of $T_{reg}$ cells, but instead to some failure in germinal center response as compared to heat aggregated Ig as described by the rise of $T_{fh}$ in that population.

**IV.2.6 Immune complexes generate a similar humoral immune response to heat aggregated Ig in the CA30 adoptive transfer model**

Thus far, I had compared the immune responses to monomeric versus heat aggregated Ig because heat aggregates had had been used historically in BGG and HGG suppression experiments as an immunogenic species. They also acted as a model for errors in manufacturing and handling of therapeutic monoclonal Ig that have been postulated to be the source of $\alpha$-Ig responses in patients. In the experiments described to this point, heat aggregated Ig produced a modest, but at times significantly increased primary CA30 proliferation in comparison to the initially surprising proliferation in response to monomeric Ig. The heat aggregates caused a small primary humoral response that could be boosted as a secondary response with subsequent injections of heat aggregates, while the monomeric Ig did not produce a primary response to monomeric Ig or secondary boost to heat aggregates. The heat aggregates drove CA30 cells to divide more in the early proliferative response. Finally, heat aggregates induced an increase in $T_{fh}$ formation by day 14 in the primary response as compared to monomeric Ig, which implied a germinal center response. Now, I wanted to explore whether these phenomena could be replicated with a species of Ig aggregate that would be a common occurrence during systemic immune responses rather than an error of exogenous Ig handling: an immune complex. The immune complex might act quite differently than the heat aggregate due to its formation; heat aggregates are generated by causing the association
of hydrophobic regions of misfolded protein, immune complexes are native Ig molecules associated into structures via binding of antigen. They would be expected to have intact and functional Fc regions, and the increase in avidity expected that comes from associating multiple Fc regions together is thought to promote Fc signaling in some cases. It must be said that there may be functional Fc available in the heat aggregate, as it has been shown that unfolding occurs at a higher temperature (71°C) than the temperature used for this aggregation (61°C) [275]. Regardless, I wanted to evaluate immune complexes using the experimental assays that I had used with the heat aggregated Ig. First, I wanted to assess whether the immune complexes generated a primary CA30 expansion and retraction over a similar time course that was used to evaluate the heat aggregated Ig. I adoptively transferred 5 x 10^4 CA30 cells and immunized with 100 µg of monomeric Ig or the equivalent of 100 µg on mAb 36-71 in premade immune complexes. I sacrificed animals on day 2, 5, 11, and 21 and stained splenocytes to identify congenically marked CA30 cells. The kinetics of CA30 T cell expansion was not different from monomeric Ig and similar to what I had initially observed with the heat aggregates (Figure 4.6A). A reevaluation at day 14 did yield a statistically significant difference between the number of CA30 T cells in immune complex treatment as opposed to monomeric Ig (Figure 4.6B). Again, the relative modesty of the response was notable, but this was a reasonable result in light of the lack of adjuvant in the primary injection.

This presence of statistical difference in cell numbers at day 14, and the general similarity in primary response to the monomeric Ig, made me believe that I would be able to generate a humoral response to the V\kappa_{36-71} with the immune complex, as I had with the
heat aggregate. To test this, I adoptively transferred $5 \times 10^4$ CA30 cells into mice and then gave a primary injection with 100 µg of monomeric Ig or the equivalent Ig in preformed immune complexes. I bled these mice at day 21 and immediately boosted with 100 µg equivalent in preformed immune complexes, and then repeated this at day 42, with a final bleed at day 63. Sera were tested for IgG $\alpha$-V$\kappa_{36-71}$ activity using the same DELFIA assay used to assess the heat aggregates (Figure 4.7C, Table 4.3). At d21, neither the mice receiving the immune complexes nor the monomeric Ig made an IgG $\alpha$-V$\kappa_{36-71}$ response (0/5, 0/5). After a boost at day 21, 4/5 mice with a primary injection of immune complex made an IgG response by day 42, which reached 5/5 by day 63 after a second boost. In all cases, the concentration of $\alpha$-V$\kappa_{36-71}$ increased post boost, albeit modestly in some cases. Mice that received a primary monomeric Ig injection did not develop an IgG response at either day 42 (0/5) after 1 boost or day 63 (0/5) after 2 boosts. This result indicated that immune complexes were able to generate a memory response, although the levels of IgG in the primary response, if any, were beneath detection. Monomeric Ig continued to fail to develop a primary response or memory responses, even after 2 additional injections with immune complexes.

If the immune complexes could lead to a humoral response, I wanted to know whether the CFSE proliferation profile generated by immune complexes would be different than that induced by monomeric Ig. To test this, I did similar adoptive transfers to what I had used previously, with $5 \times 10^4$ CFSE-labeled CA30 cells. These mice were injected with immune complexes or monomeric Ig and then sacrificed at day 5 and splenocytes were analyzed for proliferation. As opposed to the heat aggregates, the general patterns of
Table 4.3 Immune complexes prime for a memory humoral response

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mice Producing α-Vκ&lt;sub&gt;36&lt;/sub&gt;-71 in primary at day 21</th>
<th>Mean concentration IgG α-Vκ&lt;sub&gt;36&lt;/sub&gt;-71</th>
<th>Mice Producing α-Vκ&lt;sub&gt;36&lt;/sub&gt;-71 in secondary at day 42</th>
<th>Mean concentration IgG α-Vκ&lt;sub&gt;36&lt;/sub&gt;-71</th>
<th>Mice Producing α-Vκ&lt;sub&gt;36&lt;/sub&gt;-71 in tertiary at day 63</th>
<th>Mean concentration IgG α-Vκ&lt;sub&gt;36&lt;/sub&gt;-71</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomeric Ig</td>
<td>0/5</td>
<td>-</td>
<td>0/5</td>
<td>-</td>
<td>0/5</td>
<td>-</td>
</tr>
<tr>
<td>Immune Complex</td>
<td>0/5</td>
<td>-</td>
<td>4/5</td>
<td>0.768 µg/ml (SD 0.69)</td>
<td>5/5</td>
<td>3.63 µg/ml (SD 3.92)</td>
</tr>
</tbody>
</table>

proliferation were consistent between transfers with different cell numbers, while the total percentages of cells differed. My hypothesis, based upon the difference in the humoral response was that the CFSE profile would indicate a larger population of cells that proliferated to further divisions (7) when comparing the immune complex to the monomeric Ig. The qualitative CFSE profiles showed a consistent pattern in which the monomeric led to large percentages of cells in the range of 3 – 4 divisions, while the immune complexes had an increased percentage of the number of cells that reached further (7) divisions (Figure 4.6D). Using the Flowjo CFSE proliferation algorithm, I identified peaks of division and calculated percentages of CA30 cells that were currently at each stage of division (Table A2). In the mice that received immune complexes, the percentage of CD45.1+ cells in the 7<sup>th</sup> division were significantly increased in comparison to mice receiving monomeric Ig. Again, I was curious whether there might be a sequestration or proliferation of cells in the lymph nodes that I was not perceiving by focusing on splenocytes, so I adoptively transferred 5 x 10<sup>4</sup> CA30 cells into mice, and
Figure 4.6 A-C Immune complexes generate a similar primary expansion and humoral response to heat aggregates
B6AF1 mice received adoptive transfer of $5 \times 10^4$ congenic CA30.45.1 followed 1 day later (day 0) by i.p. injection of 100 µg of monomeric Ig or immune complexes in sterile PBS. Mice were sacrificed at different time points and splenocytes assayed for CD45.1+ cells. (A) Mean CD45.1+ percentage among total CD4+ of 4 mice per group per day in primary proliferative response of CA30 T cell. (B) Representative FACS plots (left) and number of CD4+ CD45.1+ (right) in splenocytes of mice at day 14 after injection of Ig species. To increase the yield of CD45.1+ cells, events were pre-gated to exclude 99% of events outside a CD4+, MHC II-, CD19-, CD8α-, F4/80- gate. Symbols represent individual mice and mean of cell percentages is denoted by the bar in the scatter plot. Statistics represent a pairwise comparison calculated by a two-tailed Student’s t-test with $p<0.05$ (*). Data are representative of three independent experiments containing 5 mice per group. (C) IgG $\alpha$-$\kappa^{36-71}$ concentration in mice injected with immune complexes or monomeric Ig. B6AF1 mice received adoptive transfer of $5 \times 10^4$ CA30.CD45.1 cells and 1 day later were bled and i.p. injected with either 100 µg of either monomeric Ig (black), or immune complex (gray) in sterile PBS. Mice were rested for 21 days, bled, and then received a booster injection of 100 µg of heat aggregated Ig in sterile PBS. Mice were bled at day 42 and received another booster injection. IgG $\alpha$-$\kappa^{36-71}$ concentration was quantified in sera using a DELFIA assay and extrapolated based upon a standard curve generated by a previously reported IgG2b $\alpha$-$\kappa^{36-71}$ (mAb 17-63). Individual mice are represented by continuous line connecting sera concentration at each time point. Each group contained 5 mice.
Figure 4.6 D–E Immune complexes generate a similar primary expansion and humoral response to heat aggregates
Figure 4.6 D-E Immune complexes generate a similar primary expansion and humoral response to heat aggregates

(D) Representative FACS plots of CFSE and CD45.1 staining in the CD4+, MHC II-, CD19-, CD8α-, F4/80- gate (top), histograms containing CFSE proliferation profile gates generated by FlowJo proliferation algorithm and applied to all samples (bottom), and mean percentages of CD4+CD45.1+CFSE+ cells in the 7th division as calculated from the gates derived by the FlowJo proliferation algorithm (right). B6AF1 mice received adoptive transfer of $5 \times 10^4$ CFSE labeled CA30.CD45.1 cells and 1 day later (day 0) received 100 µg of monomeric Ig, or immune complex. Mice were sacrificed at day 5 and splenocytes were analyzed via FACS. Histograms are gated on CD4+CD45.1+ cells. Symbols represent individual mice and mean of cell percentages is denoted by the bar in the scatter plot. Statistics represent a pairwise comparison calculated by a two-tailed Student’s t-test with p<0.05 (*). Data are representative of two independent experiments containing 4 mice per group.

(E) Representative FACS plots of percentage of CXCR5hiPD-1hi cells among CA30.CD45.1 population (left) and number of CD4+CD45.1+ CXCR5hiPD-1hi cells (right). Mice received adoptive transfer of $5 \times 10^4$ CFSE labeled CA30.CD45.1 cells and 1 day later (day 0) received 100 µg of monomeric Ig or immune complex Ig. Mice were sacrificed at day 14 and splenocytes were analyzed via FACS. To increase the yield of CD45.1+ cells, events were pre-gated to exclude 99% of events outside a CD4+, MHC II-, CD19-, CD8α-, F4/80- gate. Symbols represent individual mice, bar represents the mean, and error bars represent the standard error. Statistics represent a pairwise comparison calculated by a two-tailed Student’s t-test with p<0.05 (*). Data are representative of three experiments containing at least 5 mice per group.
then injected them IP with immune complexes or monomeric Ig and examined the axillary, brachial, right and left inguinal lymph nodes at day 5. Unlike what had been seen in the mice receiving heat aggregated Ig, there was no defect in CA30 T cell proliferation in the lymph node, but also not an enrichment (data not shown). The overall portrait of proliferation in the lymph node suggested that the immune complex was less susceptible to the mechanism that hampered heat aggregated Ig from being presented in these sites.

Finally, the humoral immune response and the increased percentage of cells in the 6th and 7th division suggested that the immune complexes might be generating a T\textsubscript{fh} response. To test this, I adoptively transferred 5 x 10\textsuperscript{4} CA30 cells into recipients and then injected them i.p. with 100 µg of monomeric Ig or the equivalent of 100 µg of mAb 36-71 in immune complexes. Mice were sacrificed on day 14 and splenocytes were analyzed for evidence of cells with CXCR5\textsuperscript{hi}PD-1\textsuperscript{hi} T\textsubscript{fh} phenotype. The percentages of T\textsubscript{fh} were significantly increased at day 14 in the spleens of the mice that received immune complexes as compared to those that received monomeric Ig (Figure 4.6E). This suggested that, similar to the heat aggregates, the immune complexes were inducing T\textsubscript{fh} development, which accorded with the data indicating a successful humoral response in animals with this treatment as compared to mice injected with monomeric Ig.

**IV.2.7 Alternative “large” heat aggregates and immune complexes induce divergent proliferative profiles at day 5**

In the course of experimentation, I generated two species of aggregated Ig that acted anomalously in regards to the generation of a humoral response. The first was a batch of heat aggregates that was aggregated at a higher temperature (65°C compared to
62°C) and incorporated a higher percentage of the Ig mass (~100% vs 66%) than the typical heat aggregates used in my experiments. The second was immune complexes generated at the equivalence point concentration of Ars-MSA rather than 4 times the concentration of the equivalence point, which was standard in the majority of my experiments. These complexes incorporated a larger component of the total Ig mass into the insoluble fraction. Neither of these species generated a primary response (0/5, 0/5) when injected into animals that had received 5 x 10⁴ CA30 cells and both displayed poor secondary responses when mice injected with the large aggregate were later challenged with “immunogenic” heat aggregated Ig (0/5) or when mice injected with large immune complexes were challenged twice (0/5 and subsequently 1/5) with immune complexes generated at antigen excess (Table 4.4). I was curious whether there was a short term test that I could have used to identify these species as unlikely to induce a response prior to using them in longer term experiments, and because CA30 proliferation in vitro to monomeric Ig was nil compared to what was observed in vivo. The difference in the proliferative patterns between the normal aggregated species (heat aggregated, Ag-excess immune complex), in comparison to monomeric Ig, and the fact that these aggregated species led to a humoral response and the monomeric Ig did not, led me to wonder whether the CFSE assay I had used previously would be a useful screening assay to identify reagents that were anomalous. To test this, I adoptively transferred 2.5 x 10⁵ CFSE-labeled CA30 cells into mice and then injected them with 100 µg of the large heat aggregate or the equivalent of Ig mass in large immune complexes or control populations (monomeric Ig, heat aggregates, immune complexes). I sacrificed these mice at day 5 and assayed their splenocytes for evidence of CFSE dilution in the CA30 cells. The
patterns of proliferation were different between the large species of the aggregates/complexes and the version used for most experiments. In the case of the large heat aggregates, there was an increased percentage of cells that did not divide at all and a decreased percentage of cells that reached the furthest measured division (7) (Figure 4.7A, Table A3). The large immune complexes had a pattern of division that was quite different from the Ag-excess immune complexes, with an increased percentage of cells that did not divide, an increased percentage of cells at divisions 1-4, and decreased percentages of cells that had undergone 5–7 divisions (Figure 4.7B). The similarity between the cell percentages that were in the 5–7th division in the monomeric Ig samples and the large heat aggregate and large immune complex groups was intriguing, because neither form of Ig produced a primary or memory immune response. This does not prove that the increased divisions are critical to the development of a humoral response, *per se*, merely that this type of analysis could be potentially useful for evaluating future batches of mAb 36-71 heat aggregates and immune complexes prior to the usage in larger experiments.

