IAP Inhibitors as Novel Immune Adjuvants for Scaffold Based Cancer Vaccines

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IAP inhibitors as novel immune adjuvants for scaffold based cancer vaccines

A dissertation presented

by

James Joseph Akin

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Immunology

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Dissertation Advisor: Dr. Glenn Dranoff

IAP inhibitors as novel immune adjuvants for scaffold based cancer vaccines

Abstract

Inhibitors of apoptosis protein (IAP) antagonists are nearing approval as promising new candidates for cancer therapy, particularly for solid tumors. Previous work had uncovered the ability of the monomer version of this drug to co-stimulate T cells and enhance anti-tumor immunity when administered systemically in conjunction with B16/GVAX, a model of melanoma immunotherapy. Further published work from multiple labs revealed the ability of these drugs to influence a variety of immune subsets. Given that IAP proteins are known to regulate alternative NFkB downstream of TNFR family members (e.g. GITR, BAFF-R, LTBR), it is evident that IAP antagonism has different effects on non-immune versus immune cells. Where drug treatment in non-immune cells sensitizes these cells to apoptosis, in contrast immune cells do not undergo cell death. We have uncovered the ability of both monomer and dimer inhibitor to enhance survival in B cells cultured ex-vivo and, in the context of stimulation, further costimulate these cells. In an effort to improve the therapeutic effect in our model we investigated local delivery of IAP antagonists via bioabsorbable poly-lactide-co-glycolide (PLG) scaffolds. Interestingly, IAP monomer, but not dimer resulted in reproducible reduction in tumor growth and increased survival of mice when delivered peritumorally in the absence of additional immunotherapy. Further studies in naive mice revealed increased cellular infiltration in monomer loaded scaffolds when compared to blank scaffolds across a month time course.
Upon FACS analysis of these cellular infiltrates, scaffolds loaded with drug contained a striking enrichment of both B cells and follicular dendritic cells, suggesting that IAP inhibitor delivered in a subcutaneous site could form tertiary lymphoid structures, an intriguing possibility given that lymphotoxin signaling, essential for secondary lymphoid organ development, occurs through alternative NFkB. Additional ex vivo experiments revealed that monomer, but not dimer could enrich for lymphoid tissue inducer cells, suggesting the possibility that IAP monomer could be used as a novel immune adjuvant delivered locally where it could impact higher order immune interactions as a therapeutic agent by itself.
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Acknowledgments

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Secondly, I am grateful to my mentor, Glenn Dranoff, for accepting me into his lab and the care with which he undertook my education as a scientist. Along these lines, I thank all of the members of the Dranoff Lab with whom I daily interacted and my fellow graduate students there. I am also deeply indebted to my advisory committee, Drs. Arlene Sharpe, Shiv Pillai and David Mooney, whose insight and advice at the bench and for my career were and are indispensable. Finally, I warmly recognize all the incredible staff of Dana-Farber Cancer Institute who support the unparalleled research mission that is undertaken there, without whom this work would have been impossible.
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Chapter 1. Cancer Immunotherapy and Design of Cancer Vaccines

A brief history of cancer immunotherapy

The first cancer immunotherapy is generally attributed to William Coley, a physician who practiced in New York City at the turn of the nineteenth to the twentieth century. He administered streptococcal (and later mixed with inactivated serratia) cultures to tumor patients with the view that activating the immune system would also result in a beneficial anti-tumor immune response[1]. These experimental therapies were not conducted, as was consistent with the times, under the auspices of an approved trial like they would be today. To put things in perspective, chemotherapy and radiation were not available as first-line therapies for cancer, and while asepsis and antisepsis had been debuted in surgical practice, by 1900 practitioners were still actively debating their use. In retrospect, that age was one of rapid advances across now specialized fields of medical practice, and a cure for cancer was not the highest medical priority. Coley’s ideas were anticipatory of research that would require the work of many lifetimes, yet he deserves credit for his “doggedness” in support of his uncommon insight[2].

In Coley’s patients, a potent immuno-stimulatory signal was likely mediated through what we now know as the toll-like receptor (TLR) family—linked to immunity in the fly in 1996[3] and in mammals in 1999[4]—the ligands of which would have been present in the bacterium. This adjuvant would have served as a strong, if non-specific, danger signal for innate immune cells and in priming an adaptive response. In the ensuing decades, eclipsing his career, Coley’s immunotherapy came under significant criticism, but the legacy of his ideas are present in the current renaissance of cancer immunotherapy. Two aspects of his work, in hindsight, seem to
have been particularly prescient: the involvement of microorganisms in carcinogenesis (for some cancers) and the idea that the immune system can suppress cancer.

In 1909, the latter idea, particularly as a direct result of the early stages of carcinogenesis, was proposed in the scientific literature by Paul Ehrlich[5]. By the 1950s, Burnet and Thomas[6] expanded this concept into a model of cancer immune surveillance. During the ensuing decades, the study of tumor immunology would ebb and flow in popularity, but by the 1990s the field gained traction with the work of Robert Schreiber, adding to the seminal “hallmarks of cancer” synthesized by Hanahan and Weinberg[7], who included immune evasion as an emerging, rather than a canonical, hallmark. Today, immune therapy is recognized as integral to the treatment of cancer, from bacillus Calmette-Guerin (BCG) adjuvant for superficial bladder cancer[8] to leucovorin treatment to protect healthy lymphocytes from the harmful effects of chemotherapy[9].

It seems, however, this is just the beginning of what targeting the immune system in cancer treatment can achieve. Interest in the next generation of immunotherapies for cancer has never been higher. For example, the Coley Pharmaceutical Group, owning proprietary rights to pharmaceutical grade TLR7, 8, and 9 ligands, was purchased by Pfizer in 2007. Since 2011, initial public offerings by biotechnology companies have generated a cumulative investment over $4 billion with cancer immunotherapy leading the optimism for innovation in this industry[10]. While this is encouraging and exciting, this new age of cancer immunotherapy is just beginning and may be risky for investors yet more importantly still, for patients. There are sure to be many new discoveries and surprises as technology matures. Now that I have introduced the genesis of
cancer immunotherapy and highlighted the excitement looking to the future, the next section will describe a major development in the field as it has taken shape over the past 20 years.

**Recent developments in designing cancer immunotherapies**

Researchers have come a long way since the nineteenth century in elucidating the complex interplay between cancer and the immune system. In order to better understand the historic trend and at the risk of oversimplification, it is useful to consider the “old” and the “new” paradigm in cancer immunotherapy. Under the old paradigm, much work focused on generating de novo immunity against a tumor that, in many cases, had been present in the patient long before it would have been clinically detectable. During this time, cancer immunologists were interested in the question of tumor antigens[11]. Do they exist? And if so, how can we make them more immunogenic? While the first human tumor antigen reactive to an antibody was identified as early as 1965[12], it wasn’t until the 1990s and the advent of new technologies that many more antibody reactive antigens were revealed[13, 14] and validated. The first human T cell tumor antigen was identified and reported in 1991[15]. Even while this identification and validation was occurring, experimental vaccines were developed on the premise that antigens would fit into the rubric “altered self”. This premise arises directly from the idea that immunologically relevant antigens would be generated by the mutating cancer cells as the disease progresses. These antigens, known as tumor associated antigens (TAAs), are generally distinguished among three types:

1. neo-antigens generated by driver and passenger mutations in a tumor
2. developmental antigens, self-antigens expressed at an earlier stage of development in normal cells

3. overexpressed antigens, self-antigens expressed at much lower levels in normal cells

While it was initially anticipated that many “new” antigens to the immune system would exist in any cancer, targeting these antigens posed practical challenges, as mutations might not be shared by multiple patients, leading to approaches using autologous cells and patient-specific vaccines. Many antigens, however, have been identified that fall into categories 2 and 3 that are shared among several cancers[16]. Along with the current effort, however, to sequence the somatic mutations in cancers, e.g. the cancer genome atlas (TGCA), the ability to engender an immune response with patient-specific neo-antigens is a re-emerging possibility[17]. Nevertheless, many current and recent experimental therapies generate antigenic responses from combining tumor antigens (autologous tumor cells, or peptides) with classical danger signals, or a more targeted adjuvant. Many of these therapies target antigen presenting cells in vivo, represent some version of a peptide vaccine (including peptides of known, relevant antigens) to target CD8 T cells in vivo, or utilize adoptive transfer of in vitro activated, or expanded lymphocytes. The clinical trials based on these therapies while encouraging yielded modest, or idiosyncratic objective responses by conventional oncology criteria (reviewed in [18] and [19], for peptide and whole cell strategies, respectively). One of the more fascinating aspects of this story is the emergence of secondary measures of immune activity in clinical trials of immunotherapy and their contested role, either to inform, or to obscure efficacy in these trials, but that subject is outside the scope of this discussion.
As more and more tumor antigens were revealed and as it was appreciated that over-expressed and developmental antigens represented many identified TAAs, immunologists considered the efficacy of the resulting immunity. After all, self-antigens would have pre-existing tolerance, whether by central (particularly with non-mutated peptides), or peripheral mechanisms. In clinical trials, experimental vaccines often engendered enhanced adaptive responses in the presence of progressing disease. It seemed the vaccines were activating the immune system as hoped, but it wasn’t enough to overcome the momentum of the disease. Alternatively, the tumor might be enforcing some form of tolerance that costimulatory (discussed later) approaches were not able to overcome. At this time, discoveries in post-activation T cell biology were shedding light on important mechanisms of peripheral tolerance and exhaustion[20, 21]. From this work, new targets emerged for enhancing the tumor-specific responses that had been engendered with the previous therapy.