**Table 4.4 Large heat aggregates and large immune complexes do not prime for a memory humor response**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mice Producing α-Vκ36-71 in primary at day 21</th>
<th>Mean concentration IgG α-Vκ36-71</th>
<th>Mice Producing α-Vκ36-71 in secondary at day 42</th>
<th>Mean concentration IgG α-Vκ36-71</th>
<th>Mice Producing α-Vκ36-71 in tertiary at day 63</th>
<th>Mean concentration IgG α-Vκ36-71</th>
</tr>
</thead>
<tbody>
<tr>
<td>Large Heat Aggregate</td>
<td>0/5</td>
<td>-</td>
<td>0/5</td>
<td>-</td>
<td>0/5</td>
<td>-</td>
</tr>
<tr>
<td>Large Immune Complex</td>
<td>0/5</td>
<td>-</td>
<td>0/5</td>
<td>-</td>
<td>1/5</td>
<td>1.76 µg/ml</td>
</tr>
</tbody>
</table>
IV.2.8 The lack of a functional Fc diminishes presentation of a peptide in the F\textsubscript{ab} region

The concept of using this CFSE proliferation assay to evaluate reagents intrigued me and I wanted to use this methodology to ask another question about presentation that had puzzled me since my initial observation that monomeric mAb 36-71 induced robust proliferation: did this presentation somehow rely on the Fc portion of the Ig? To test this, I generated F(ab')\textsubscript{2} and Fab fragments using the enzyme ficin and purified them by size-exclusion chromatography with the help of Fran Crawford in the Kappler/Marrack lab. It was important to generate both species because of the capacity for the F(ab')\textsubscript{2} to crosslink the receptor on a B cell, which the literature had suggested could induce a humoral response when injected into mice without adjuvant. This was attributed to a capacity to crosslink the B cell receptor without engaging the inhibitory Fc\gammaRIIB. Conversely, a Fab α-IgD reagent has been shown to tolerize B cells when transferred, rather than induce a productive humoral response. I hypothesized that this capacity to crosslink the receptor of B cells would allow the F(ab')\textsubscript{2} to be presented to the CA30 T cell, while the Fab would not be efficiently presented. I adoptively transferred 2.5 x 10^5 CA30 cells into recipient mice and then injected the mice with 100 µg of the monomeric Ig, or the equivalent amount of antigenic V\kappa\textsubscript{36-71} epitope in the form of F(ab')\textsubscript{2} (73 µg) or the Fab (66 µg). After 5 days, I sacrificed the mice and assessed the splenocytes for CFSE proliferation of the CA30 cell. When I calculated the CA30 percentage in the total CD4+ population, I realized that my hypothesis was incorrect. Neither the Fab nor the F(ab')\textsubscript{2} induced CA30 proliferation; their CA30 percentages were significantly lower.
Figure 4.7A-B Large heat aggregates and large immune complexes drive fewer divisions than expected
Mice received adoptive transfer of $5 \times 10^4$ CFSE labeled CA30.CD45.1 cells and 1 day later (day 0) received either heat aggregated Ig or large heat aggregated Ig (experiment in A) or immune complex or large immune complex (experiment in B). Mice were sacrificed at day 5 and splenocytes were analyzed via FACS. Representative histograms containing CFSE proliferation profile gates generated by FlowJo proliferation algorithm and applied to all samples. Histograms are gated on CD4+CD45.1+ cells. Histograms are representative of two experiments with 4 mice per group.
than the monomeric Ig and, in fact, equivalent to the cell populations in mice that received the negative IgG\textsubscript{1} (Figure 4.8A). When I used the Flowjo CFSE proliferation algorithm to identify division peaks, I discovered that both the Fab and the F(ab’)\textsubscript{2} had increases in the cell percentages at 0 and 1 division and had decreased percentages of cells in later divisions (Figure 4.8B, Table A4). On top of having a decreased percentage of CA30 cells overall in comparison to monomeric Ig treated mice, the cells that were remaining were had not divided much. Out of curiosity, I had also included an additional group which received F(ab’)\textsubscript{2} complexed with Ars-MSA. As I had normalized the amount of F\textsubscript{ab} region (and antigenic peptide) that I was injecting into these mice, I decided to complex the 73 µg of F(ab’)\textsubscript{2} with the same amount of excess antigen, Ars-MSA, that I would use to complex the roughly equivalent amount of F\textsubscript{ab} in 100µg of mAb 36-71. The F(ab’)\textsubscript{2} complexes rescued the CA30 percentage of total CD4+, returning it to statistically equivalent with the monomeric population and rising it back above the negative IgG\textsubscript{1} control (Figure 4.8C). When I examined the cell proliferation patterns qualitatively and quantitatively, I saw a pattern that reminded me of the heat aggregate or the immune complex. There was a decrease in the percentage of cells that were in the middle stages of division (2 – 5) that were followed by an increase of the percentage that were in division 6 or 7 as compared to the monomeric Ig. The rescue in total CA30 percentage had associated with an increase in the population that was at division 6 or 7, which was very similar to what had been seen in both the heat aggregate and the immune complex.
IV.2.9 B cells are an important source of presentation for heat aggregated Ig antigen to CA30, but not critical for presentation of immune complex or monomeric Ig

The observation that complexing of F(ab’)2 could rescue the proliferation of CA30 was interesting in that it implied that there was probably some aspect of increase in antigen size that could lead to increased presentation. It was possible that the Ars-MSA contained a sufficient amount of LPS or other adjuvant that was responsible for the increase in antigen presentation, however the negative result with the large immune complexes, which only contained 4-fold less antigen than the F(ab’)2 immune complex made this seem unlikely. Given that the antigen size argument was more intriguing, I was curious whether the presentation of the immune complex or the heat aggregates could be influenced by targeting the cell that I believed might benefit from an increase in avidity of the V\textsubscript{\kappa}36-71 antigen, the B cell. It has been shown that marginal zone B cells transport antigen to the follicles to help them to reach follicular dendritic cells\cite{282-284}. Recent work has shown that low affinity B cells are important for the transport of virus-like particles into the follicle, which allows for higher affinity B cells to obtain antigen and interact with primed T cells at the T-B border \cite{285}. To test this hypothesis, I contacted Genentech and obtained the antibody clone 5D2, an IgG2a that binds mouse CD20 and leads to B cell depletion, the mouse version of the human antibody Rituximab. A day before adoptively transferring CA30 T cells, I injected mice IP with 500 µg of this antibody, 5D2, or an isotype control IgG2a with a kappa light chain acquired from Sigma Aldrich. The next day, I adoptively transferred 5 \times 10^4 CFSE-labeled CA30 T cells, and a day later injected with either 100 µg of negative control IgG\textsubscript{1}, monomeric Ig, heat
Figure 4.8 The lack of a functional Fc diminishes presentation of a peptide in the Fab region but an F(ab')₂ complex can rescue presentation.
**Figure 4.8** The lack of a functional Fc diminishes presentation of a peptide in the Fab region but an F(ab’)2 complex can rescue presentation

Mice received adoptive transfer of 5 x 10^4 CFSE labeled CA30.CD45.1 cells and 1 day later (day 0) received 100 µg of negative control Ig, monomeric Ig, 73 µg of F(ab’)2 or F(ab’)2 complex or 66 µg of Fab. Mice were sacrificed at day 5 and splenocytes were analyzed via FACS. (A) Mean percentage of CD45.1+ cells among total CD4+ cells for monomeric Ig, F(ab’)2, Fab, and negative control Ig (B) Representative histograms containing CFSE proliferation profile gates generated by FlowJo proliferation algorithm and applied to all samples for monomeric Ig, F(ab’)2 and Fab. (C) Mean percentage of CD45.1+ cells among total CD4+ cells for monomeric Ig and F(ab’)2 complex (left) and Representative histograms containing CFSE proliferation profile gates generated by FlowJo proliferation algorithm and applied to all samples for F(ab’)2 complex. (right) Histograms are gated on CD4+CD45.1+ events. Symbols represent individual mice and mean of cell percentages is denoted by the bar in the scatter plot. Statistics represent a pairwise comparison calculated by a two-tailed Student’s t-test with p<0.05 (*).
aggregates, or the equivalent in the form of immune complexes. I waited for five days and then sacrificed the mice and evaluated the CFSE proliferation of CA30 cells by assaying mouse splenocytes. My expectation was that this α-CD20 Ig would significantly decrease the CA30 T cell proliferation in mice treated with both the heat aggregate and the immune complex, given that both antigens were probably large enough to necessitate B cell transport to reach the follicles and initiate antigen-specific B cell presentation to T cells. I thought might be the source of the additional proliferation based upon the F(ab’)_2 phenomenon. The actual result was somewhat more complicated.

First, I assessed the B cell depletion in the mice that had received 5D2 and saw a 3 fold reduction in CD19+ cells within the spleen compared to isotype control (Figure 4.9A). Upon examining the CA30/CD4 ratios, there was no significant difference between the mice that had been injected with monomeric Ig and received the isotype control or 5D2; similarly, the mice injected with Ag-excess immune complexes that had received either isotype or 5D2 and immune complexes did not have significantly different percentages (Figure 4.9B). However, the mice that received the α-CD20 and heat aggregates had a significantly decreased percentage of CA30 T cells compared to mice that had received the isotype control and heat aggregates, although this was still above mice injected with negative control IgG1. In the isotype control group, there was no statistical difference in the CA30/CD4 ratios among the different antigens, but when α-CD20 therapy was examined, the group that was exposed to monomeric Ig had significantly higher CA30/CD4 percentages (4.9C). Thus, I was observing two effects in the mice treated with α-CD20: there was a significant decrease in the heat aggregate CA30 percentage as seen between the two heat aggregate groups, and a significant
increase in CA30 percentages compared to immune complexes and heat aggregated Ig in mice that received α-CD20 and monomeric Ig.

To put these findings in context, I examined the CFSE proliferation of each group of the mice that had received the monomeric Ig and the isotype control. Qualitatively, it appeared that α-CD20 led to more CA30 cell divisions in mice injected with monomeric Ig, approximated by a division profile shifted 2-3 divisions forward (4.9D). The heat aggregate and α-CD20 treated mice appeared to have an increased percentage of CA30 cells that had not divided and decreased percentages of cells in the later division cycles, in contrast to what I had seen previously with heat aggregates. Both immune complex samples appeared to follow a similar pattern to what I had seen previously, with relatively low populations of cells that had not divided and an increase in the percentage of cells that were in further division stages. When I used the Flowjo CFSE proliferation algorithm to assign division peaks, I found that these qualitative observations were fairly accurate (Table A5, Figure 4.9E). The mice receiving monomeric IgG had a small percentage of cell reach the 7th division when treated with isotype control, but a high percentage of cells if treated with α-CD20. The mice receiving heat aggregated Ig had a decreased number of cells reach the 7th division when treated with either the isotype control, where it was decreased from what I had seen in other experiments, or α-CD20, which eliminated most of the proliferation from this antigen. Finally, the immune complex treated mice had CA30 T cells that maintained a roughly consistent amount of proliferation whether treated with isotype control or α-CD20. This was markedly different than what had been seen in the heat aggregate treated mice.
Figure 4.9A-C α-CD20 therapy diminishes proliferation of CA30 T cells in response to heat aggregated Ig
Figure 4.9A-C α-CD20 therapy diminishes proliferation of CA30 T cells in response to heat aggregated Ig

Mice received i.p. injection of 500 µg of α-CD20 (mIgG2a5D2) or isotype control (UPC 110). The next day (day -1), mice received adoptive transfer of 5 x 10⁴ CFSE labeled CA30.CD45.1, and 1 day later (day 0) received 100 µg of negative control Ig, monomeric Ig, heat aggregated Ig, or immune complex. Mice were sacrificed at day 5 and splenocytes were analyzed via FACS. (A) Mean B220+ percentage of splenocytes of mice receiving isotype control or α-CD20. Events were in B220+CD4- gate. (B) Comparison of mean percentage of CD45.1+ cells among total CD4+ cells for groups receiving a similar antigenic Ig species but different B cell depletion treatment. (C) Comparison of mean percentage of CD45.1+ cells among total CD4 for groups receiving same B cell depletion treatment but different antigenic Ig species. Symbols represent individual mice and mean of cell percentages is denoted by the bar in the scatter plot. Statistics represent a pairwise comparison calculated by a two-tailed Student’s t-test with p<0.05 (*), p<0.005 (**). Data is representative of two experiments with 4 mice per group.
Figure 4.9D-E α-CD20 therapy diminishes proliferation of CA30 T cells in response to heat aggregated Ig
Figure 4.9D-E α-CD20 therapy diminishes proliferation of CA30 T cells in response to heat aggregated Ig

Mice received B cell depletion treatment and CFSE transfer as described in 4.9A. (D) Representative histograms containing CFSE proliferation profile gates generated by FlowJo proliferation algorithm and applied to all combinations of B cell depletion therapy (side) and Ig antigen (top). Histograms are gated on CD4+CD45.1+ events. (E) Mean percentages of CD4+CD45.1+CFSE+ cells in the 7th division as calculated from the gates derived by the FlowJo proliferation algorithm. Symbols represent individual mice and mean of cell percentages is denoted by the bar in the scatter plot. Data is representative of two experiments with 4 mice per group.
IV.3 Discussion

The initial objective of the work in this chapter was to test the hypothesis that monomeric Ig would not be perceived by CA30 cells in vivo and not generate a productive CA30 expansion and subsequent humoral immune response, while aggregated species would be perceived, generate expansion, and lead to a humoral response. To test this hypothesis in vivo, I used an adoptive transfer model in which I typically transferred $5 \times 10^4$ congenically-marked (Thy1.1$^a$ or CD45.1) CA30 cells into B6AF1 mice and injected 100 µg of whole mAb 36-71, or a $\text{V}_\text{κ}$36-71 epitope molar equivalent of Fab or F(ab’)$_2$ in various physical states. This was the first comprehensive analysis of the primary response kinetics and phenotype of an individual CD4+ clone, specific to antigen contained within an Ig, to monomeric Ig or variants of that Ig, to include heat aggregates, immune complexes, F(ab’)$_2$, and Fab species.

At the outset of the experiments, I was surprised to find that ultracentrifuged, monomeric Ig was perceived rapidly and induced a primary proliferative response of the CA30 T cell in vivo and that the magnitude of this primary response mirrored closely by the injection of heat aggregated Ig or immune complex. In contrast to Ig with the adjuvant alum, which induced massive expansion in the primary response, the heat aggregate and the immune complex induced expansions that were significantly but modestly different than from the response to monomeric Ig and never greater than about 2 fold in terms of CA30 cell numbers or CA30 percentages. The IgG response to $\text{V}_\text{κ}$36-71 elicited by immune complex or heat aggregate was similarly existent but modest, generally requiring a secondary boost to be consistently measured and never reached a serum concentration of more than 10 µg/ml. From a global perspective, I was examining
the response of an Ig-reactive CD4+ T cell in a primary response that was teetering on the edge of the line between immunity and non-immunity, and trying to discern whether this non-immunity was a function of tolerance or ignorance. By using heat aggregate or immune complex that was not augmented with a large amount of adjuvant, I was attempting to model the usage of therapeutic Ig in the clinic in which the reagents are generally assumed to be made with good manufacturing practice. The proviso to this statement is that, as was seen in the first chapter, my reagents contained an insoluble fraction that, particularly in the case of the heat aggregates, was a visibly particulate species that would ostensibly be captured by IV filters during infusion in the clinic. All of this being said, the use of heat aggregated Ig as a positive control was well documented in the early investigation of the immunogenicity or tolerogenicity of Ig species.

Once it was established that the immune complexes and heat aggregates could generate a humoral response that was boosted in a secondary response, but the monomeric Ig did not, I needed to address a potential mechanism to explain this difference. At first, I relied upon the published observation that increased presentation and CD4+ division was associated with an immunogenic response, although there is some thought to the contrary, and examined CA30 proliferation through the use of CFSE [280]. I found a pattern of CA30 division that was similar between heat aggregates and immune complexes and different than monomeric Ig; the species that generated the humoral response also tended to have increased percentages of cells in the furthest division (7) observed using the FlowJo algorithm for defining peaks of proliferation. This increased percentage of cells that were in a late stage of division was particularly
interesting in the context of the observation that there was an increased percentage of CXCR5^{hi}PD-1^{hi} T follicular helper cells in the CA30 population at day 14 in mice that received either heat aggregated Ig or immune complexes. Recent work has suggested that prolonged antigen presentation is required for the development of the T_{fh} and that this presentation may be initiated by dendritic cells (DC) and then require subsequent B cell presentation when antigen is limiting or further DC stimulation if antigen is not limiting [286-289]. I believe that this manifestation of T_{fh} is more likely related to the development of the humoral response and the lack thereof in the mice receiving the monomeric Ig rather than an active suppression generated by the cells treated with monomeric Ig, as I did not observe a significant increase in a CD25^{+}FoxP3^{+} T_{reg} population in these cells at day 7, 10, or 14.

The CFSE data involving the species that were anomalous aggregates (the “large” species), without an Fc (F(ab’)_{2} or Fab), and the mice treated with α-CD20 are also interesting in this regard. The large immune complexes and large heat aggregates acted differently than the smaller species of each type, not generating a detectable secondary response to an immunogenic stimulus and displaying different patterns of CFSE proliferation. In both cases, aggregates within the samples were assumed to have been created with a greater mass of immunoglobulin based upon their formation, and perhaps this is related to decreased uptake by the B cell compartment and decreased presentation to CA30 T cells. The summary of the mass distribution of these samples in chapter III supports this hypothesis in the case of the large immune complex, which was predominantly insoluble and lacked much in the way of small particulate or soluble Ig. However, the distribution of mass in the large heat aggregate was similar to that of the
immunogenic immune complexes, so this simplistic analysis requires refinement. The decrease in the presentation of the F(ab’)2 and the Fab as measured by CFSE dilution implies that the Fc is important in generating the efficient proliferation seen with monomeric Ig. Although, it is important to note that this may also be an expression of the volume of distribution of these smaller species, which would be expanded, and the knowledge that an intact Fc has been shown to be important for its role in recycling and preventing the elimination of monomeric Ig from the blood. The fact that presentation of the F(ab’)2 could be rescued by complexing it with Ars-MSA further supports the idea that increasing the size of antigen may be important in inducing some APC population to participate in its presentation. Finally, the α-CD20 presentation data adds another view of the role of the B cell in this early presentation of the Ig to CD4+ T cells. The two most important observations are the increase in percentage of cells reaching further divisions in the mice receiving monomeric Ig and the notable decrease in CA30 percentage in the total CD4+ population in the mice treated with heat aggregated Ig. The latter finding suggests an important role for B cells in the promoting the presentation of the aggregates, which may be a function of direct presentation by Ag-specific B cells or the nonspecific transport of aggregates to other APC by B cells. I would speculate that the important B cells in this context may be marginal zone B cells, as the heat aggregation of Ig might be expected to create structures of repetitive antigen that could stimulate these cells in a T-independent 2 type manner which would be similar to other work involving rheumatoid factor immune complexes and B cell stimulation [195, 196, 290]. The pattern of proliferation also appeared to show decreased percentages of cells reaching further divisions in the mice that received isotype control and heat aggregated Ig, although the
percentage of CA30 T cells in total CD4+ was not decreased. This may also be a clue to the role of B cells in the presentation of heat aggregated Ig antigen, as the injection of large amounts of an isotype control Ig 2 days before the inclusion of heat aggregated Ig may have been sufficient to disrupt some of the presentation. Although the α-CD20 was stored in low-aggregation pharmaceutical buffer, it was not ultracentrifuged prior to injection. The increase in the percentage of CA30 cells reaching further divisions with treatment of monomeric Ig and α-CD20 is more perplexing. It is possible that this was a function of the decrease in the B cell compartment and an increase in resources and “space” for proliferation of the CA30 cell, similar to homeostatic proliferation seen in lymphopenic environments. Conversely, it could show a role for B cells in the suppression of CD4+ T cell response to an antigen with the potential to be tolerogenic. The monomeric Ig weakly bind and tolerize Ag-specific B cells in a manner similar to previous work with rabbit α-IgD Fab, and perhaps these B cells would negatively effect the proliferation of the CA30 T cell. This would also similar to another model in our lab, in which anergic B cells suppressed humoral responses and are poor stimulators of T cells that are presented with hapten conjugates of protein-peptide antigen [291]. This suppression could also occur non-specifically due to uptake of monomeric Ig through Fc receptors, although the inhibitory FcγRIIB would be unlikely to bind very much monomeric Ig[183].

In summary, the work in this chapter provided a unique view into the perception-of by an antigen-specific CD4+ and subsequent humoral response-to monomeric Ig in comparison to aggregated species. For the first time, it demonstrated the expansion and contraction of a specific CD4+ T cell in response to monomeric Ig without a subsequent
humoral response. These cells did not become $T_{\text{reg}}$ at an increased percentage, as had been previously hypothesized. Instead, both heat aggregates and immune complexes generated humoral responses against antigen within the Ig. The difference in this response appeared to be in its increased percentages of CD4+ T cells that had reached 7 divisions by day 5 and an increased percentage of $T_{\text{H}}$ at day 14. The chapter also contains data suggesting that the Fc region is important for presentation of monomeric Ig and that the response to heat aggregated Ig may require B cells to aid in the early stages of antigen presentation.
CHAPTER V

THE EFFECT OF IG FORM ON THE EXPANSION OF MEMORY

CA30 T CELLS

V.1 Introduction

The hallmark finding of the literature base initiated by Dresser in 1961 and extended by Chiller, Habicht, and Weigle in the early 1970s was the lack of a humoral immune response to an immunogenic form of Ig, whether heat aggregated or emulsified in complete Freund’s adjuvant (CFA), when the animals in question had been treated previously with ultracentrifuged, monomeric Ig[43, 68, 69]. That the tolerance extended to the memory response was critical for the separation of the phenomenology into its constituent cellular components. For Chiller, Habicht, and Weigle, this meant that tolerized thymocytes could be mixed with normal bone marrow, or tolerized bone marrow mixed with normal thymocytes, and injected into irradiated mice to create a “central lymphoid organ chimera”, milieus with predominantly tolerized or normal B and T cell compartments. At the dose of Ig that I typically used in my experiments, 100 µg, the monomeric HGG-tolerized thymocytes did not support the formation of antibody secreting cells (ASCs) from normal bone marrow upon two challenges with heat aggregated HGG, whereas HGG-tolerized bone marrow only instituted 9% of the tolerance in terms of ASC formation that could be achieved with a higher dose of tolerogen (2.5 mg) [69]. In this example, the thymocytes were tolerized in some way, although the exact mechanism, be it active suppression by a regulatory cell population, deletion, or anergy, was unidentified. In the experiments described in the preceding chapter, I observed that the adoptively transferred CA30 T cells expanded and contracted
in vivo in response to the injection of ultracentrifuged, monomeric mAb 36-71 and that this general pattern of expansion and contraction was mirrored closely, with significant differences, by CA30 cells exposed to heat aggregate or immune complex. However, the monomeric Ig did not yield an IgG primary response nor an IgG secondary boost to heat aggregates, or secondary and tertiary responses to immune complexes, as assessed using antigenic Vk_{36-71} light chain as an antigen. In contrast, both the immune complex and heat aggregate did induce a humoral response that was particularly notable after a boost with the same antigen. This lack of a memory humoral response by the monomeric Ig led me to wonder whether I had generated tolerized T cells. If so any CA30 cells remaining in these mice after the primary response should not respond to secondary injection of antigen.

To test this, I adoptively transferred 5 x 10^4 congenic CA30 cells into B6AF1 mice, primed them with various Ig antigen, and then injected them with a secondary stimulus 30 days after the primary injection. Five days after the secondary injection, I sacrificed mice and assayed for congenic CA30 cells in the spleen, comparing their frequencies to those of mice that had received a primary treatment but had not received the secondary injection. Due to the long period of time required for mice to incubate with these treatments, the number of mice required for boost/non-boost comparisons, my own time constraints, and the complexity of the data, these experiments were carried out at the beginning and the end of my studies with a long pause in the middle, meaning that the data that I present in this chapter raises more questions than it provides answers. However, I believe that the results are interesting in light of the data presented in other chapters, and I believe that the presentation of these novel data is important. The result
of the work provides the first demonstration that an individual CD4+ T cell clone could be exposed in vivo to a monomeric Ig and subsequently expanded in response to a secondary boost of a corresponding antigenic peptide. However, the magnitude of this expansion varied based upon both the primary and the secondary stimulus, which may have important implications for future experiments considering the primary and secondary presentation of Ig antigen to the T cell compartment.

V.2 Results

V.2.1 CA30 T cells exposed to monomeric Ig can expand in a memory response, but this expansion is similar to that of CD4+ T cells exposed to a tolerogen

The first publication involving adoptively transfer of transgenic CD4+ T cells into mice also documented the observation that the injection of soluble, antigenic peptide without adjuvant led to rapid expansion, contraction, and a minor recall upon secondary stimulation [276]. In comparison to the response to peptide delivered with an adjuvant, this response was deemed to be tolerogenic, as the adjuvant treated mice maintained a larger residual population of T cells during the phase of gradual contraction, and these cells were more responsive to a secondary stimulus. Given that CA30 T cells expanded and contracted in a primary response, I was curious whether their response to a secondary stimulus would be more similar to the expansion seen in mice that received a primary tolerogenic stimulus than in mice that received adjuvant with the initial injection. To test this, I adoptively transferred 5 x 10^4 congenically marked CA30 T cells into B6AF1 mice and IP injected these mice with either 10 µg of Vκ^{36-71} peptide in PBS, 100 µg of ultracentrifuged, deaggregated mAb 36-71 in PBS, 10 µg of Vκ^{36-71} peptide plus 10 µg of bacterial LPS in PBS, or 100 µg of ultracentrifuged, deaggregated mAb 36-71 plus 10 µg
of bacterial LPS in PBS (Figure 5.1A). After 30 days, I boosted all groups with 10 µg of \( \text{V}_\kappa^{36-71} \) peptide plus 10 µg of bacterial LPS in PBS or PBS alone, sacrificed the animals 5 days later and assayed for CA30 T cells in spleens by flow cytometric analyses (Figure 5.1B). The adjuvant LPS was used in the secondary stimulus on the recommendation of Dr. Megan Macleod, a member of the Kappler/Marrack lab at the time, who advocated for the usage of LPS to increase the expansion of cells in the memory response.