The “new” approach to cancer immunotherapy represents a re-focusing from engendering more potent, antigen-specific immunity to blocking any suppressive mechanism withholding that response, employed potentially by the tumor. Active tolerance by the immune system is emphasized as well as the context of the tumor where lymphocytes encounter cognate antigens. In a 2007 review of clinically tested immunotherapies, Gajewski observes there are two major barriers that make patient tumors resistant to immunotherapies that largely target cytotoxic T cell responses: suboptimal recruitment of circulating T cells to the tumor and suboptimal effector stimulation in the tumor microenvironment[22]. In recent years, the latter obstacle has received much attention, and mechanisms identified experimentally can be classified into the following 3 categories:
1. cell intrinsic mechanisms, e.g. loss of MHCI expression on tumor cells
2. cell extrinsic mechanisms, e.g. expression of TGF-beta cytokine by tumor cells
3. immune cell dysregulation, e.g. tumor promotes T regulatory cell differentiation

Notably, two therapies, based on this shift in focus, have catalyzed the promise of next-generation immunotherapies in the minds of clinicians and researchers interested in the field. A trial reported in 2003 showed provisional efficacy of anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) antibody in late stage melanoma and ovarian carcinoma patients who had prior immunotherapy[23]. A further phase III trial (including antibody with peptide vaccine) reported dramatic results for metastatic melanoma[24], a cohort of patients with no therapeutic options. These patients showed an increased survival with treatment (10.1 versus 6.4 months), and the 2.5 year survival was double in groups receiving the antibody. These results prompted the FDA to approve anti-CTLA-4 (ipilimumab) for this cohort, but also as a first line therapy.

A 2012 phase I trial of anti-programmed cell death 1 (PD-1) antibody (candidate BMS-936558) showed a 20-25% objective response across three cancers: non-small cell lung cancer, renal cancer and melanoma. Importantly, tumor tissue was collected and imaged to determine level of receptor ligand expression (PD-L1) which is a known mechanism of tumor evasion[25]. Interestingly, out of 25 patients with PD-L1 positive tumors, 9 showed an objective response while no patient with PD-L1 negative tumors showed an objective response. These results suggest that, while PD-L1 expression in patients might be necessary to respond to this treatment, it is not sufficient. Other mechanisms leading to incomplete activity of anti-PD-1 treatment include expression of the additional PD-1 ligand, PD-L2, or involvement of another inhibitory signal like CTLA-4, or a pathway that is yet to be discovered. Combination therapy including, for
example anti-CTLA-4, might be beneficial. In addition, eight patients (not counted in the objective response group) had reduction in index lesions in the presence of a new lesion. Such a pattern of response is typical of immunotherapy[26] where lymphocytes activated by the therapeutic disruption, traffic not only to the indexed lesion, but also to metastases that were previously undetected. Due to the increased infiltration, these lesions are then detected in follow-up.

One of the most interesting aspects of this study is that all the patients with an objective response had prior immunotherapy. This, combined with results from the anti-CTLA4 trials, suggests that bimodal immunotherapy might be optimal. Borrowing a term from classical immunology, one could prime a patient response with initial therapy and followup with therapy intended to remove, or mitigate, tumor generated inhibition. The fact that no patients with colon, nor prostate cancer achieved an objective response underscores the fact that not all cancers will respond the same to blocking inhibition, as the immunological context may be different. Nevertheless, both of these trials prompted by blockade of inhibition (also known as checkpoint blockade) studies have signaled the new potential of immunotherapy. Important questions remain about how best to prime and then block inhibition and the optimal combination of immunomodulatory agents, or even their combination with traditional cancer therapeutics, either approved or under development.

Although PD-1 and CTLA-4 are classified as co-inhibitory molecules that potentiate a normal T-cell response, they fit into a broader category of co-expressed molecules that can also intensify, or sustain a T-cell response. These have been historically known as costimulatory molecules. Targeting these molecules are a mainstay of cancer vaccine design and will be discussed in the next section.
The importance of costimulation

In conventional immunity, the importance of costimulation in the adaptive immune system cannot be overstated. The requirement for costimulation represents a major evolutionary bulwark that keeps adaptive immunity from uncontrolled tissue damage. The original model proposing a “second signal” for lymphocytes emerged when experimenters were trying to reconcile B cell self-tolerance and the ability of these cells to undergo somatic hypermutation as a late-stage maturation event[27]. In this context, a “second signal” described simultaneous activation of a cognate CD4+ helper T cell as necessary to generate a productive immune response. While it is interesting the original costimulatory signal was the tumor necrosis factor superfamily (TNFSF, discussed below) member, cluster of differentiation [number] 40 (CD40), the term costimulation has since been extended to other contexts. It describes any second signal a lymphocyte can receive in addition to its primary stimulus, often the engagement of a co-expressed stimulatory receptor (or, by extension, co-inhibition through an inhibitory signal). By convention, a lymphocyte receiving only a primary signal, lacking a second signal (e.g. in sterile immunity) would be expected to become anergic, or be deleted. In order to break this peripheral tolerance, researchers endeavor to provide potent costimulatory signals in their experimental tumor vaccines. Table 1 summarizes some of these approaches as introduction to murine pre-clinical models.
Table 1-1. Summary of various approaches used to co-stimulate an anti-tumor immune response in murine tumor models.

<table>
<thead>
<tr>
<th>Author and Reference</th>
<th>Year</th>
<th>In-vivo Model(s)</th>
<th>Intended Immune Target</th>
<th>Costimulator 1</th>
<th>Costimulator 2</th>
<th>Costimulator 3</th>
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<tr>
<td>Martin-Fontecha [29]</td>
<td>2000</td>
<td>TS/A</td>
<td>CD4 T-cell</td>
<td>CD28</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dredge [31]</td>
<td>2002</td>
<td>CT26 / B16</td>
<td>T-cell</td>
<td></td>
<td>thalidomide analog</td>
<td></td>
</tr>
<tr>
<td>Zuberek [33]</td>
<td>2003</td>
<td>MethA / B16F1</td>
<td>CD4 T-cel</td>
<td>CD28</td>
<td></td>
<td>ICOS</td>
</tr>
<tr>
<td>Yan [34]</td>
<td>2004</td>
<td>AGN2a</td>
<td>CD8 T-cell</td>
<td></td>
<td>4-1BB</td>
<td></td>
</tr>
<tr>
<td>Chen [36]</td>
<td>2006</td>
<td>P815</td>
<td>adaptive response</td>
<td>CD28</td>
<td>IL-12</td>
<td></td>
</tr>
<tr>
<td>Murata [37]</td>
<td>2006</td>
<td>FVB MMTV-neu</td>
<td>CD8 T-cell</td>
<td>GM-CSF</td>
<td>OX40</td>
<td></td>
</tr>
<tr>
<td>Aigner [38]</td>
<td>2008</td>
<td>MCF-7/humanized mouse</td>
<td>T-cell</td>
<td>viral antigen (HN)</td>
<td>CD28</td>
<td>IL-2</td>
</tr>
<tr>
<td>Sharma [39]</td>
<td>2009</td>
<td>TC-1 / 3LL</td>
<td>adaptive response</td>
<td>4-1BB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shi [40]</td>
<td>2009</td>
<td>P815 / B16</td>
<td>T-cell</td>
<td>DcR3/TR6</td>
<td>BCG</td>
<td></td>
</tr>
<tr>
<td>Cho [41]</td>
<td>2013</td>
<td>B16 / TC-1</td>
<td>CD8 T-cell</td>
<td>polyl:C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xie [42]</td>
<td>2013</td>
<td>B16</td>
<td>CTL</td>
<td>CD40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Berezhnoy [43]</td>
<td>2014</td>
<td>B16 / GVAX</td>
<td>CD8 T-cell</td>
<td>GM-CSF</td>
<td>4-1BB</td>
<td></td>
</tr>
</tbody>
</table>
This collection of murine vaccine studies, while far short of exhaustive, shows the breadth of costimulatory approaches that are being pursued in pre-clinical efforts. One can see nearly all studies aim to costimulate a T cell response (sometimes indirectly through APC) and mainly that of cytotoxic CD8 T cells. Researchers are targeting different classes of costimulatory molecules to include integrins, B7 family, tumor necrosis factor receptor super family (TNFRSF) and classical TLR ligand adjuvants. In addition, approaches include recombinant versions of endogenous protein, agonistic antibodies, and small molecules. As can be seen in the table, many studies combine multiple costimulatory agents in hopes of overcoming the tumor-enforced immune suppression. It is important to note, as well, these studies represent different tumor models, especially when viewed from an immunological perspective.

As can be seen in the table, a popular mouse model is B16 (also the primary model used in the present work), a syngeneic melanoma cell-line injected (or delivered intravenously) into a C57BL6 wild-type, female mouse which reproducibly generates rapidly growing tumors in the subcutaneous tissue (or liver, spleen and lung when delivered intravenously). The pathology of the tumor follows human melanoma in certain respects, particularly melanin expression and early 2D expansion followed by later stage growth out of that plane. Likewise, this model is
resistant to many therapies and has low immunogenicity[47], as compared to other transplantable models (EL4, or 3LL).

In order to understand the low immunogenicity of this model, a paper from 2011 is illustrative[48]. Researchers tried multiple combinations of both innate and adaptive stimuli. They found they could therapeutically treat tumor bearing mice with the following components in their model and vaccine:

1. ovalbumin (OVA) expressing B16 cell line used to generate tumor
2. anti-CD40 agonist antibody
3. EDA (TLR4 agonist)-OVA
4. polyI:C (TLR3 agonist)
5. Imiquimod (TLR7 agonist)

Components 2, 3 and 4 were administered intratumorally, while imiquimod was applied topically above the tumor site. This combination represents a potent immunostimulatory strategy recruiting both adaptive and innate arms of the immune system. One would expect strong adaptive immunity to be present to the OVA xeno-antigen, perhaps generated during the initial tumor challenge and restimulated in the therapeutic treatment. Notably, the antigen is actually conjugated to the TLR4 agonist, EDA. This is important because studies have shown that antigen and danger signal must be taken up by the same APC, and perhaps even present in the same endosome to engender cross-presentation, considered essential for a cytotoxic CD8 T-cell response[49]. It is also interesting to note, all of these treatments were given locally. If anti-tumor immunity is present at, but sequestered by, the tumor, the local treatment could re-stimulate
this antigen-specific immunity. Nevertheless, only 50% of mice survived long-term. This is a testament to the low immunogenicity of this transplantable model. Clinicians would be reluctant to recommend such a potent immunostimulatory therapy in human patients since the likelihood of severe toxicity would be high. Nevertheless, while the B16 model may be less immunogenic than other transplantable models, a therapy found to be effective in B16 would be extendable to other settings where increased immunogenicity may further increase efficacy (e.g. other mouse models, or patients with clinical correlates of immunogenicity).

The aforementioned trials of anti-CTLA-4 and anti-PD-1 have shown the benefit of targeting co-inhibitory pathways in addition to costimulatory pathways. Both of these molecules belong to the CD28 family of proteins (reviewed in [50]), the most well-known class of co-stimulatory molecules. In the conventional model, during priming, CD28 would be upregulated on the T cell and receive a signal from B7-1 (CD80), or B7-2 (CD86) upregulated on the surface of an activated, mature dendritic cell, which presents the primary stimulus via an MHC-antigen complex. Such costimulatory events are assumed to occur directly following, if not simultaneous, to the primary stimulus. The course of this priming reaction can take several days, however, and it is unclear which costimulatory signals are redundant, or perhaps temporally related[51, 52]. After lymphocytes are activated, another important class of co-stimulatory molecules are known to be expressed, proteins in the TNFR superfamily related to TNFRII. These molecules are currently the subject of intense study in both the fields of cancer immunology and autoimmunity. As costimulators they can potentiate an adaptive response, yet it is unclear if multiple members of this family are redundant, or play distinct roles. Furthermore, researchers are trying to
determine the best way to target these receptors to fine-tune an immune response. The next section will discuss these costimulatory receptors in greater depth.

**Costimulation in lymphocytes- TNFR superfamily**

Members of the TNFR superfamily are known to be expressed in lymphocytes and, for some members, up-regulated after activation. The members of this family are summarized in table 2 below. These proteins are classified into three broad categories based on the nature of their signaling: death domain containing, TRAF associating and ligand competing.

**Table 1-2. TNFR super family members and genetic deletion phenotypes.**

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
<th>Category</th>
<th>Mutant Phenotype</th>
<th>Type of Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD95</td>
<td>CD95L</td>
<td>death domain</td>
<td>Lymphadenopathy, autoimmunity [53]</td>
<td>lpr/lpr</td>
</tr>
<tr>
<td>DR3</td>
<td>TL1A</td>
<td>death domain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DR4</td>
<td>TRAIL</td>
<td>death domain</td>
<td>lymphoid malignancy (TRAIL) [54]</td>
<td>-/-</td>
</tr>
<tr>
<td>DR5</td>
<td>TRAIL</td>
<td>death domain</td>
<td>lymphoid malignancy (TRAIL) [54]</td>
<td>-/-</td>
</tr>
<tr>
<td>DcR2</td>
<td>TRAIL</td>
<td>death domain</td>
<td>lymphoid malignancy (TRAIL) [54]</td>
<td>-/-</td>
</tr>
<tr>
<td>EDAR</td>
<td>EDA</td>
<td>death domain</td>
<td>hair and skin defects [55]</td>
<td>a/a</td>
</tr>
<tr>
<td>TNFR1</td>
<td>TNF, LTα</td>
<td>death domain</td>
<td>susceptible to infection (l. monocytogenes) [56]</td>
<td>-/-</td>
</tr>
<tr>
<td>DcR3</td>
<td>CD95L, TL1A, LIGHT</td>
<td>ligand competing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DcR1</td>
<td>TRAIL</td>
<td>ligand competing</td>
<td>lymphoid malignancy (TRAIL) [54]</td>
<td>-/-</td>
</tr>
<tr>
<td>OPG</td>
<td>TRAIL, RANKL</td>
<td>ligand competing</td>
<td>osteoporosis [57]</td>
<td>-/-</td>
</tr>
<tr>
<td>XEDAR</td>
<td>EDA</td>
<td>TRAF binding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Receptor</td>
<td>Ligand(s)</td>
<td>TRAF Binding</td>
<td>Effect</td>
<td>Reference</td>
</tr>
<tr>
<td>----------</td>
<td>-----------</td>
<td>--------------</td>
<td>--------</td>
<td>-----------</td>
</tr>
<tr>
<td>TNFR2</td>
<td>TNF, LTα</td>
<td>TRAF binding</td>
<td>Increased resistance to TNF-induced apoptosis</td>
<td>[58]</td>
</tr>
<tr>
<td>LTBR</td>
<td>LTα/β, LIGHT</td>
<td>TRAF binding</td>
<td>Multiple immune structural defects</td>
<td>[59]</td>
</tr>
<tr>
<td>HVEM</td>
<td>LTα, LIGHT, BTLA</td>
<td>TRAF binding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD27</td>
<td>CD27L</td>
<td>TRAF binding</td>
<td>Reduced CD4/CD8 compartment following infection</td>
<td>[60]</td>
</tr>
<tr>
<td>CD30</td>
<td>CD30L</td>
<td>TRAF binding</td>
<td>Elevated thymocyte numbers, reduced negative selection</td>
<td>[61]</td>
</tr>
<tr>
<td>CD40</td>
<td>CD40L</td>
<td>TRAF binding</td>
<td>No class switch recombination, no germinal centers</td>
<td>[62]</td>
</tr>
<tr>
<td>4-1BB</td>
<td>4-1BBL</td>
<td>TRAF binding</td>
<td>Reduced CD8 proliferation (4-1BBL)</td>
<td>[63]</td>
</tr>
<tr>
<td>OX40</td>
<td>OX40L</td>
<td>TRAF binding</td>
<td>Reduced CD4 proliferation</td>
<td>[64]</td>
</tr>
<tr>
<td>GITR</td>
<td>GITRL</td>
<td>TRAF binding</td>
<td>Enhanced T-cell activation</td>
<td>[65]</td>
</tr>
<tr>
<td>RANK</td>
<td>RANKL</td>
<td>TRAF binding</td>
<td>Lack peripheral LN, reduced splenic B cells, osteoporosis</td>
<td>[66]</td>
</tr>
<tr>
<td>BAFFR</td>
<td>BAFF</td>
<td>TRAF binding</td>
<td>Defective B cell differentiation</td>
<td>A/WySnJ</td>
</tr>
<tr>
<td>TACI</td>
<td>BAFF, APRIL</td>
<td>TRAF binding</td>
<td>Uncontrolled lymphoproliferation, autoimmunity</td>
<td>[68]</td>
</tr>
<tr>
<td>BCMA</td>
<td>BAFF, APRIL</td>
<td>TRAF binding</td>
<td>Reduced plasma cell survival</td>
<td>[69]</td>
</tr>
<tr>
<td>Fn14</td>
<td>TWEAK</td>
<td>TRAF binding</td>
<td>Hyperactive innate response, expanded T cells (TWEAK)</td>
<td>[70]</td>
</tr>
<tr>
<td>TROY</td>
<td></td>
<td>TRAF binding</td>
<td>No abnormality reported (skin)</td>
<td>[71]</td>
</tr>
<tr>
<td>RELT</td>
<td></td>
<td>TRAF binding</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Bold typeface indicates ligand binds multiple receptors as indicated.**
Upon ligand binding and activation, the death domain containing members recruit intracellular adapters also containing the death domain. These assemblies ultimately lead to a caspase cascade which results in apoptosis. In contrast to other members in this category, TNFRI signaling also results in transcription (through NFkB activation) of protective proteins which act to inhibit apoptosis. Therefore, cell types in which NFkB is an active pathway apoptosis will only occur if the balance between pro-survival (e.g. Bcl-xL) and death complex signaling is skewed towards the latter. In naïve T cells, for example, TNF is a potent autocrine, costimulatory, rather than apoptotic signal, especially in early stages of activation[72].