At day 5 post-boost, the group primed with \( \text{V}_\kappa^{36-71} \) peptide plus LPS appeared to give the greatest recall response (Figure 5.1C, D). The CA30 cell percentage of the total CD4+ population was relatively constant among all of the groups that did not receive a booster injection. In mice primed with \( \text{V}_\kappa^{36-71} \) peptide plus LPS there was a significantly increased CA30 cell percentage of the total CD4+ cells after the boost relative to that of any other group, and no substantial differences among the other groups. Mean percentages of CA30 cells in the total CD4+ population were compared between unboosted and boosted mice in each treatment group to provide an estimate of fold change in percentages as a measure of expansion in response to the secondary injection (Table 5.1). The group initially injected with monomeric mAb 36-71 produced a fold change in CA30 cell percentages similar to that of the group receiving \( \text{V}_\kappa^{36-71} \) peptide alone, the treatment predicted to be tolerogenic. Moreover, addition of LPS to the mAb 36-71 increased the fold change in percentage relative to that of the group that was initially injected with monomeric Ig alone. However fold change in CA30 number for the group initially injected with the \( \text{V}_\kappa^{36-71} \) peptide plus LPS primary was the greatest of all groups. The quantitative disparity between this group and the one that received a
Figure 5.1 CA30 T cells exposed to monomeric Ig expand in a memory response, but this expansion is similar to that of CD4+ T cells exposed to a tolerogen.
Figure 5.1 CA30 T cells exposed to monomeric Ig expand in a memory response, but this expansion is similar to that of CD4+ T cells exposed to a tolerogen

Mice received experimental injections as in (A). Mice received adoptive transfer of 5 x 10^4 CA30.Thy1.1 cells and 1 day later (day 0) received injection with 100 µg of monomeric Ig with or without 10 µg of LPS or 10 µg of Vκ36-71 peptide with or without 10 µg of LPS. 30 days later, they received a booster injection of 10 µg Vκ36-71 peptide plus 10 µg LPS. Mice were sacrificed at day 5 post-booster (day 35) and splenocytes were analyzed via FACS. (B) Representative FACS plots from mice of different primary treatments (top) and boost status (side) expressing the percentages of Thy1.1+ cells in the CD4+, MHC II-, CD19-, CD8α-, F4/80- gate. (C) Mean percentage of Thy1.1+ cells among total CD4+ cells in mice that were not boosted. (D) Mean percentage of Thy1.1+ cells among total CD4+ cells in mice that were boosted with Vκ36-71 peptide. Statistics represent a one way analysis of variance ANOVA with p<0.05 (*).
Table 5.1 CA30 T cells exposed to monomeric Ig expand in a memory response, but this expansion is similar to that of CD4+ T cells exposed to a tolerogen

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean Thy1.1% of Total CD4 day 35 (Unboosted)</th>
<th>Mean Thy1.1% of Total CD4 day 35 (10 µg α-Vκ36-71 peptide + 10 µg LPS)</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptide</td>
<td>0.0389 SD 0.03467</td>
<td>0.2383 SD 0.2780</td>
<td>6.1</td>
</tr>
<tr>
<td>Peptide + LPS</td>
<td>0.1032 SD 0.1342</td>
<td>1.702 SD 1.604</td>
<td>17</td>
</tr>
<tr>
<td>Monomeric Ig</td>
<td>0.0368 SD 0.0439</td>
<td>0.1471 SD 0.0630</td>
<td>4</td>
</tr>
<tr>
<td>Monomeric Ig + LPS</td>
<td>0.0375 SD 0.0183</td>
<td>0.1627 SD 0.1500</td>
<td>4.3</td>
</tr>
</tbody>
</table>

Primary injection of mAb 36-71 plus LPS was somewhat surprising, as I had expected the inclusion of LPS with the mAb 36-71 to drive a more robust secondary expansion.

V.2.2 The primary expansion of residual CA30 T cells in negative control mice is larger than that of mice treated with various mAb 36-71 species

The result from this initial memory experiment was intriguing, but I wanted to screen multiple conditions to set the stage for future experimentation. I had expected a more substantial CA30 T cell expansion in mice that had received mAb 36-71 and LPS, and was curious whether this expansion could be augmented by using an alternative form of the Ig. The original paper characterizing the primary response of adoptive transgenic CD4+ T cells had used CFA as an adjuvant to induce a productive immune response with peptide, and I wanted to know whether I could replicate these results with the Vκ36-71 peptide or the mAb 36-71[276]. I had also recently made Ars-MSA (mouse serum albumin) and wanted to see if immune complexes between this and mAb 36-71 behaved similarly to the monomeric Ig alone. Finally, I wanted to include a test of the primary response of a residual population of CA30 T cells at day 30 in mice that received no
primary injection of mAb 36-71 or corresponding peptide, as expansion of such cells could confound my interpretations.

To these ends, I adoptively transferred 5 x 10^4 congenic CA30 cells into B6AF1 mice and then i.p. injected them with 100 µg of ultracentrifuged, monomeric mAb 36-71, 100 µg of mAb 36-71 emulsified in CFA, 10 µg of Vκ36-71 peptide emulsified in CFA, 100 µg of immune complexed mAb 36-71, or 100µg of the negative control IgG1 (Figure 5.2A). After 30 days, I boosted these mice with 10 µg of Vκ36-71 peptide plus 10 µg of bacterial LPS or with PBS. 5 days after the boost, I sacrificed these animals and assayed their splenocytes by flow cytometry for congenic CA30 T cells (Figure 5.2B). At day 5 post-boost, the mice that had received the negative control IgG1 appeared to give the most substantial proliferative response (Figure 5.2C,D). In the unboosted mice, the percentage of CA30 of the total CD4+ population was highest in the mice that had received the mAb 36-71 emulsified in CFA. This was unexpected, as I had predicted that mice primed with the Vκ36-71 peptide emulsified in CFA would have an equivalent or greater percentage of residual CA30 T cells. Similar to that of the peptide plus LPS group in the preceding experiment, the residual population in the peptide CFA group was indistinguishable from that of the monomeric Ig group. Also, the residual CA30 percentage in the mice receiving the immune complexes was also indistinguishable from that of mice receiving the monomeric 36-71. The unboosted mice that received the negative control IgG1 had very low percentages of cells remaining. After the mice were boosted, those receiving primary injections of Ig plus CFA or peptide plus CFA developed the highest percentages of CA30 T cells in the CD4+ population, while the negative control IgG1, monomeric mAb 36-71, and immune complexes appeared to
achieve similar levels. Mean percentages of CA30 cells among total CD4+ cells were compared between unboosted and boosted mice in each treatment group to provide an estimate of fold change in percentages of cells (Table 5.2).

The results were somewhat surprising; even though the mice treated with mAb 36-71 and CFA had the highest residual and boosted CA30 population by percentage, the fold change post-boost was less than half of the fold change for the mice receiving the Vk^{36-71} peptide and CFA. Also, it was less than a third of the primary expansion of the CA30 cell seen in the negative control IgG1-receiving mice, although this can be largely explained by the few residual cells present in this group prior to the secondary injection and greater clonal expansion reported in primary versus secondary responses. The mice receiving the monomeric mAb 36-71 and the immune complexes both had fold changes in CA30 percentages that were similar to what had been seen in the previous experiment for the tolerogenic peptide and the mAb 36-71. Although the mAb 36-71 group showed a slightly decreased fold expansion in this specific experiment and was only half of that induced by the immune complexes, both were low compared to those of other groups.

V.2.3 **Monomeric Ig is a poor inducer of memory expansion of CA30 T cells**

Results from the previous two experiments were interpreted in the shadow of a recall stimulus of the Vk36-71 peptide plus LPS. In a scenario weighted towards inducing a secondary boost, I had induced one, but was this the correct model for an example of therapeutic Ig, where the secondary stimulus is another infusion of the monomeric Ig without any exogenous adjuvant. When I performed the time course comparing monomeric, heat aggregated, and alum precipitated Ig presented in Chapter IV, I had an opportunity to test another recall scenario.
Figure 5.2 The primary expansion of residual CA30 T cells in negative control mice is larger than that of mice treated with various mAB 36-71 species.
Figure 5.2 The primary expansion of residual CA30 T cells in negative control mice is larger than that of mice treated with various mAB 36-71 species
Mice received experimental injections as in (A). Mice received adoptive transfer of 5 x $10^6$ CA30.Thy1.1 cells and 1 day later (day 0) received injection with 100 µg of negative control Ig, monomeric Ig, immune complexes, Ig emulsified in CFA, or 10 µg of $\kappa_{36-71}$ peptide emulsified in CFA. 30 days later, they received a booster injection of 10 µg $\kappa_{36-71}$ peptide plus 10 µg LPS. Mice were sacrificed at day 5 post-booster (day 35) and splenocytes were analyzed via FACS. (B) Representative FACS plots from mice of different primary treatments (top) and boost status (side) expressing the percentages of Thy1.1+ cells in the CD4+, MHC II-, CD19-, CD8α-, F4/80- gate. (C) Mean percentage of Thy1.1+ cells among total CD4+ cells in mice that were not boosted. (D) Mean percentage of Thy1.1+ cells among total CD4+ cells in mice that were boosted with $\kappa_{36-71}$ peptide.
Table 5.2 The primary expansion of residual CA30 T cells in negative control mice is larger than that of mice treated with various mAb 36-71 species

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean Thy1.1% of Total CD4 day 35 (Unboosted)</th>
<th>Mean Thy1.1% of Total CD4 day 35 (10 µg Vκ36-71 peptide + 10 µg LPS)</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Control Ig</td>
<td>0.0038 SD 0.0038</td>
<td>0.1297 SD 0.0681</td>
<td>34.1</td>
</tr>
<tr>
<td>Vκ36-71 peptide in CFA</td>
<td>0.0717 SD 0.0819</td>
<td>1.9700 SD 1.295</td>
<td>27.5</td>
</tr>
<tr>
<td>Monomeric Ig</td>
<td>0.1235 SD 0.2021</td>
<td>0.1665 SD 0.5084</td>
<td>1.3</td>
</tr>
<tr>
<td>Ig in CFA</td>
<td>0.4460 SD 0.0600</td>
<td>4.6780 SD 2.048</td>
<td>10.5</td>
</tr>
<tr>
<td>Immune Complex</td>
<td>0.0468 SD 0.0825</td>
<td>0.1578 SD 0.0350</td>
<td>3.4</td>
</tr>
</tbody>
</table>

I had transferred 5 x 10⁴ congenic CA30 cells into mice and injected them i.p. with 100 µg of negative control monomeric IgG₁ (mAb 36-65), monomeric mAb 36-71, mAb 36-71 precipitated in alum, or mAb 36-71 heat aggregates. 30 days later, I boosted some mice with 100 µg of monomeric mAb 36-71 in PBS (as opposed to Vκ36-71 peptide plus LPS) and other mice I did not boost (as they were a component of the time course) (Figure 5.3A). 5 days later, I sacrificed the mice and assayed their splenocytes via flow cytometry for congenic CA30 cells (Figure 5.3B). At day 5 post-boost, all groups had poor recall proliferative responses (Figure 5.3C, D). In the unboosted mice, the mice that had received mAb 36-71 precipitated in alum had the highest percentage of CA30 T cells in the CD4+ population. The unboosted groups that received monomeric Ig, the negative control, or the heat aggregated Ig all possessed low percentages of CA30 T cells. After the boost, CA30 cell populations increased in all groups the cell populations increased in all groups, but only slightly. As before, mean percentage of CA30 cells among total CD4+ cells were compared between unboosted and boosted groups to provide an estimate of fold change (Table 5.3). This appearance of a weak secondary manifested itself in two notable observations. First, the mAb 36-71 precipitated in alum primary injection gave
the weakest proliferative response after the secondary mAb 36-71 stimulus, which seemed incongruous with the use of adjuvant in the primary. Second, the mice that received negative control Ig, and as such were making a primary response after the booster injection, had a fold change in the CA30/CD4 ratio equivalent to the mice that had initially received an injection of monomeric Ig. In comparison to the previous experiment, in which the primary responders boosted with peptide and LPS made the largest expansion compared to the residual population of cells, the expansion in response to a secondary injection of mAb 36-71 was essentially equivalent to what had been observed in mice tolerized with peptide or monomeric Ig.

**V.2.4 Memory expansion of CA30 T cells primed with aggregated Ig species does not appear to be dependent upon rechallenge with the same antigen**

While examining the humoral response to mice with adoptively transferred CA30 T cells and immune complexes, I made a perplexing observation (Table 5.4). In an experiment based upon the adoptive transfer of $5 \times 10^4$ CA30 T cells into B6AF1 mice, priming with monomeric Ig or immune complex, and then two boosts with immune complex (day 21, day 42), I had included a pilot group of mice that received a primary injection of 100µg of heat aggregated Ig followed by the two boosts with immune complex. While the mice receiving a primary injection of monomeric Ig never made a response to the booster injections, and 4/5 of the mice receiving immune complexes made a response after the secondary injection, the mice receiving a primary injection of heat aggregated Ig made a poor response after the secondary injection with immune complex (1/5 mice) and then a better response after the tertiary injection (3/5).
Figure 5.3 Monomeric Ig is a poor inducer of memory expansion of CA30 T cells
Figure 5.3  **Monomeric Ig is a poor inducer of memory expansion of CA30 T cells**
Mice received experimental injections as in (A). Mice received adoptive transfer of $5 \times 10^4$ CA30.CD45.1 cells and 1 day later (day 0) received injection with 100 µg of negative control Ig, monomeric Ig, heat aggregated Ig, or Ig precipitated in alum. 30 days later, they received a booster injection of 100 µg of monomeric Ig. **Mice were** sacrificed at day 5 post-booster (day 35) and splenocytes were analyzed via FACS. (B) Representative FACS plots from mice of different primary treatments (top) and boost status (side) expressing the percentages of CD45.1+ cells in the CD4+, MHC II-, CD19-, CD8α-, F4/80- gate. (C) Mean percentage of CD45.1+ cells among total CD4+ cells in mice that were not boosted. (D) Mean percentage of CD45.1+ cells among total CD4+ cells in mice that were boosted with Vκ$^{36-71}$ peptide.
Table 5.3 Monomeric Ig is a poor inducer of memory expansion of CA30 T cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean CD45.1% of Total CD4 day 35 (Unboosted)</th>
<th>Mean CD45.1% of Total CD4 day 35 (100 µg Monomeric Ig)</th>
<th>Fold Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative Control Ig</td>
<td>0.0020 SD 0.0009</td>
<td>0.0067 SD 0.0030</td>
<td>3.4</td>
</tr>
<tr>
<td>Monomeric Ig</td>
<td>0.0115 SD 0.0085</td>
<td>0.0404 SD 0.0469</td>
<td>3.5</td>
</tr>
<tr>
<td>Ig in Alum</td>
<td>0.3359 SD 0.2246</td>
<td>0.4488 SD 0.3009</td>
<td>1.3</td>
</tr>
<tr>
<td>Heat Aggregated Ig</td>
<td>0.0056 SD 0.0018</td>
<td>0.0204 SD 0.0155</td>
<td>3.6</td>
</tr>
</tbody>
</table>