The TRAF binding members are mainly expressed on activated lymphocytes. These proteins are known to signal through alternative NFkB (p52 translocation to the nucleus) and lead to immune response, albeit under protracted kinetics compared to canonical NFkB. As can be seen in the table, the deletion of these genes in mouse models often results in some form of immune dysregulation, especially weakened adaptive responses. However, these costimulatory molecules may exert fine control over later stages of a developing adaptive response, or may operate at multiple points in differentiation programs. In cases where the temporal context of the costimulatory signal is critical to its function, a germ-line deletional mutant may not reveal the entire function. The study of these TNFRII related proteins is a rapidly advancing and dynamic field.

As an example, in the case of BCMA, after the deletion mutant mouse was reported to have no immunological phenotype upon initial study[73], it was assumed this receptor played a redundant role with BAFF-R since it was also known that BAFF bound both receptors. However, further study revealed a defect in long term survival of plasma cells in the bone marrow[69].
suggesting a non-redundant role with BAFF-R. In addition, some controversy surrounds the Fn14/TWEAK axis. The first reported phenotype in Fn14 null mice was reduced proliferation in liver progenitor cells upon induced damage[74], but it is now a target in autoimmunity.

These TNFRSF members are classified as TRAF binding, because the first step after ligand binding is recruitment of TRAF second messenger molecules to the cytoplasmic tail of these proteins. Cellular inhibitor of apoptosis (cIAP) 1 and 2 were first identified in a co-immunoprecipitation of TNFRII. It was later shown that cIAP1 and cIAP2 (in complex with TRAF2 and TRAF3) constitutively bind NFkB inducing kinase (NIK) resulting in its ubiquitination and proteasomal degradation. Inhibition of IAPs, therefore, can generate a costimulatory signal in lymphocytes through alternative NFkB activation that mimics binding of receptor to endogenous ligand[75]. Small molecule IAP antagonists were developed originally as a cancer therapeutic, as some cancer cells overexpress these proteins to avoid apoptosis, and several candidates are nearing FDA approval. These molecules were designed from a tetra-peptide motif found in the endogenous regulator of IAP proteins, second mitochondria-derived activator of caspases/direct IAP binding protein with low pI (SMAC/DIABLO)—leading to the alternative name of these compounds SMAC mimetics. The immune-modulatory ability of these small molecules emerged as an unintended consequence, and it is now appreciated that IAP proteins regulate signaling in a variety of immune subsets. While it is possible these costimulatory effects are mediated largely through the inhibition of cIAP1 and cIAP2, it is important to note there are 9 identified mammalian IAPs containing the IAP family defining baculovirus inhibitory repeat (BIR) domain which can be bound by the IAP antagonist (and endogenously binds to the tetrapeptide motif on SMAC/DIABLO). The versions of the IAP antagonists that are being pursued in clinical trials are
dimer versions of the drug, as the dimers bind with greater affinity to their targets, and exhibited less toxicity in initial trials[76]. In addition, it has been shown that dimer is able to degrade X-linked inhibitor of apoptosis protein (XIAP), the only mammalian IAP protein shown to directly inhibit caspases, and therefore apoptosis. In the current study, we use both monomer and dimer versions of the drug.

**The importance of vaccination site**

A recent review[77], argues that many of the immunotherapies under investigation today, while focusing on key aspects of the effector phase, like costimulation, are unlikely to show clinical benefit because of the potent immune suppression prevalent at the intended site of the effector phase, the tumor microenvironment. Tumors are well-known for their ability to disarrange the tissue vasculature to promote tumor growth and survival. Tumor growth typically outpaces its angiogenic ability, leaving a chronic deficit of oxygen in the tumor tissue. While total volume of blood supplied to the expanding tissue would increase over time, the oxygen deficiency preferentially inhibits, or kills immune cells which are more sensitive to hypoxia than the tumor cells.

This and other limitations of effector immune cells at the tumor site is a significant form of immune suppression. A logical therapeutic strategy, therefore, is to create an immune-sufficient site in proximity to the tumor, otherwise known as peritumor delivery. Under this mode, the immune response will be generated *in situ*, or within the endogenous tissue of the animal, without recourse, for example, to adoptive transfer, and it will be generated near the tumor, not distal to it. In recent years, local immune structures, in the perimeter space of tumors
have been increasingly studied and characterized. The immune system, composed largely of cells in circulation, is broadly categorized into primary and secondary structures based on how these structures interact with the circulating cells. These structures are defined as follows:

1. primary lymphoid organs, or sites of lineage generation: bone marrow, thymus
2. secondary lymphoid organs, or sites of lineage differentiation: spleen, peripheral lymph nodes, follicles in mucosa-associated lymphoid tissue (MALT)

Not commonly included in the canonical description of the immune system given above, a tertiary lymphoid structure (TLS) is an association of immune cells, similar to what is found in secondary tissue, but ectopic, located in peripheral tissue (not including lymph nodes, or MALT). These structures are often associated with sites of persistent inflammation, e.g. connective tissue of joints in rheumatoid arthritis. Since an expanding tumor often mechanically disrupts the surrounding tissue, chronic inflammation is often associated with a growing tumor margin. In fact, this clinically evident aspect of some cancers was an early connection made between model tumor behavior and the immune system, the concept of a “smoldering wound” and the long-standing idea that wound-healing inflammatory mechanisms can be co-opted to support tumor growth.

Recent, exciting findings have illuminated the clinical phenomena that patients with the same grade and stage cancer, can present with tumors of a strikingly different nature when viewed from an immunological perspective. Galon and colleagues coined the term “immune contexture” to describe the different immunological profiles observed in colorectal tumors[78] and revealed the prognostic utility of these profiles, showing that in multivariate analysis,
conventional staging did not add any predictive value above that provided by their immune variables. A previous study had shown increased CD3+ CD45RO+ infiltrates (both in central tumor and invasive margin regions) were significantly correlated with increased survival[79], validating an early finding in a mouse model where the tumor protective role of memory T cells was confirmed in horizontal transfer experiments[80]. On a final note, most of the experimental strategies discussed have focused on T cell responses. But, as the work on immune contexture has revealed, B cells may be just as important. As an example, a recent mouse study has revealed a novel mechanism for endogenous anti-tumor immunity mediated by antibody [81]. Using a mouse with a mutation in MHC class I allele Qa-1 that does not allow the binding of CD8 Treg, it was shown that the titer of IgG antibodies for several known autoantigens (e.g. ANA, dsDNA, Ang-2) was increased in the context of GVAX, compared to vaccinated WT mice. This underscores the aforementioned point that, while GVAX promotes anti-tumor immunity, there is a stringent tolerogenic threshold in the B16 model that must be overcome to impart survival. Furthermore, this work highlights a potential role for anti-tumor antibodies as Qa-1 mutant mice also showed reduced angiogenesis and increased tumor infiltration of macrophages which was correlated with the deposition of anti-tumor IgG at the tumor site.

Initial work in our lab had shown an effect of systemic IAP inhibitor in a mouse B16 tumor model. While systemic IAP inhibitor treatment alone had no effect, systemic treatment with IAP inhibitor in the context of a GVAX therapeutic vaccine delayed tumor growth and increased survival. This work also showed an increase in tumor specific CD8 T-cells in draining lymph nodes of the vaccine site. Therefore, we were enhancing immunity, but these activated cells could be
otherwise suppressed at the margins of the tumor. We wondered if delivering the drug in a peritumor location via bioabsorbable scaffold would increase the immune efficacy of the drug.

Conclusion

The recent focus on blockade of inhibition has captured the attention of clinicians and promulgated cancer immunotherapy to a much wider audience. It would be going too far, however, to focus entirely on this latest mode and ignore the work leading up to it. While models of immune surveillance and tumor evasion have been discussed throughout the last century, blockade of inhibition represents the natural progression of the last few decades which have been particularly exciting in identifying tumor antigens and pursuing a great variety of costimulatory strategies, seemingly while the basic immunology of these pathways was still under investigation. Looking across the field, one can appreciate the potential of a combined approach, where de novo antigenic response and checkpoint blockade can be pursued in series, or in the same treatment. Much will be learned, no doubt, about which combinations are most effective and novel methods to better control an immune response that could well impact fields beyond cancer immunotherapy.

I have highlighted some of the history, the present and the excitement for the future. It is certain that harnessing the full power of the immune system, to include humoral and cellular adaptive responses, to overcome “endemic” tolerance and suppression at the tumor site, while preserving beneficial self-tolerance requires a great deal of knowledge about the immune system, but may also require the involvement of multiple, diverse disciplines. This represents the impetus of this work. We wanted to take a small molecule, known to have novel costimulatory
potential, and deliver it over time in the peritumoral space. The first step was optimizing the
delivery method for the drug, a bioabsorbable poly-lactide-co-glycolide (PLG) scaffold, discussed
in detail later. As I have highlighted in the previous sections, not just understanding the temporal
manipulations and differentiation of lymphocytes, but also understanding the fine-tuned
compartmentalization in the immune system is necessary to better control immune response.
The next chapter focuses on delivering the vaccine, where I will take the perspective of creating
a specialized compartment for manipulating immune response.
Chapter 2. Novel Materials for Vaccine Delivery

Introduction

In conventional prophylactic vaccination against pathogens, the vaccine formulation intends to provide antigen exposure and costimulatory signal. While the latter was discussed in the previous chapter, I will now turn my attention to the former parameter and note that 10th century Chinese and Indian methods of variolation (a prophylactic treatment engendering immunity to smallpox) used scabs of infected individuals that were ground up and administered to the nasal cavity (Chinese technique), or scratched into the skin (Indian technique)[82]. It is not understood why these techniques did not lead to deadly smallpox infection (rather, exhibiting superior prophylaxis compared to vaccination) in the treated individual while the major risk was infection of others who came in contact with the treated individual[83]. One proposed idea is that the fibrous scab material provided a dense matrix to sequester virus[84]. Therefore, these variolation materials could be viewed as involving primitive vaccine delivery devices.

It is well known that duration of antigen in a vaccine is a critical determinant to longevity of a memory response in conventional vaccination against pathogens[85]. This is one of the driving factors behind booster vaccinations; they provide an additional boost of antigen. The persistence of antigen is most closely related to duration of IgG class switched antibody titer, and mechanistically this persistence is carried out by antibody-antigen complexes on follicular dendritic cells (FDCs) [86, 87]. This represents an important endogenous depot of antigen for the highly sought after long-term memory, and in the germinal center FDCs are required for plasmablast differentiation, an important source for high affinity, persistent IgG antibodies.
While therapeutic, tumor vaccination typically focuses on the cytotoxic T cell response as mentioned in chapter 1, it is important to consider that a natural response in the body is likely not as compartmentalized as this. All arms of immunity likely participate in a real world vaccination.

Materials approaches typically aim to create an antigen depot, mimicking the role of FDCs identified in immunological studies, but also to incorporate costimulatory signals (especially in tumor vaccination). The implications for sequencing, or temporally modifying a costimulatory signal (or blockade of inhibition), are an exciting new horizon which can now be explored at the growing interface of immunology and materials research. The following discussion will introduce particulate delivery approaches, which can be seen as an extension and elaboration upon conventional vaccine strategies, followed by an argument for three-dimensional approaches which are used in the current work.

**Particulate delivery approaches**

Many approaches have been pursued in various delivery systems. The majority of studies have been conducted with particulate delivery systems as these are more convenient in production and in administration. It is important to note, conventional vaccine adjuvants which developed in practice before their immunomodulatory mechanisms were understood, often involved micro- and nano-scale particles (e.g. alum). Focusing on particulate systems (reviewed in [83]), they are generally categorized according to size, with the following being a typical division:

1. virus-like particles/nanoparticles 20-200 nm
2. virosomes/liposomes 100-200 nm
3. microparticles/liposomes/alum/whole cell 100 nm-20 um

Virosomes are considered to be like liposomes, but incorporate viral factors that enhance fusion of the particle with target cells. They are designed to mimic the fusion properties of the virus and delivery of antigens, and do not try to mimic any other pathogenic, or replicative function of the virus. Size is a critical determinant that influences the resultant immunity. Larger particles can lead to frustrated phagocytosis, while smaller sizes can allow uptake in the lymph node capillary beds and direct delivery to LN resident DCs[88].

In addition to size, geometry of particles play an important role in how they ultimately interact with cells of the immune system[89]. The approaches vary significantly and allow a great flexibility in design, from using an oblong, hyaluronic acid coated surface to create “backpacks” on the outside of macrophages[90], to irregular-shaped glycerylmonostearate nanoparticles that preferentially accumulate in the spleen (when given systemically)[91].

In a B16 model, researchers have shown that peptide loaded PLG nanoparticles (PLG-NP) can delay tumor growth in a prophylactic setting, and this effect can be enhanced by including TLR4 agonist MPLA and IFN-gamma to inhibit tumor escape through downregulation of MHCI, suggesting that the immunity is a result of cytotoxic T cell activity[92]. While the nanoparticles can be efficiently taken up by dendritic cells, it is important to note that PLG itself (at least in soluble form) has been shown to activate NLRP3 inflammasomes, and this may explain why NPs in these experiments loaded with antigen alone showed a response; the delivery particles were providing a second signal.
Gelatin-chondroitin sulfate microspheres have been used to delivery GM-CSF in a tumor model[93] over twenty years ago. In these experiment, microsphere encapsulation led to superior tumor protection in a prophylactic setting compared to bolus injection. In another interesting use of poly-lactide particles, researchers encapsulated IL-12, an important cytokine for generating a pro-inflammatory Th1 response, and injected them intratumorally in a BALB/c lung carcinoma model[94]. This delivery lead to greater survival of mice compared to bolus injection, or systemic delivery.

Finally, in contrast to the aforementioned particulate approaches that target T cell responses, researchers, also using PLG-NPs, have shown that including multiple TLR ligands in such particulate systems can enhance an antibody response[95]. Curiously, in this system, early phase plasmablast response was not impacted by additional TLR ligands even though long term antibody response was increased, comparing to single TLR adjuvant formulations.