These data stood in contrast to those of a similar experiment that I had done in which both primary and secondary injections were with aggregated Ig, which induced all mice to produce an IgG humoral response after just one booster injection. This was interesting to me based upon recent reports of a requirement of B cells for successful CD4+ T cell memory response in several models. The exact mechanism of this need for B cell involvement in the memory recall is still speculative, and there is some disagreement over the need for various functions, such as antigen presentation to mediate this effect with different pathogens[292-294]. I was curious if this need for B cells to facilitate the expansion of memory CA30 cells might play a role in the inconsistent humoral response by the mice in the group that received a heat aggregated Ig primary injection and booster injections with immune complex boosts. To test this, I adoptively transferred 5 x 10⁴ congenic CA30 T cells into B6AF1 mice and injected them with either 100 µg of heat aggregated Ig or the equivalent Ig in immune complexes. I allowed the mice to rest for 30 days, at which point I provided a booster injection of heat aggregated Ig, immune complex, or a negative control boost with PBS (Figure 5.4A). 5 days later, I sacrificed the mice and assayed their splenocytes via flow cytometry for congenic CA30
cells (Figure 5.4B). At day 5 post-boost, all groups had responded poorly (Figure 5.4C). Unboosted CA30 cells that had been exposed to heat aggregated Ig in the primary had a slightly larger residual CA30 cell percentage of total CD4+ than those that had been exposed to immune complexes. Although the proliferative responses were weak, in both cases they seemed stronger in mice boosted with immune complex. Mean percentages of CA30 cells among total CD4+ T cells cells were compared between unboosted and boosted mice in each treatment group to provide an estimate of fold change (Table 5.4). I had expected that a secondary antigen that matched the primary antigen (heat aggregated/heat aggregated) would be superior to a mismatched primary and secondary (heat aggregated/immune complex). However, this experiment showed that the immune complex was superior to the heat aggregated Ig as an antigen at eliciting a secondary expansion in the case of either type of priming antigen. It is important to note that the expansion was similar in all cases to what had been seen previously in regards to monomeric Ig or tolerogenic peptide, but the fold changes in this experiment suggested that the heat aggregated Ig was not as good in eliciting a memory CA30 T cell expansion.

V.3 Discussion

Although the experiments described in this chapter should be viewed as preliminary due to the high variability in the data as evidenced by the standard deviations of the cell percentages and the fact that the experiments were only performed once, several of the observations are worthy of consideration in the context of future plans. This work provided the first demonstration that an individual CD4+ T cell clone could be exposed in vivo to an antigenic monomeric Ig and then subsequently recalled for secondary expansion by a secondary injection of a corresponding antigenic peptide.
Figure 5.4 Memory expansion of CA30 T cells primed with aggregated species does not appear to be dependent upon rechallenge with the same antigen
Mice received experimental injections as in (A). Mice received adoptive transfer of 5 x 10^4 CA30.CD45.1 cells and 1 day later (day 0) received injection with 100 µg of heat aggregated Ig or immune complex in sterile PBS. 30 days later, they received a either received a booster injection of 100 µg of heat aggregated Ig or 100 µg of immune complex or PBS. Mice were sacrificed at day 5 post-booster (day 35) and splenocytes were analyzed via FACS. (B) Representative FACS plots from mice of different primary treatments (top) and boost status (side) expressing the percentages of CD45.1+ cells in the CD4+, MHC II-, CD19-, CD8α-, F4/80- gate. (C) Mean percentage of CD45.1+ cells among total CD4+ cells. Top label describes primary injection, bottom label describes booster injection.
Table 5.4 Memory expansion of CA30 T cells primed with aggregated species does not appear to be dependent upon rechallenge with the same antigen

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean CD45.1% of Total CD4 day 35 (Unboosted)</th>
<th>Mean CD45.1% of Total CD4 day 35 (100 µg Heat Aggregated Ig)</th>
<th>Fold Change Heat Aggregated Boost</th>
<th>Fold Change Immune Complex Boost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat Aggregated Ig</td>
<td>0.0711 ± 0.0289</td>
<td>0.0944 ± 0.06456</td>
<td>1.3</td>
<td>2.2</td>
</tr>
<tr>
<td>Immune Complex</td>
<td>0.02987 ± 0.0062</td>
<td>0.0531 ± 0.0148</td>
<td>1.8</td>
<td>4.2</td>
</tr>
</tbody>
</table>

However, the expansion of CA30 T cells exposed to monomeric Ig in the primary response and then recalled with antigenic peptide plus LPS was not detectably different than that of cells from mice given a primary injection of Vκ36-71 peptide alone, a classically tolerogenic stimulus. CD4+ T cell expansion and contraction to a tolerogenic stimulus, and subsequent failure to expand robustly or produce IL-2, has been repeatedly demonstrated since the initial adoptive transfer of transgenic CD4+ cells[277].

While the OVA peptide was used as the tolerogen in this initial set of experiments, induction of tolerance in CD4+ T cells by soluble OVA has been demonstrated as well, and as such it is reasonable to postulate that the failure of the CA30 T cell exposed to monomeric Ig to expand in a secondary recall could be related to its status as a soluble protein rather than an unique effect of a structural component of Ig.

The increased fold change seen in CA30 cells in mice receiving primary injections with Vκ36-71 peptide, compared to mAb 36-71 when both are delivered with adjuvant is more intriguing. I would have expected that the Ig in adjuvant would have been as effective, if not more effective, in inducing a memory CD4+ population due to
the probably generation of a germinal center. The mice exposed to mAb 36-71 with
adjuvant during the primary have differential retention of cells. The group that received
the Ig emulsified in CFA had an increased percentage of CA30 among total CD4+ cells
in the unboosted group at day 35, while the mice that received the Ig with soluble LPS
have residual percentages of CA30 similar to that of mice treated with monomeric Ig.
The difference could be easily explained by a depot effect due to the CFA. However,
even if this is the case, why is the memory expansion of the cells exposed to mAb 36-71
in CFA only half as much as that the cells in mice that received Vκ^{36-71} in CFA as a
primary? One hypothesis that would be suggested by some recent publications, and is
related to the work in Chapter IV, is that compared to peptide the mAb 36-71 may
present more of an opportunity for the development of a T follicular helper subset in the
CA30 cells because of its increased capacity to crosslink antigen receptors and activate B
cells [295-297]. B cell activation and germinal center formation have been shown to be
important for the formation of T\textsubscript{fh}, and I have shown that the CA30 cell can adopt a T\textsubscript{fh}
phenotype in response to species of mAb 36-71[287, 289, 298]. There is increasing
evidence for the formation of memory cells derived from the T\textsubscript{fh} component of a CD4+
response, however these cells may be less responsive in terms of proliferation in
comparison to more classical central memory T cells (T\textsubscript{cm}). Perhaps this observed
differential between the fold expansions of CA30 T cells initially exposed to a peptide
plus adjuvant versus whole Ig plus adjuvant was actually a manifestation of T\textsubscript{fh}
development in the context of humoral immunity induced by whole Ig. While the mAb
36-71 in LPS did not generate a primary IgG humoral response, all mice receiving mAb
36-71 emulsified in CFA did have measurable IgG anti-Vκ^{36-71}. There was no detectable
anti-V\textsubscript{k}\textsuperscript{36-71} response for the animals that received the peptide with adjuvant, although the assay used to assess for the response utilized an IgM containing the peptide in the \(\kappa\)-light chain rather than an assay against the peptide itself.

The poor proliferation in all groups upon secondary injection with monomeric Ig, heat aggregated Ig, or the immune complex, all without adjuvant, was both vaguely surprising and affirming. It has been shown that a toll-like receptor stimulus is not necessary for activation of CD4\(^+\) memory cells, although such a stimulus does increase the response [299]. That the monomeric Ig was unable to stimulate a greater fold change in the mice that had been treated with mAb 36-71 adsorbed to alum suggests that it was not teeming with TLR ligands.

An intriguing question emerged from the poor response elicited by a late post transfer injection of monomeric Ig: if monomeric Ig is proficient at inducing CA30 cell proliferation in spleen as demonstrated in mice injected with it shortly after adoptive transfer, why was there such a weak fold change at day 5 in the CA30 cells that had been incubated in the mouse for 30 days prior to Ig injection. In an earlier experiment, V\textsubscript{k}\textsuperscript{36-71} peptide plus LPS had been quite proficient as a secondary stimulus to induce a robust fold change/primary response in these cells. This raises questions regarding the localization of monomeric Ig or its processed peptides in the spleen and the trafficking of the CA30 T cells as a function of time after adoptive transfer. The localization of immune complexes in the lymph nodes has been demonstrated using intravital microscopy, and the role of complement association with immune complexes, complement receptor 3 on the surface of subcapsular sinus macrophages, and complement receptor 2 on naïve B cells have been detailed in the transport of antigen to
follicular dendritic cells[284, 300-305]. However, we still do not know which cell is presenting mAb 36-71 peptides \textit{in vivo}, and how this cell is acquiring the Ig. The B cell depletion experiments in Chapter IV would suggest that it is not a B cell dependent process, at least for monomeric Ig. Both monocytes and macrophages express the activating FcγRIII and inhibitory FcγRIIB, which can bind IgG, however they are not high affinity and are generally thought bind immune complexes rather than monomeric Ig [306]. Numerous additional hypotheses can be expressed to predict the source of this uptake, including some mechanism for aggregation after transfer into the animal with subsequent complement deposition or lectin binding of glycosylated residues.
CHAPTER VI
DISCUSSION

VI.1 Context and Discussion of the Findings

The primary objective of this thesis was to explore the behavior of a CD4+ T cell that perceives an immunoglobulin-derived peptide in the context of MHC II when adoptively transferred cells were challenged with an antigenic IgG\textsubscript{1} \textit{in vivo}. Based upon a review of the literature, the initial hypothesis was that this cell would not perceive the IgG\textsubscript{1} if it was initially introduced in a monomeric form, but that it would perceive and respond with a robust primary response if the IgG\textsubscript{1} was introduced in an aggregated form, even in the absence of adjuvant. To investigate this hypothesis, I sought to produce and characterize various forms of Ig, test the primary response of the CA30 T cell to these species in an adoptive transfer model, and perform initial experiments examining the capacity to recall the CA30 T cell in a memory response after primary exposure to some of these reagents in the adoptive transfer model. The immunogenicity or tolerogenicity of Ig for CD4+ T cells has vast implications for the future of monoclonal antibody therapy, and this work presents novel data generated \textit{in vivo} that increases our understanding of the how these therapeutics may initiate immune responses.

VI.1.1 Chapter III: Are particle mass and LPS contamination as important as immune complexes with regard to immunogenicity?

In Chapter III, I discussed the production and characterization of the aggregation status and adjuvanticity of the Ig species analyzed in the thesis, with an emphasis on monomeric Ig. This analysis was primarily descriptive, but sought to bridge a chasm between historical immunological literature about the immunogenicity of Ig and the
modern high-resolution techniques of pharmaceutical analyses for assessing structural characteristics of protein solutions, which are on the path to becoming adopted as part of the regulatory apparatus surrounding these therapeutics. Studies using animal models have indicated that aggregation enhances the immunogenicity of specific proteins used in human therapies, and these data have been extrapolated to postulate that defects in manufacturing or handling contribute to the immunogenicity of protein therapeutics, including Ig [231, 258, 261, 307, 308]. Similarly, there is great concern surrounding the contamination of these reagents with toll-like receptor agonists such as LPS, which is typically assayed using some derivative of the *Limulus* amebocyte lysate test. The earliest literature about tolerance induced by BGG and HGG was produced at a time when assessing particle size was limited to ultracentrifugation, size exclusion chromatography and mass conservation analyses. At that time, investigators did not assess their protein for LPS content, and yet they were able to achieve tolerance or immunogenicity with relatively small amounts of injected Ig. This is not to say that there is not a substantial immunological literature investigating the role of protein aggregation, immune complexes, LPS contamination, and other size and structural characteristics of antigen in its immunogenicity; in fact, the literature base for this type of assessment is broad to the point of being overwhelming. The global point is that pharmaceutical scientists are developing increasingly high resolution techniques to study structural abnormalities for which they want to predict immunogenicity and immunologists are developing increasingly high resolution techniques to study the immune response for which they need sophisticated antigen. The confluence of these aims is in its infancy.
The findings of my pharmaceutical analyses painted an interesting profile for monomeric Ig, heat aggregated Ig, and immune complexes. The ultracentrifuged Ig was indeed predominantly monomeric (82% of the ideal mass, 98.5% of the recovered mass) with very small fractions of dimer and particles with radii in either the nanometer or micron range that may have been a consequence of the sterile PBS used to dilute these samples. The heat aggregated Ig showed a vastly different pattern, with nearly all of the detectable mass contained in particles with micron scale radii (21.5% of the ideal mass, 99% of the recovered mass) and an undetectable 78.5% of ideal mass presumably sequestered in dense insoluble complexes. The immune complexes demonstrated a third pattern, with a more equitable distribution of mass amongst many particle types including soluble aggregates (39.7% of the ideal mass, 44% of the recovered mass), micron-sized particles (42.2% of the ideal mass, 47% of the recovered mass), and an assumed insoluble fraction (10.5% of the ideal mass). This disparity between the mass distribution in the heat aggregated Ig and the immune complex was also manifested qualitatively by distinct appearances of the two species due to differences in the micron sized particles. In the context of the results discussed in Chapter IV, the assumptions going into the thesis about the role of monomeric Ig in tolerance development and the capacity for aggregated species to behave in an immunogenic fashion, may have been correct in spirit but complicated in their mechanics. The monomeric Ig, which would eventually lead to a proliferative primary response of the T cells but little evidence of humoral immunity, was mostly comprised of monomeric protein rather than detectable particulate species, making the ready presentation in the in vivo model ever more intriguing. The heat aggregated Ig and the immune complex both led to productive humoral responses,
mirrored by increased percentages of CA30 cells converting to T_{fh}, even though these species look decidedly different when compared using these modern techniques for structural evaluation. This divide is further interesting in regards to other differences in the biological effects of these species that will be discussed further in the context of of Chapter IV data, which suggest that the mechanisms of perception of these antigens by the immune system are quite different. The inclusion of the analysis of the “large” heat aggregated Ig and “large” immune complex species, neither of which were effective in inducing a humoral response, raises the question of what extent these analytical techniques are identifying an immunogenic population as opposed to identifying structural abnormalities without functional consequence. The mass profile of the “large” heat aggregated Ig was more similar to that of my Ag-excess (small) immune complex, while the “large” immune complex mass profile more closely mirrored the smaller, immunogenic heat aggregated Ig. While not without flaws, this initial characterization raises an important question for the future of research into the immunogenicity of monoclonal antibody therapeutics: are the pharmaceutical scientists inordinately focused on the immunogenicity of a rare species obtained as a manufacturing or handling defect and ignoring highly immunogenic immune complexes, which are a natural consequence of their action in vivo. With another therapeutic protein, for example recombinant growth hormone, the capacity for the pre-administration handling of the protein to cause aggregation and activate some the adaptive immune system may be entirely relevant as these species do not naturally directly interact with effector components of the immune system, unlike with an Fc component that binds to receptors on immune cells.
Analyses of the adjuvanticity of monomeric Ig, and to an extent the heat aggregate and immune complex, were complicated by the method of purification, which used a cellulose resin and could be the cause of the positive Limulus amebocyte assay result for the some of the reagents. This will be discussed again in the consideration of limitations of this thesis. However, the results of these assays should be considered in the context of the in vivo data. In the LAL assay, monomeric Ig and heat aggregated Ig gave different results using three kits. The monomeric Ig tested as being the equivalent of sterile water once and then tested as having greater than 1.0 EU/ml twice. The heat aggregated Ig tested as having slightly more endotoxin than sterile water once (0.592 EU/ml) and then twice tested as containing less endotoxin (~0.14 EU/ml) than the sterile water threshold (0.5 EU/ml). The immune complexes and Ars-MSA used to complex the Ig were tested only with the second and third kits, and tested as having greater than 1.0 EU/ml. The monomeric Ig was used to test for adjuvant activity in an in vivo assay to see if it could increase OT-II proliferation in response to OVA peptide, which it did not. The positive control in this assay was emulsified in CFA, which may have been an unduly strong positive control for potential LPS in the monomeric Ig, but there was no increase in cell proliferation between the mice receiving the OVA peptide alone or the OVA peptide and monomeric Ig. While it will be important in the future to consider alternative methods of purification or alternative assays to assess LPS and other TLR agonists within Ig samples, the disparity between the LAL test results for the monomeric Ig and heat aggregated Ig implies to me that differences in endotoxin contamination between the monomeric Ig and the heat aggregated, and probably the immune complex, are not likely to be the cause of the differential results observed in the in vivo assays. Heat aggregated
Ig that tested very low in the LAL led to a humoral response in mice with transferred CA30 T cells, and monomeric Ig that tested as high in the LAL elicited primary proliferation, but not humoral responses to secondary or even tertiary boosts. In either case, these results are interesting: monomeric Ig, with a modicum of endotoxin and in an environment with an high precursor frequency of potential helper T cells, was unable to elicit a humoral immune response, while heat aggregated Ig and immune complexes were able to do so, even though the heat aggregate contained lower measured levels of endotoxin and the immune complex is made with the monomeric Ig. Perhaps endotoxin is less important as an adjuvant than something about the size or some other physical characteristics of the aggregate populations. It seems unlikely that the aggregate populations are specifically enriched for LPS compared to monomeric Ig. In fact, the immunostimulatory capacity of soluble immune complexes has been established previously, although in the context of adjuvanticity for the targeted antigen rather than for the Ig itself [309-311]. It would be interesting to repeat the humoral response experiments including a group with monomeric Ig plus LPS as a primary stimulus and then inject these animals with booster vaccinations of either immune complex or heat aggregate to see if this adjuvant would be sufficient to induce a break in tolerance.

VI.1.2 Chapter IV: Monomeric Ig versus immune complexes: differential inducers of Th₁ and Th₂ development, or tolerogenic versus immunogenic stimuli?

In Chapter IV, I described an in vivo characterization of the primary response of the CA30 T cell to the various Ig species and the capacity to generate a memory humoral response, as assessed by production of detectable anti-Vκ36-71 Ig. These analyses were a novel extension of a canon of work dating from the early 1960s investigating the capacity
for ultracentrifuged HGG or BGG to induce tolerance against subsequent injections of the
gamma globulin with adjuvant or in an aggregated form. My work also was aimed at
improving our understanding of the behavior of T cells reactive to Ig-derived peptides in
secreted immunoglobulin as much prior work focused on the response of these cells to
auto-presented BCR-derived peptides by B cells in adoptive transfer models, bone
marrow chimaeras, or intact transgenic mice [246, 248, 255-257, 312-317]. My
hypothesis at the outset was that the monomeric Ig would not be perceived by the CA30
T cell in vivo and as such not lead to a humoral response, while I believed that the heat
aggregated Ig and the immune complexes would induce proliferation and a productive
humoral response.

I quickly discovered that my hypothesis was only partly correct in that the
monomeric Ig stimulated no humoral Ig response but virtually the same primary
proliferation, in terms of CA30 T cell percentages and cell numbers, as the heat
aggregated Ig, while the heat aggregated Ig induced both a primary Ig response with
memory and a stronger CA30 recall proliferative response. Immune complexes induced
a primary expansion and contraction of CA30 T cells that was similar to that induced by
monomeric Ig and heat aggregated Ig, and also induced a detectable anti-Vk\textsuperscript{36-71} IgG
secondary response but not a primary response. The heat aggregated Ig and immune
complexes were perhaps less immunogenic than I had expected, which was exhibited by
relatively low concentrations of anti-Vk\textsuperscript{36-71} in the sera of mice, even after a booster
injection. This also manifested itself in the observation that mice that received adoptively
transferred CA30 cells and then received heat aggregated Ig 22 days later did not make a
detectable IgG anti-Vk\textsuperscript{36-71} response 21 days after injection of the Ig. Experiments in the
early literature involving heat aggregated Ig assayed for an anti-gamma globulin response, by elimination of injected radioactive iodinated-gamma globulin or the formation of gamma-globulin specific ASC[43-45, 49, 61, 62, 66, 68-70]. In both cases, it is possible that these methods were primarily detecting an IgM response. However, indirect ASC (plaque) assays also showed positivity in some cases, indicating that IgG contributed to these immune responses. It is important to recognize that the BGG and HGG used in these early experiments were polyclonal-fractions of gamma globulin and would contain a large number of potential antigenic epitopes because they are foreign in mice and because of idiotypic diversity. When assessing the immune response to mAb 36-71, I measured the response to the Vκ36-71 rather than the full idiotype of the monomeric Ig. It is not unreasonable to suspect that the number of precursor B cells within the mouse that would respond to the full idiotype of a single Ig, let alone the light chain alone, would be much smaller than the number of precursor B cells for heterologous Ig.

To investigate potential explanations of why heat aggregates and immune complexes elicited humoral Ig responses where monomeric Ig did not, despite only a modest, but significant increase in cell numbers during expansion/contraction of the primary responses; I used an in vivo CFSE assay to explore the proliferative pattern of the cells. The following trends emerged: a high percentage of the CA30 cells that were exposed to monomeric Ig in vivo divided at least once by day 5, with the highest percentage of cells found at the 2nd – 4th division before dropping to a lower percentage of cells in the 6th – 7th division and complete dilution of the CFSE. CA30 cells exposed to heat aggregated Ig divided more extensively than cells responding to monomeric Ig.
Many reached the $7^{th}$ division or lost detectable CFSE. However, the CA30 T cells in the lymph nodes tended to show less proliferation and reached lower numbers of division in response to this species, which may indicate that an i.p. injection of heat aggregated Ig is being sequestered in the peritoneum, the spleen, or eliminated. Immune complexes produced the highest percentages of cells in the $6^{th} - 7^{th}$ division and beyond the range of CFSE detection. Therefore, there were two major division profiles the cells exposed to monomeric Ig tended to have large percentages of the CA30 cells in the $2^{nd} - 4^{th}$ division, while the heat aggregated Ig and the immune complex tended to have larger percentages of cells in the $6^{th}$ and $7^{th}$ division or having diluted their CFSE entirely. The simplest interpretation of this data is that the monomeric Ig is being presented by a tolerogenic APC population, such as immature dendritic cells, which have been shown to temper CD4+ responses and induce the production of CD25+Foxp+ T$_{reg}$ as well as FoxP3-regulatory populations such as the T regulatory-1[318, 319]. The fact that I did not identify high conversion of CA30 T cells into FoxP3+ on day 7, 10, or 14 would seem to preclude these cells from being the regulatory aspect of this initial CFSE proliferation. I did not assay for IL-10 production by the CA30, so it is not possible for me to assess whether this may be a factor in the limited CFSE proliferation, but it may be worthwhile to consider in the future.

An alternative view based on the increased divisions of the cells in mice stimulated by immune complexes or heat aggregated Ig and the limited divisions in mice receiving monomeric Ig may be related to two patterns are similar to the two models of CD4+ proliferation discussed in the literature. The proliferation of CA30 cells that were exposed to monomeric Ig looked similar to the proliferation of OT-II cells in response to
in vivo infection with a recombinant Listeria monocytogenes expressing a truncated OVA[278]. When this paper was published, the authors sought to demonstrate that CD8 cells divided rapidly and robustly to antigen, while CD4+ cells went through a limited number of divisions. A Listeria infection should induce a strongly Th1 inflammatory environment which is unlikely to be generated by a monomeric Ig injected without adjuvant, however an exploration of the characteristics of the CD4+ cells activated by this Listeria infection may be insightful in understanding the limited divisions in response to the monomeric protein, given that it probably did not initiate a Th2 profile response.

Conversely, the heat aggregated Ig and the immune complexes tended to induce a division profile more similar to that reported for Tg CD4+ cells activated in a doxycycline-induced model of persistent antigen presentation by dendritic cells. Increased division of the Tg CD4+ cells was seen either by prolonged, pharmaceutically-enforced antigen presentation by resting DC or by brief antigen-presentation by CD40-activated DC [279-281]. If activated DCs are responsible for the presentation of the V\kappa^{36-71} epitope to the CA30 T cell in the case of the immune complex or the heat aggregated Ig, this could provide insight into the eventual humoral response. Based upon the results of my α-CD20 analysis, this DC mediated presentation may be more important for immune complexes than heat aggregates, which may rely on B cells for antigen presentation, or simply transport of heat aggregates to additional APCs. It has been shown that dendritic cells that have been activated with LPS for a prolonged period of time and become “exhausted” are more likely to induce CD4+ cells to take on Th2 or undifferentiated Th0 phenotypes upon antigen presentation [320]. This model in which soluble Ig drives Th1 polarization while immune complexes and heat aggregated Ig, drive
a Th$_2$ response is in agreement with results of two other models involving suppression of autoimmune disease. The research group of Habib Zaghouani has published several studies involving an Ig-delivery system of T cell epitopes to either induce tolerance, or ameliorate manifestations of model autoimmune diseases [129, 130, 140, 141]. This Ig-delivery system incorporates peptides perceived by T cells in complementarity determining region 3 (CDR3) of an IgG2b/k antibody. They used adoptive transfers of CD4$^+$ Tg T cells and subsequent injections of Ig-peptide constructs to describe the effect of these Ig on the progression of model autoimmune diseases. Using an EAE model of multiple sclerosis, they found that injections of an aggregated Ig containing the myelin oligodendrocyte glycoprotein (MOG) could suppress the induction or progression of disease. When they examined the cytokine profile induced in CD4$^+$ Tg cells specific for MOG peptide by this aggregated Ig, they found that the cells preferentially secreted the Th$_2$ cytokines IL-4 and IL-5 as opposed to the Th$_1$ cytokine IFN$_\gamma$, which was more likely to be secreted if these T cells were exposed to a soluble form of the Ig-MOG and a TLR agonist (CpG). In a model of type I diabetes (TID), they used an alternative Ig-epitope construct, this time incorporating a peptide from glutamic acid decarboxylase 2 (GAD2) into IgG2b/k. If this antibody was injected in a soluble form, it could delay the onset of fulminant TID once mice have reached the stage of insulitis and it could restore normoglycemia to hyperglycemic mice. Based upon subsequent experiments, they attributed the tolerogenic effect in this model to Ig-GAD2 induction of the Th$_1$ cytokine IFN$_\gamma$, which in turn downregulated the production of the proinflammatory cytokine IL-17 by Th$_{17}$ CD4$^+$ cells. It is interesting that these two models using the injection of Ig-peptide constructs for tolerization regimens report mechanisms that are related to two
models of antigen presentation that have similarities to my CFSE findings. It is important to note two differences about the Ig in the Zaghouani model: first, the handling of the soluble Ig is not described prior to IP injection and there is no specification of ultracentrifugation or any other methodology to ensure monomeric Ig as opposed to potential aggregated species. Their use of the term “soluble” implies that they are either not perceiving or deliberately removing any insoluble component of the Ig, although this is not described explicitly. Second, the inclusion of the T cell epitopes in the CDR3 region ablates binding of the IgG2b, κ to the hapten p-azophenylarsonate, so they were unable to incorporate immune complexes into their EAE study. In addition, the aggregates they used were generated by precipitation with ammonium sulfate rather than by heat. This aggregated Ig is neither effective in inducing presentation in Fcγr−/− APC in vitro, nor in inducing tolerance in the EAE model in vivo in Fcγr−/− mice, so it is assumed that the predominant APC uptake occurs through Fc receptors. However differences between the physical structures and consequent biological activity of my immune complexes and these ammonium sulfate precipitated Ig are yet undetermined.