**Rationale for three-dimensional in situ approaches**

A recent approach takes the antigen depot a step further. By recognizing that particulate systems, depending on their size, can either be 1) taken up by phagocytes, or 2) provide ample surface area for diffusion and release of internal, active components, researchers attempt to create a more persistent site, in situ, as an antigen depot. In particular, Ali, et al [96] showed that PLG microspheres fused and coalesced into a 3D network that included void space (this process will be detailed in the next chapter), could enrich the local implantation site for dendritic cells (or inflammatory monocytes, CD11c+ CD11b+) by incorporating GM-CSF in addition to providing an antigen (in this case B16 tumor lysate). In the proposed model, dendritic cells would take up
antigen, mature and then travel to the draining lymph node (dLN) to initiate an adaptive response.

This approach is akin to targeting dendritic cells by other means, mentioned here because of important considerations governing antigen dose. When researchers found the protein DEC-205, with restricted expression on dendritic cells and thymic epithelia, was involved in antigen processing and loading on MHC molecules, it was shown that conjugating antigen to an anti-DEC-205 antibody (a non-material based targeting) could reduce the amount of antigen needed for a T cell response by 100 fold[97]. When using the same conjugated approach in vivo, antigen doses could be reduced 400-1000 fold, compared to what was necessary in a conventional vaccination[98, 99].

Whether one brings the dendritic cells to the antigen, or brings the antigen to the dendritic cells, the effect of targeting the immunotherapy to the immune subset of interest is the desired goal. Of key importance is that antigen be delivered in an appropriate amount for the desired target cell. It is well known that persistent, ubiquitous antigen (especially in the absence of a danger, or costimulatory signal—see chapter 1) can enhance peripheral tolerance. In this context, therefore, it is advisable to engineer the vaccine such that, regardless of the absolute amounts of antigen and danger signal included, both are present in relatively commensurate amounts. There is enough danger signal to be paired with antigen and no excess remaining of either. Like most things in the immune system, or more generally biological systems which tend to exhibit, to borrow a term from aerodynamic design, static stability, there is probably a well-defined window for all the parameters researchers wish to permute, and falling outside this
window for any single parameter will initiate a mechanism to return the system to the stable state, even a mechanism the researcher is not aware of, or intending to invoke.

When considering 3D matrices, additional avenues for immune-modulation may be possible compared to particulate approaches. Providing a template of connections that would more readily be replaced by an endogenous fibrin, or collagen scaffold might provide even more persistent, or effective modulatory power. By the same token, an implantable device will cause a surgical disruption to the targeted tissue. While this might be desirable for a pro-inflammatory application, it may not be ideal for a pro-tolerogenic one. The implantation procedure, while conducted with aseptic technique, will induce a wound healing response at the very least. For this reason, all \textit{in vivo} experiments discussed herein include blank scaffolds as a control for the implantation process itself, as well as for the PLG as material vehicle.

\textbf{Conclusion}

Delivery materials for vaccination have an ancient history, but today we possess more specific knowledge about how materials interact with the immune system and how the immune response might be optimally manipulated. There is likely a lot of therapeutic potential at the interface of materials and immunology. In the current work, we have chosen a non-toxic, bioabsorbable 3D system to deliver a small molecule we know to have novel costimulatory ability. Using this system we have unlocked novel effects of this drug in our local delivery system. The next chapter will detail our results in optimizing the scaffold manufacturing process in our laboratory where all the scaffolds were made for the experiments described herein.
Chapter 3. Optimizing PLG scaffold for delivery of IAP inhibitors

Background and Introduction

Poly(lactic-co-glycolic acid) (PLGA, or PLG implying the polymerization) based polymer scaffolds are a popular choice in drug delivery devices for the following reasons:

1. PLG is an approved material by the FDA for several device applications
2. PLG is bioabsorbable and its persistence time in the body can be selected
3. PLG (and in particular our manufacturing process) is shown to be non-toxic

The US Food and Drug Administration (FDA) first approved PLG as a material in a bioabsorbable suture in 1970 with the release of the product Dexon™ (currently made by Covidien). While bioabsorbable sutures are nothing new, even at that time, they are used routinely to suture internal surgical wounds to avoid future invasive procedures for removal. PLG is increasingly used in devices and in drug depots.

One of the key differences in this 3D scaffold system is the foaming of the particles into a 3D network by carbon dioxide equilibrium and release. Carbon dioxide, in contrast to conventional, wet processing of polymers dissolves out of the material and would not persist in toxic amounts.

Manufacturing PLG scaffolds

The manufacture of PLG scaffolds in the standard biomedical laboratory setting proceeds with the following four steps:
1. preparation of aqueous-phase-containing microspheres by double emulsion

2. admixing of PLG microspheres with porogen and compression

3. dissolving carbon dioxide into compressed tablets followed by formation of polymer network by foaming

4. leaching of porogen out of networked polymer to create void space

The main purpose of the first step is to create, embedded within the polymer (that will eventually become a 3D porous network), aqueous micro-domains in which hydrophilic components (such as GM-CSF, or other biologic) can be sequestered, thereby increasing their half-life \textit{in vivo}. Once inside the body, these microdomains will be released over time as a result of 1) diffusion of the domains to the surface of the polymer and 2) the degradation and contraction of said polymer surface by spontaneous hydrolysis of PLG. The double emulsion process is depicted in figure 3-1.
Figure 3-1. Schematic of double emulsion process, from left to right, polymer is dissolved in ethyl acetate, combined with water, sonicated to create an emulsion, combined with second water phase and vortexed to create second emulsion, and finally added to larger volume of water to allow for evaporation of ethyl acetate and condensation of organic polymer.
The double emulsion process is a popular method for creating PLG microspheres because it requires little in the way of special equipment and can be done under standard laboratory conditions. This process has been well studied in the literature[100, 101], but is described in brief to familiarize the reader. In our laboratory, the microspheres are prepared in a batch process, with each batch yielding enough microspheres to manufacture 2.5 scaffolds (using current practice). In the initial step, PLG stock copolymer is dissolved in organic solvent (in this case, ethyl acetate) at a concentration of 5% by weight. This polymer solution is prepared in 1 mL volumes for emulsification with aqueous phase. The aqueous phase is added at 10% by volume (or 100 uL), and the separation is sonicated with a horn sonicator for 10 seconds. Immediately following this, a second aqueous phase is added (creating the transitory water-oil-water emulsion), vortexed and then added to a 100X larger volume of water including ethyl acetate at a lower percentage (0.07 percent volume). Throughout this process it is important to keep the emulsified suspensions moving at all times, as suspensions naturally coalesce when allowed to sit. This suspended WOW emulsion is stirred vigorously for 3 hours allowing for the solvent evaporation to occur, whereby ethyl acetate first partitions out of the PLG solution and into the unsaturated, larger volume solution, and then from there slowly volatilizes out of solution. During this controlled evaporation, the PLG will condense into microspheres inside of which are now trapped submicron aqueous domains containing our hydrophilic component.

The second major step in manufacturing a scaffold involves, at a minimum, combining the lyophilized PLG microspheres (now containing an aqueous phase) with a porogen. It is also at this step that other components (such as CpG condensate, lysate solution, or small molecule drug) can be admixed. Figure 3-2 exhibits a typical porogen (sucrose), microspheres adsorbed with B16
tumor lysate and CpG condensate preparation that would be used to create a single GVAX PLG scaffold. One can observe the slight brown tint to the melanin containing lysate used as antigen, coating the microspheres. One can also observe these powders are macroscopically different in size and homogeneity.
Figure 3-2. Photo illustrating the constituents of scaffold vaccine prior to mixing and fabrication into a 3-D mold, then foaming with carbon dioxide.

Figure 3-3. Photo illustrating a manufactured scaffold (right) compared to a standard US dime for size comparison. Photo credit: Molly Akin
The admixed powders (plus additional components) are compressed into a mold of arbitrary geometry that in our lab is a small disk, 8 mm in diameter. A picture of a manufactured scaffold is given in Figure 3-3. In the penultimate step of manufacture, compressed scaffolds are placed in a pressure chamber that is subsequently charged with 800 psi carbon dioxide and allowed to equilibrate for 18 hours. After this time period, the gas is released rapidly from the chamber, and as the carbon dioxide separates from its dissolved state in the bulk polymer particles it causes their foaming and interconnection around the admixed porogen particles. In addition, the admixed components will be mixed and trapped within the expanding, or fusing polymer network. The final step allows the porogen to be removed from the scaffold. Foamed scaffold devices are suspended in water, and the porogen is allowed to dissolve out leaving void space in its place. This leaching process takes 12 hours using a ratio of 10 milliliters of water per scaffold. Prior to implantation, or use in cell culture, scaffolds are disinfected with 70% ethanol and washed with PBS.