If this thread of logic is valid, it is not surprising that my CA30 cells differentiated into Tfh cells in higher numbers in mice exposed to immune complexes and heat aggregates as opposed to monomeric Ig. The presence of a humoral response matched with a CFSE profile that is more similar to a Th2 response is consistent with Tfh development in mice that received immune complexes or Ig aggregates. It has been shown that activated DCs initiate differentiation towards a Tfh phenotype in CD4+ cells and have the capacity to progress and maintain this differentiation in some models.[287,
An alternative model is that this initiation by DCs is followed by required cognate interactions between nascent T<sub>fh</sub> and B cells to progress and maintain this status.

If the most important differentiation between my work and the early work on tolerization by monomeric Ig is that others were observing tolerance and immunogenicity of a large, heterologous, polyclonal population of antigen as opposed to my monoclonal cells, the second most important differentiation is that the Chiller, Habicht, and Weigle observation of tolerance induction was differentiated using centrally derived cells, i.e. from the bone marrow and thymus, rather than peripherally derived cells. It is not unexpected that multivalent immune complexes and heat aggregates made with foreign Ig should induce a B cell response, but to what extent should we expect tolerization of the B cell compartment by the monomeric Ig? The previous work by the lab of David Parker would suggest that tolerance induction in B cells is more likely to occur with a monovalent antigen, such as a Fab, rather than with a divalent antigen of monomeric Ig [321]. However, poor crosslinking of the B cell receptor by a divalent antigen and without a danger signal such as a TLR agonist might also lead to impaired presentation of antigen to T cells with insufficient co-stimulation. I think it is unlikely that all Vk36-71 specific peripheral B cells are deleted due to monomeric Ig, although I have not shown this. In the Chiller, Habicht, and Weigle work, they demonstrated that the tolerization through the injection of similar amounts of immunoglobulin to my experiment only tolerized the bone marrow compartment by 10% [69]. Of course, it is always important to point out that the expected precursor frequency for their foreign, diverse antigen is quite different than my monoclonal reagent. Perhaps the difference in T<sub>fh</sub> development between the mice injected with multivalent forms of Ig as opposed to monomeric Ig is the
reflection of a stoichiometric threshold, in which the multivalent species are more likely to activate more B cells to cross a threshold level of B cell Ag-presentation required to maintain the T<sub>fh</sub> phenotype. Based upon a previous study in our lab that showed a propensity for CA30 cells to adopt T<sub>fh</sub>-like characteristics in an adoptive transfer system designed to investigate regulation between the CA30 cell and B cells expressing V<sub>k</sub><sup>36-71</sup> in the germinal center, we knew that the CA30 T cell had the capacity, and perhaps the propensity to differentiate into T<sub>fh</sub>[257]. There will be a lengthier discussion of CD4+ Tg T cell behavior in a further exploration of the limitations of this study, but a recent paper has suggested that individual CD4+ Tg cells tend to adopt patterns of differentiation when stimulated in vivo, and this was not the case in regards to the T<sub>fh</sub> phenotype in my model when I injected monomeric Ig, despite previous evidence that the CA30 cell may be likely to enter this differentiation pathway [322].

In the latter part of Chapter IV, I wanted to investigate the bounds of my phenomena in the CFSE proliferation to assay for the importance of Fc in presentation and the role of B cells in early CD4+ proliferation. These analyses paint divergent pictures of the presentation of immune complexes and the heat aggregated Ig. Eliminating the F<sub>c</sub> region of the mAb 36-71 led to decreased CA30 CFSE proliferation, which is consistent with a potential F<sub>c</sub> mediated presentation of monomeric Ig. The fact that complexing the F(ab’)<sub>2</sub> with antigen restored the proliferation profile seen in treatments with immune complexes was not as easily interpreted. It could indicate that these structures can be bound by lectin receptors for sugars in the hinge region in the F(ab’)<sub>2</sub>, that the increased valence of the idiotypic epitope enhanced binding to Ag-
specific B cells or in a less interesting scenario, that the Ars-MSA used to complex the 
F(ab’)_2 contained a TLR agonist.

Eliminating B cells with the anti-CD20 therapy led to a decrease in overall CA30 
proliferation to heat aggregated Ig, although this may have also been affected when the 
mice were treated with a large amount of isotype control. The decrease in the CA30/CD4 
ratio between the mice treated with heat aggregated Ig and α-CD20 was significant 
compared to those treated with the isotype control, but the CA30 proliferation profile was 
also shifted to the right in mice receiving the isotype control. The proliferation of the 
cells in mice receiving the immune complexes stayed the same and the proliferation 
increased in mice receiving monomeric Ig, although this may have been a function of the 
induced lymphopenic environment in the spleen. If the effective presentation of heat 
aggregated Ig relies on the activation of a population such as B cells in the marginal zone, 
it is possible that the infusion of a polyclonal control IgG2a 2 days prior to injection of 
heat aggregated Ig could have sequestered these cells with potential to react to 
aggregates, thus diminishing the difference between control and experimental groups. 
This idea also raises comparisons to a recent model examining mice expressing the 
AM14 rheumatoid factor transgene, in which B cells expressing the receptor can be 
recruited into extrafollicular clusters if they are exposed to immune complexes generated 
by an IgG2a anti-chromatin antibody [195, 196]. It has been shown that the B cells in 
these extrafollicular clusters do not require CD4+ T cells to class switch and secrete 
antibodies, but that the secretion of antibody by these cells can be augmented by CD4+ T 
cell help. A critical component of this model is the TLR agonist activity of the chromatin 
in the immune complex that costimulates B cells to secrete antibody in the absence of a
traditional interaction with a T cell. If my heat aggregated Ig is contaminated with a TLR agonist, it could be possible that the eventual secretion anti-Vk$^{36-71}$ antibody and the enhanced presentation for the CA30 cells is reliant on a similar mechanism.

**VI.1.3 Chapter V: Evidence of T$_{fh}$ memory or a critique of my experimental protocol?**

The results presented in Chapter V are preliminary in their findings, but two observations particularly intrigued me. The first is that mice that received a primary injection of monomeric Ig emulsified in CFA had a poorer fold increase in CA30 cells after a challenge with V$k^{36-71}$ plus LPS at day 30 than mice that received a primary injection of V$k^{36-71}$ peptide emulsified in CFA. This was despite the presence of a much larger residual population of CA30 cells at day 35 in Ig-CFA injected mice that were not boosted. Similarly, in mice injected with monomeric Ig plus LPS the increase in CA30 T cells upon a secondary challenge was less than that of mice primed with peptide and LPS. In this case, both residual CA30 cell populations were similar at day 35 in mice that were not boosted. A potential caveat is that the dose of peptide was roughly 10 nanomoles of peptide versus 1.3 nanomoles of peptide in 100 µg of monomeric Ig. However, in the mice receiving peptide, CA30 T cells contracted back to a level well below that of mice that received Ig-CFA and in line with the level seen in the group that received Ig-LPS.

There are two explanations that I have considered for this increased expansion of the CA30 cells in mice injected with peptide as opposed to monomeric Ig. The first explanation is based upon reports that CD4$^+$ T cells in early stages of proliferation due to suboptimal antigen stimulation are the most likely to differentiate into memory cells [323, 324]. It is possible that 10 nanomoles of peptide are cleared rapidly in the mice
receiving the soluble peptide and LPS, or dispersed sporadically to the systemic circulation when emulsified in a CFA depot. On the other hand, it is tempting to consider that the difference is instead related to recent reports that T\(_{fh}\) memory cells undergo less robust memory expansion in comparison to classical T\(_{cm}\) cells [296]. The monomeric Ig should present a superior epitope for B cell recognition in comparison to the peptide, and B cells may play a critical role in maintenance and expansion of T\(_{fh}\) cells. The use of the adjuvant CFA or LPS could explain the difference between the fold changes in these populations receiving emulsified Ig and populations receiving monomeric Ig without additional adjuvant. While the observations are not proof of this phenomenon, the T\(_{fh}\) data from Chapter IV does make it seem like an interesting alternative explanation for the phenomenon.

The other observation from Chapter V that I find interesting is the poor CA30 response to a secondary injection of monomeric Ig regardless of the first injection, and particularly a failure in expansion of CA30 cells that were exposed to a negative control IgG\(_1\) as a primary antigen. When similarly primed mice were boosted with peptide and LPS in the secondary response, there was the largest fold increase in CA30 cells of all treatment groups. The presence of two variables (Ig/peptide, no LPS/LPS) makes it difficult to interpret whether the difference between the peptide and the monomeric Ig was truly responsible for this phenomenon. However, it does raise a question that concerns me when considering this model from a physiological perspective. Most of my experimental protocols involved the adoptive transfer of congenic CA30 cells on day -1 and injection of the antigen on day 0, 24 hours later, to allow the cells to enter lymphoid organs prior to stimulation. I did not undertake time courses to see whether the primary
proliferation of cells that I could measure would diminish at a rate faster than the actual loss of cells. It is possible that this failure to respond to a booster injection indicates a tier of regulation that involves differential trafficking of memory CD4+ T cells and Ig. Perhaps my adoptive transfer model is weighted towards generating a primary CA30 response due to a confluence of the trafficking of cells and antigen that is artificially concocted by the specific time course used. From the perspective of generating future models of CD4+ perception of exogenously transferred or newly-generated Ig epitopes, this type of antigen availability question may be important.

VI.2 Limitations

To this point, I have discussed some limitations within the work in the chapters at varying points when analyzing the results of the experiments. I would describe many of these limitations as primarily technical rather than thematic. Examples of these are particularly evident in Chapter III include: the potential for crossreaction of cellulose-derived glucans in the Limulus amebocyte lysate assay misleading me to believe that some of my Ig preps had endotoxin, and an inability to separate the Ig samples into individual mass category components to test them individually for immunogenicity. In Chapter IV, the fact that the CA30 Tg is present on an A/J genetic background and requires the MHC II I-A<sup>k</sup> for recognition of the antigenic peptide makes it prohibitively difficult to breed any genetic deficiency onto recipients or donors. There are two limitations to this work that I want to highlight as concerns for future experiments addressing questions deriving from the results in this thesis: the use of I-A<sup>k</sup>-restricted transgenic T cells and the use of a single Ig isotype.
VI.2.1 The use of the CA30 CD4+ TCR Tg T cell

In the last year, the Journal of Immunology reprinted the original study examining the primary response of a CD4+ TCR Tg T cell in an adoptive transfer model in their Pillars of Immunology series, and this status as a venerable paper in the history of immunology is well deserved[276, 325, 326]. The ability to know the specificity of an individual CD4+ cell and the capacity to track that cell using congenic markers or tetramer staining or any of a number of labeling techniques are powerful tools that have been used in a vast number of adoptive transfer studies since the first report. The adoptive transfer aspect of these studies is an important point, as this original paper, and many others after it, have argued that the number of Tg cells present in any given study may have significant effects on the progression and characteristics of the immune response. I generally transferred $5 \times 10^4$ CA30 cells into mice, which if one assumes a 10% seeding rate for cells, could lead to anywhere from 10 – 500-fold more cells reactive with the Vκ^{36-71} epitope than would be found in the natural wildtype repertoire based upon studies of other CD4+ epitopes [327]. For the purpose of improving resolution, these CA30 T cell numbers were detectable by staining for congenic markers in the spleen and lymph nodes of adoptive recipients. By consistently transferring the same number of CA30 cells and injecting the same amount of Ig, I could compare experiments for consistency of responses, which was generally quite good. That same reproducibility may be a major caveat to this work. A recent publication from the lab of Marc Jenkins, the same lab that produced the first adoptive transfer of CD4+ Tg T cells, has suggested that CD4+ Tg T cells follow specific and reproducible effector differentiation patterns when stimulated with antigen [322]. Using single cell adoptive transfers, the group
stimulated T cells and examined patterns of differentiation into Th\(_1\), Th\(_{\text{fh}}\), and GC-Th\(_{\text{fh}}\) phenotypes. Unlike naïve, wildtype CD4+ cells from a polyclonal repertoire, the CD4+ TCR Tgs produced predictable distributions of differentiation, which the group argues were dictated by the TCR transgene. The initial intent of the work in this dissertation was to examine the effect of antigenic, soluble Ig at the level of a single CD4+ cell, and this was to be accomplished using the resolution provided by the CA30 TCR Tg cell.

The CA30 TCR transgene was cloned from a T cell hybridoma fused from mice injected with the mAb 36-71 in adjuvant that elicited an immunogenic response. The CA30 T cell has been shown previously to take on a Th\(_{\text{fh}}\) like phenotype in a co-adoptive transfer model with V\(\kappa\)\(^{36-71}\) Tg B cells. In a way, it would have been surprising if antigenic Ig, i.e. the heat aggregated Ig or immune complexes, had not generated an increased Th\(_{\text{fh}}\) phenotype. It was unlikely that the activated CA30 cell would have differentiated in any way other than what had been previously observed with this cell, and the fact that the original TCR was cloned from a hybridoma fused after an immunogenic response to the mAb 36-71 made it similarly unlikely that this cell would ever have adopted a regulatory phenotype \emph{in vivo}. While I suppose this is not impossible, I believe that our use of the CA30 Tg T cell had weighted us towards a specific phenotypic result, and that future considerations of such questions should be targeted towards a more physiological, but still definable, polyclonal T cell repertoire.