As can be seen from the brief overview given above, the manufacturing process includes numerous parameters (e.g. sonicator power, vortex time, stir speed, evaporation time and mixing method, just to name a few) that can vary from preparation to preparation. While making scaffolds by this process is approachable and convenient for a standard laboratory setting, to make scaffolds reproducibly requires detailed training and practice. In transitioning this technology from the engineering to the biomedical laboratory we learned valuable lessons not just about scaffold manufacture, but also about transitioning technology across disparate fields.
Results from in-house process

To validate our in-house manufacturing process, initial work focused on creating a GM-CSF-containing vaccine identical to what was published previously[96]. To assay for bioactivity, prophylactic protection against challenge with B16 tumor was selected. Initial scaffolds implanted (with tumor later injected distal to the implantation sites) did not impart protection against tumor challenge with all mice exhibiting the same tumor growth and lack of survival, whether they were treated with GM-CSF-containing scaffold vaccines, with blank scaffolds, or left untreated. Since these scaffolds did not exhibit the expected bioactivity we investigated the details of our manufacturing process to determine if a discrepancy existed between our process and the published process. We first validated that we were achieving similar release of GM-CSF from our scaffolds and biological activity of scaffolds with CpG incorporated.
Figure 3-4. GM-CSF release and bioactivity of CpG loaded scaffolds, as determined by ELISA analysis of supernatants incubated with microspheres for 48 hours (top), and CpG loaded scaffolds (n=3) show expected bioactivity when cultured with mouse splenocytes *ex vivo* for 5 days (bottom). Experiments have been repeated at least once with similar results.
Figure 3-5. Isolation of microsphere process in scaffold manufacture leads to no protection in a prophylactic treatment and B16 tumor challenge. Scaffolds were manufactured in our laboratory while microsphere material was sourced from either our laboratory, or the Mooney laboratory. Mice were challenged with 100,000 B16 tumor cells 7 and 14 days after receiving scaffold vaccines containing GM-CSF, CpG, and tumor lysate as described in text.
In addition to analyzing the GM-CSF and CpG incorporation, we initiated an experiment in collaboration with the Mooney lab, isolating microspheres from both labs, while steps 2-4 in the manufacturing process would occur in our lab for all scaffolds. In this experiment, shown in figure 3-5, all mice exhibited rapidly growing tumors and were euthanized, or otherwise succumb to tumor burden. This result indicated that what was non-optimal about our process was likely not our microsphere manufacture, but another later step in the manufacturing process. At this time, we also were able to characterize cellular infiltrates in our scaffold devices.

In the previous report, the three dimensional structure was shown to be necessary to elicit immune protection. Not only was the porous structure necessary for cellular infiltration of dendritic cells, but in the therapeutic tumor challenge experiment microspheres containing vaccine components, which had not been fabricated into the final device structure, were used as a control and exhibited no protection. These data suggested the importance of the three dimensional structure for the bioactivity of the devices. Additionally, the three dimensional structure represented an accumulation of the parameters of the manufacture. It is plausible that three dimensional structure could be impacted by numerous variables in the multi-stage manufacturing process. Therefore, surface porosity was selected as a “gold standard” for controlling the quality of the manufacture. We analyzed the porosity of the surface by scanning electron microscopy. Not surprisingly, given the repeated failure for scaffolds to protect against tumor challenge, our surface structure exhibited low porosity, and the following defects were not uncommon:

1. surface skin present over more than 50% of scaffold surface
2. bubbling out of concentrated polymer domains

3. differential porosity on either side of the device

In total, these defects suggested that the powder components were suboptimally mixed. For example, if a large conglomerate of PLGA microspheres was not broken up and not mixed homogeneously with porogen, when that polymer foamed it would erupt out from the surface in a large bubble. If it was mixed thoroughly with porogen, the expansion of the more dilute grains would be confined to the proximity of the porogen particles. Both surface skins and the differential porosity on opposite sides of the device indicate a settling of polymer particles to one side, separated from porogen. Given these surface defects, we doubled our mixing time prior to compression from 30 seconds per scaffold to 1 minute per scaffold.
Figure 3-6. Scanning electron micrographs showing the effect of mixing time on surface porosity of scaffolds, while all other manufacturing variables were the same. Each image is representative of at least 5 scaffolds. Experiment was repeated at least two additional times.
Discussion

It is important to note that while proper mixing is necessary for ensuring good porosity it is not sufficient. Other factors play an important inter-dependent role. In particular the relative size of the admixed powders is an intrinsic parameter that governs homogeneous mixing. When dealing with particles of disparate size, they will necessarily migrate into separate domains according to the well-studied phenomena of granular convection (counterintuitive, at first glance, to the principle of increasing entropy). For convection to take place, a force would have to occur, either within the system, or placed upon the system. Care is taken in the manufacturing process to limit any vibrations, or temperature fluctuations after the powders receive their final mix prior to compression. Other factors that influence this mixing behavior include the shape of the particles, the net charge of the particles, the surface roughness of the particles, and their collision behavior. Factors not intrinsic to the powders themselves include the shape of the mixing container and the roughness of its surfaces, the method of mixing, and the ambient humidity. As one can see, the mixing time is but one factor of many that could be explored for optimization. However, with making this one change, we were able to observe an increase in the surface porosity of the manufactured scaffolds. Scaffolds manufactured in the lab (GVAX) were shown to have bioactivity in a therapeutic tumor experiment with 15% of mice showing tumor regression and long term survival (conducted at Wyss Institute)[102].

Conclusion

We have optimized our PLG scaffold manufacture to ensure reproducible surface porosity by focusing on the mixing time of admixed components. The next step was incorporating
monomer compound and analyzing its release which will be discussed in chapter 5. From here I will discuss our experiments delivery IAP inhibitors in vivo and in a peritumor location where we revealed activity of monomer, but not dimer drug. Before discussing those in vivo results, chapter 4 explores activity of monomer and dimer in several ex vivo systems to better understand the effects of IAP antagonism in adaptive immunity.
Chapter 4. IAP antagonism in adaptive immunity

Introduction

Based on previous findings that IAP monomer could costimulate a T cell response and contribute to enhancing and antitumor vaccine, we wanted to establish if dimer compound could further enhance and costimulate a response. We investigated dimer compound, in comparison to monomer compound treatment in several ex vivo systems, to include erythrocyte-depleted splenocytes culture, purified B cell culture, T-cell independent B cell stimulation, T-cell dependent B cells stimulation and T cell stimulation. Following results that will be presented in chapter 5, we returned to our ex vivo system to interrogate the ability of monomer and dimer to enrich for lymphoid tissue inducer cells (LTi).