\textbf{VI.2.2 The use of a single isotype}

The work in this dissertation relies upon a single antigenic Ig, the mAb 36-71, which has an IgG\(_1\) isotype. I believe that this is both a strength and a limitation of my approach. In terms of strength, a single isotype is that I believe that this work has
suggested that there are physical characteristics of monomeric Ig, heat aggregated Ig, and immune complexes that may mitigate the perception of antigen by CD4+ T cells \textit{in vivo}, whether the Ag is derived from the Ig itself or another protein within the complex. Early in the project, there had been plans to compare responses to IgM carrying the \( \text{V}_\kappa^{36-71} \) peptide with those elicited by mAb 36-71. If this work had followed the pattern of a previous study of the immunogenicity of IgM and IgG, it is possible that I would have seen activation of the CA30 cell by IgM that looked similar to what I eventually saw when I used IgG\textsubscript{i} immune complexes. This result would have been interesting, and will continue to be a question worth pursuing, but I think that it would have taken me, and perhaps specifically me, down the wrong path. Ultimately, this thesis addresses antigen size, antigen valency, \( F_c \) availability, in CD4+ cell activation and differentiation. If had initially compared IgG to IgM instead of complexes and heat aggregates, I doubt that I would have addressed the influence of physically-oriented characteristics that I eventually landed upon, or whether I would have been seduced by the difference in the \( F_c \) alone between the two isotypes. I do believe that it is a limitation of this work that I only looked at IgG\textsubscript{i}, as it binds to a specific set of \( F_c \gamma \)Rs, it is less inflammatory and opsonogenic than IgG\textsubscript{2} species, it has a lower valence than IgM and IgA; there are any number of reasons to consider looking at alternative isotypes, not the least of which is that the majority of monoclonal antibody therapeutics for humans would actually equate to a mouse IgG\textsubscript{2a/c} isotype. However, I would caution future researchers to think carefully about the fundamental physical questions such as how a specific isotype will aggregate rather than simply thinking of it from the perspective of a magical activator of FcRs.
VI.3 Recommendation for future work

VI.3.1 Building and transitioning to the B6.3kλ Tg mouse as a source of Ig

Two of the limitations mentioned previously were the difficulty in breeding genetic deficiencies onto the donors or recipients in the CA30 Tg TCR CD4+ model and the potential for skewing of the phenotypic repertoire by utilizing a monoclonal TCR Tg population. During the time of this dissertation, I initiated work on constructing a novel immunoglobulin light chain containing a CD4+ epitope that could be used to create a new transgenic mouse strain to overcome both of these limitations. The three criteria that I considered were that I wanted the peptide of choice to be immunogenic for a polyclonal T cell repertoire in the context of the MHC II I-A\(^b\), found in the C57BL6 strain, to allow for ease of breeding genetic deficiencies, I wanted the peptide of choice to have a documented I-A\(^b\)-peptide tetramer construct that had been verified and produced in a consistent way, and I wanted the immunoglobulin light chain to be able to pair with a heavy chain in the B6 repertoire to produce an antibody with a known specificity. These criteria were fulfilled by the peptide-3K model of the Kappler/Marrack lab, which has been used to study TCR/MHCII binding and memory cells from a polyclonal T cell repertoire in C57BL6 mice, and the B1-8 immunoglobulin model derived from the injection of the hapten (4-hydroxy-3-nitrophenyl)acetyl (NP) protein conjugates into C57BL6 mice [328, 329]. I developed a plan to insert the 3K peptide onto the carboxy-terminal end of the Igλ constant region 1, the constant region used in the V\(\gamma\)1Jλ1Cλ1 light chain paired with the heavy chain B1-8 for NP-specificity. I did some initial *in vitro* testing indicating that a light chain construct containing the 3K peptide could pair with a B1-8 heavy chain and bind NP (data not shown), after which point my mentor, Larry
Wysocki, designed a genomic construct with a GFP reporter that will eventually be used for the generation of a transgenic mouse that can either be bred to B1-8 knock-in mice to create NP-specific B cells, or injected with NP-protein conjugates to fuse hybridomas and obtain a panel of 3K-containing, NP-specific antibodies for future experiments. These 3K-containing, NP-specific antibodies could be utilized to investigate questions about the acquisition and presentation of antigen from Ig as well as the activation and differentiation of the Ig-specific T cells themselves using genetic deficiencies, genetic reporter mice, or other mice expressing genetic constructs for the depletion of specific cells in a rapid and systematic fashion. The possibilities are myriad: FcR knockouts of multiple types, CD11c-DTR mice for the elimination of dendritic cells, FoxP3 reporter mice to assay for 3K-specific, T_{reg} development, or µMT mice for the elimination of B cells. As the Ig produced by these hybridomas should be NP-specific, these mice could be injected with monomeric Ig, immune complexes, or heat aggregates in a manner similar to which I have already done. One of my favorite speculative questions is whether any strains of autoimmune mice on the C57BL6 background, for example the B6.Sle1Sle2Sle3 mouse, may have increased sensitivity to Ig immunogenicity in the T cell compartment, which could be a source of the generation of rheumatoid factor antibodies, although the current AM14 rheumatoid factor model would suggest that this is TLR agonist dependent rather than Ig dependent per se. If and when this transgenic mouse is finally produced, the potential for rapidly feasible, intriguing experiments into Ig-peptide immunogenicity will increase exponentially.
VI.3.2 The immunogenicity of immune complexes in monoclonal antibody therapy

Prior to the completion of the B6.3Kλ Tg mouse, I think that the current CA30 TCR Tg system is amenable to several types of experiments. From a clinical perspective, I think that one of the most important observations to come from this work is that the pharmaceutical researchers investigating the possibility for production or handling errors to lead to immunogenic, aggregated mAb are probably correct that this could be a source of some immunogenicity, but that immune complexes may be just as, if not more, concerning for the development of anti-mAb antibodies. There are quick experiments that could help establish this idea further. First, I think we need to assess the isotype of the anti-Vκ^{36-71} Ig being made in the B6AF1 mice with transferred CA30 cells. I am curious whether the mice that received heat aggregated Ig may be making more of a T-independent response as compared to the mice that received immune complexes. Then, I think we need to establish whether B6AF1 mice will make anti-Vκ^{36-71} IgG in response to heat aggregated Ig or immune complexes without the transfer of CA30 T cells. The question remains whether the increase in T cell precursor frequency was necessary to induce the memory production of anti-Vκ^{36-71} IgG. If we can show that the response to heat aggregated Ig is different than the response to immune complexes, which I think the anti-CD20 experiments already suggest, this would be an interesting development and suggest that the rejection of monoclonal antibody may develop due to mishandling in some cases and much more commonly to immune complexes in others. Assuming that this is true, I think that we should repeat CFSE experiments with the creation of immune complexes in vivo rather than prior to injection, but injecting in antibody and Ars-MSA independently. If we see a similar CFSE profile in response to this in vivo generation of
complexes, I think it makes the case against in vivo complexes during therapy to be even more compelling. Based upon the original tolerization experiments with monomeric Ig, we have always wondered whether we might be able to use an Ig product to tolerize the T cell repertoire in a potential patient prior to the injection of the mAb. Based upon my data with the Fab and F(ab’)2, my CFSE data surrounding the monomeric Ig, and the type 1 diabetes data of Zaghouani’s group, I would be very interested to test an Fc-Fab fusion protein as a potential tolerizing agent for CA30 cells in vivo. Based upon my humoral response data as well as my CFSE data, I think that monomeric Ig is tolerizing in so far as it is not immunizing, but that it cannot be used to tolerize patients about to receive the same mAb, because they will make immune complexes with the intended endogenous target protein. My concern about using the monovalent Fab alone is that between the larger volume of distribution, and the less efficient uptake due to the lack of an Fc, that it may not achieve sufficient presentation to ablate a productive CD4+ T cell response. Assuming that there is not a vast amount of aggregation, an Fc-Fab fusion would allow for the efficient presentation mediated by the Fc, have the Fab component containing the variable region that is likely to be immunogenic, and be unable to make immune complexes due to its monovalency. This is definitely a speculative idea, but one that I find intriguing for its potential to alleviate rejection of mAbs, which I believe may be a function of the natural action of the antibody, making complexes.

VI.3.3 The tendency of immune complexes to generate Th2 responses

I am intrigued by the idea that immune complexes may be activating DCs, and perhaps rapidly “exhausting” them, and that this activation may be stimulating the DCs to present to T cells in such a way that they are more likely to adopt a Th2 cytokine profile.
With the current CA30 cell CFSE assay, I think that we could enrich for CD4+ and then use FACS sorting to acquire these cells from different CFSE divisions, with an emphasis on an early (3rd division) and late (7th division) from each population. We could restimulate these cells in culture with the Vκ36-71 peptide and assess for their production of IFN-γ (Th1) or IL-4 or IL-5 (Th2). Assessing cytokine transcript production in these cells using real time PCR might be an alternative way to assess for polarization. If we can establish that the immune complexes are more likely to induce cells to divide more and take on a Th2 phenotype, then we can attempt to replicate this effect in a different model. The most obvious candidate in the short term would be to acquire or produce B1-8 Ig and use it to complex NP-OVA or NP-OVA-3K. These immune complexes could be used to replicate the results of the CA30 cell proliferation in a different cell population, either the OT-II TCR Tg cell or the F508 TCR Tg cell. It has been shown previously that immune complexes increase the presentation of T cell epitopes in the antigen, and that they also increase the production of anti-antigen IgG, but addressing this question from the perspective of cell division and Th2 versus Th1 polarization has not been shown explicitly to my knowledge. This type of result would have implications for our understanding of how Ig-regulates CD4+ activation during the progression of an immune response. There are papers that have sought to model Ig-regulation of Ig-production in the germinal center via high affinity masking of epitopes, but my proposed approach would attempt to understand the progression of Ig-regulated CD4+ activation, presumably by dendritic cells, during the progression from antigen excess, and the presence of high numbers of immune complexes, to Ig excess, in which
case there would be more available monomeric Ig, which could disrupt the further polarization of CD4+ cells towards a Th2 phenotype.
REFERENCES


55. Weigle, W.O., Studies on the termination of acquired tolerance to serum protein antigens following injection of serologically related antigens. Immunology, 1964. 7: p. 239-47.


APPENDIX A

PERCENTAGES OF CD45.1+ CELLS IN EACH CFSE DIVISION FOR

PROLIFERATION EXPERIMENTS

Every table in this appendix contains mean values for every FlowJo designated CFSE division in the CD4+, CD8α-, CD19-, MHC II-, F4/80-, CD45.1+ gate. In each case, n=4 for each group and was generated in the same experiment, except for Table A3, which is composite data from 2 independent experiments.

Table A1 Mean number of divisions: monomeric and heat aggregated Ig

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<thead>
<tr>
<th>Treatment</th>
<th>Number of Divisions</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Monomeric Ig</td>
<td></td>
</tr>
<tr>
<td>(SD 1.12)</td>
<td>(SD 5.07)</td>
</tr>
<tr>
<td>Heat Aggregated Ig</td>
<td></td>
</tr>
<tr>
<td>(SD 6.67)</td>
<td>(SD 0.72)</td>
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Table A2 Mean number of divisions: monomeric and immune complex

<table>
<thead>
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<th>Treatment</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Monomeric Ig</td>
<td></td>
</tr>
<tr>
<td>(SD 1.12)</td>
<td>(SD 5.07)</td>
</tr>
<tr>
<td>Immune Complex</td>
<td></td>
</tr>
<tr>
<td>(SD 0.81)</td>
<td>(SD 6.26)</td>
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</table>

Table A3 Mean number of divisions: large head aggregated Ig and large immune complex

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<td></td>
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<tr>
<td>Large Heat Aggregated Ig</td>
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</tr>
<tr>
<td>(SD 14.65)</td>
<td>(SD 1.695)</td>
</tr>
<tr>
<td>Large Immune Complex</td>
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</tr>
<tr>
<td>(SD 5.8)</td>
<td>(SD 3.14)</td>
</tr>
</tbody>
</table>
### Table A4 Mean number of divisions: F(ab')2, Fab, and F(ab')2 complex

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<td></td>
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<tr>
<td>F(ab')2</td>
<td>40.8</td>
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<tr>
<td>(SD 16.37)</td>
<td>(SD 6.62)</td>
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<tr>
<td>Fab</td>
<td>20.21</td>
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<tr>
<td>(SD 13.91)</td>
<td>(SD 7.59)</td>
</tr>
<tr>
<td>F(ab')2 complex</td>
<td>3.07</td>
</tr>
<tr>
<td>(SD 2.83)</td>
<td>(SD 2.43)</td>
</tr>
</tbody>
</table>

### Table A5 Mean number of divisions: Isotype control and α-CD20 mIg2A5D2

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<th>Treatment</th>
<th>Pretreatment</th>
<th>Isotype</th>
</tr>
</thead>
<tbody>
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<td>0</td>
<td>1</td>
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<tr>
<td>Monomeric Ig</td>
<td>0.97</td>
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</tr>
<tr>
<td>(SD 0.56)</td>
<td>(SD 2.27)</td>
<td>(SD 2.15)</td>
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<tr>
<td>Heat Aggregated Ig</td>
<td>13.82</td>
<td>9.025</td>
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<tr>
<td>(SD 15.97)</td>
<td>(SD 2.97)</td>
<td>(SD 2.75)</td>
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<tr>
<td>Immune Complex</td>
<td>6.85</td>
<td>5.95</td>
</tr>
<tr>
<td>(SD 1.52)</td>
<td>(SD 1.93)</td>
<td>(SD 2.28)</td>
</tr>
<tr>
<td>Pretreatment a-CD20</td>
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<td></td>
</tr>
<tr>
<td>Monomeric Ig</td>
<td>0.28</td>
<td>0.30</td>
</tr>
<tr>
<td>(SD 0.27)</td>
<td>(SD 0.25)</td>
<td>(SD 0.93)</td>
</tr>
<tr>
<td>Heat Aggregated Ig</td>
<td>25.4</td>
<td>7.86</td>
</tr>
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<td>(SD 11.66)</td>
<td>(SD 4.22)</td>
<td>(SD 1.49)</td>
</tr>
<tr>
<td>(SD 0.67)</td>
<td>(SD 1.29)</td>
<td>(SD 1.28)</td>
</tr>
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</table>