Results

We wanted to investigate the differential activity of IAP inhibitors on various immune subsets. In our first system, we cultured splenocytes, depleted of erythrocytes, ex vivo for 4 days in the presence of vehicle (DMSO), monomer or dimer inhibitor. After this timepoint, several hematopoietic subsets are no longer present as can be seen in figure 4-1. In particular, we see a relative diminution of myeloid and granulocytic lineages, broadly identified by surface expression of CD11b/c integrin chains and Gr-1 respectively, while lymphocyte lineages persist under these conditions, as characterized by CD19, CD4 and CD8. It is known over 90% of cells in the spleen are lymphocytes, and our results are consistent with this, where close to 60% of cells are characteristic CD19+ B cells and another 35% are either CD4+ or CD8+ T cells at day zero.
Figure 4-1. Hematopoietic subsets differentially persist in splenocytes cultured ex vivo for 4 days as determined by flow cytometry analysis of indicated surface markers. Dot plots are representative of three mice per condition. Experiment has been repeated once with similar results.
As can be seen, after 4 days, under vehicle treatment, CD4+ cells exhibit a relative increase in frequency compared to CD8+ and CD19+ subsets, the latter of which actually decreases in frequency. In figure 4-2, we compare ex vivo cultured splenocytes in the presence of vehicle, monomer or dimer inhibitor. As can be seen, CD19+ cells now show an increase in frequency with monomer and, then, dimer treatment. At the same time, the CD4+ subset shows a decrease, while the CD8+ subset frequency remains essentially constant. Other myeloid and granulocytic subsets do not show a change in frequency upon IAP inhibitor treatment. In summary, it was apparent that B cells were persisting in ex vivo culture at the relative expense of CD4+ cells with IAP inhibitor treatment and that dimer exhibited an increased effect compared to monomer.
Figure 4-2. IAP inhibitor treatment leads to increased persistence of B cells in \textit{ex vivo} cultured splenocytes after 4 days as determined by flow cytometry analysis of indicated surface markers. Dot plots are representative of three mice per condition. Experiment has been repeated once with similar results.
We extended our *ex vivo* culture to 7 days, and these results are reported in figure 4-3. After 7 days of *ex vivo* culture there is more than a 2.5 fold difference in total numbers of viable cells, as determined by hemacytometer counts of trypan negative cells, comparing monomer and dimer treated cultures to vehicle treated cultures. There was no statistically significant difference (by students t test, confidence level of 0.05) between monomer and dimer treated wells. Assaying these splenocytes for surface expression of CD19 and CD3, IAP inhibitor treatment showed a relative increase in frequency of B cells compared to vehicle treatment with a concurrent decrease in frequency of T cells (bottom).
Figure 4-3. IAP inhibitor treatment leads to more viable splenocytes cultured *ex vivo* for 7 days compared to vehicle (top) as determined by hemacytometer counts of trypan negative cells and the majority of these cells are B cells (bottom) as determined by flow cytometry analysis of indicated surface markers. Results are representative of three independent experiments.
In order to better understand these effects in our *ex vivo* system, we conducted time course experiments, following the persistence of cells sourced from individual mice at both day 4 and day 7. These results are presented in figure 4-4. As can be seen clearly in this experiment, most cells die over the course of culture which is not surprising given they have been removed from their physiological context. In the case of IAP inhibitor treatment, less cells die compared to vehicle treatment (figure 4-4, top), indicating that IAP antagonism increases survival of hematopoetic cells. When analyzed by flow cytometry for CD3 and CD19 surface expression, B cells preferentially survive in these cultures upon IAP inhibitor treatment. At the day 4 timepoint, at least in the case of monomer treatment, more T cells are also present compared to dimer and vehicle treated cells.
Figure 4-4. IAP antagonism leads to persistence of B cells over a 7 day timecourse of ex vivo cultured splenocytes, total viable cells determined by hemacytometer counts of trypan negative cells (top) and B and T cell subsets (middle and bottom) calculated from frequencies determined by flow cytometry analysis of appropriate surface markers. Error bars represent the standard
(Figure 4-4 cont’d.) error of the mean calculated from 3 mice per condition. Experiment has been repeated once with similar results.

Although most cells in the cultures were dying over the time course, we wanted to know if IAP antagonism was leading to less cell death in the B cell population, or if it was leading to increased proliferation. We stained splenocytes first with CFSE and cultured ex vivo for 7 days. We analyzed CFSE dilution by flow cytometry, and these results are presented in figure 4-5. Within the CD19+ gate, most cells are still CFSE high suggesting there is no proliferation engendered by IAP inhibitor treatment in this subset, nor does treatment enhance homeostatic proliferation as a result of these cells being removed from their physiologic context (vehicle treated cells exhibit higher frequency in the CFSE diluted gate). While this result argues against IAP antagonism resulting in proliferation of bulk CD19+ cells, it does not rule out the possibility that a rare subset of B cells is responding to IAP antagonism by proliferating.
Figure 4-5. IAP antagonism does not lead to increased B cell proliferation in splenocytes cultured ex vivo for 7 days as determined by CFSE dilution and analysis by flow cytometry. Experiment has been repeated once with similar results.
Given that our splenocyte cultures include a range of freshly explanted hematopoetic lineages at least at the outset of ex vivo culture, we wondered whether the increased persistence of B cells was a result of IAP antagonism in the B cells themselves, was the result of IAP antagonism in a bystander cell, or was a combination of both. In order to investigate the first possibility we sorted CD19+ cells from B6 spleens and cultured these purified cells ex vivo in the presence of vehicle, monomer or dimer inhibitor. The results for total viable cell counts by hemacytometer are given in figure 4-6 and show that IAP antagonism in B cells is sufficient to result in their persistence, but this does not rule out additional effects through a bystander cell in the splenocyte cultures. We further characterized these purified B cell cultures by flow cytometry analysis of surface markers for major splenic B cell subsets as shown in figure 4-7. Marginal zone (MZ) B cells are described by high expression of CD21 (also known as complement receptor 2) and no expression of CD23 (a c-type lectin, also known as the low affinity receptor for IgE, or FcεRII), while follicular (FO) B cells are described by intermediate expression of CD21 and high expression of CD23. The double negative population includes recent bone marrow emigrants and immature B cells. Similar to our timecourse results in whole splenocytes, most cells in these purified cultures were dying, again likely due to the extra-physiological conditions.
Figure 4-6. IAP antagonism also leads to increased persistence in purified B cells cultured ex vivo for 7 days as determined by hemacytometer counts of trypan negative cells. Results are representative of three independent experiments.
Figure 4-7. Follicular, but not marginal zone B cells are present at much higher frequency in purified B cells treated with IAP inhibitors, compared to those treated with vehicle after 7 days of ex vivo culture, as determined by flow cytometry analysis. Results are representative of three independent experiments.
It was likely, therefore, that IAP antagonism led, either to the persistence of follicular B cells, or the conversion of another subset to a follicular phenotype. The latter possibility suggests that IAP antagonism could effect the lineage differentiation of B lymphocytes, in addition to their survival. In order to investigate this, we isolated immature versus mature B cells according to both CD93 and IgD expression, exposed these cells to IAP inhibitors, or vehicle and analyzed the subsets. In these experiments (see appendix I), whether B cells were immature, or mature did not effect the resultant difference in follicular B cells, suggesting that it was the persistence of, rather than conversion to the follicular subset that IAP antagonism impacted. Alternatively, the artificiality inherent in our ex vivo system precluded the ability to investigate true differentiation under these conditions. When we returned to our purified B cell cultures, however, we observed a dramatic increase in the rare population of pre-plasmablasts, characterized by CD138+ MHCII high surface expression, as shown in figure 4-8. These data did suggest IAP antagonism could impact differentiation in addition to survival, as plasma cells represent a distinct transcriptional and functional fate for an activated B cell.
Figure 4-8. Purified B cells cultured *ex vivo* for 7 days show a dramatic increase in MHCII high, CD138+ cells when treated with IAP inhibitor compared to vehicle. Results are representative of three independent experiments.
Given the potential for IAP antagonism to effect activation, we endeavored to understand the effect of IAP antagonism in B cells under stimulatory conditions. In initial experiments, we stimulated B cells three different ways: recombinant CD40L (CD40), anti-IgM (BCR), and CpG (TLR9). In looking at total viable cells (figure 9), both IAP inhibitor monomer and dimer lead to a 2.5 fold increase in cells at 7 days of stimulation, while CD40L and anti-IgM stimulation were largely unaffected by presence of inhibitor. In order to simulate more physiological stimulation conditions, we combined B cell agonists and assayed both CFSE dilution and total cell counts at 7 days ex vivo culture, and these results are shown in figure 4-10.
Figure 4-9. IAP antagonism leads to increased total B cells 7 days post stimulation in the context of CpG stimulation, but not in the context of CD40, or BCR stimulation as determined by hemacytometer counts of trypan excluded cells. Results are representative of at least three independent experiments.
Figure 4-10. IAP antagonism increases \textit{ex vivo} stimulation of B cells particularly in conditions mimicking a T cell independent response as determined by total cell counts 7 days post stimulation (A) and by CFSE dilution (B). Results are representative of at least two independent experiments.
As can be seen in the figure, both conditions containing CpG exhibited robust proliferation as indicated by CFSE dilution where distinct peaks represent specific rounds of proliferation. While it seemed adding CD40L obscured the costimulatory enhancement of IAP antagonism and combined with the single-agonist experiment, it was unclear whether anti-IgM stimulation was providing robust stimulation in our system. Both of these initial B cell stimulation experiments, however, revealed a potential difference with previous work investigating CD40 signaling which will be addressed in the discussion. We next assayed CpG only stimulation after 4 days of ex vivo culture to determine unambiguously the potential of IAP antagonism to co-stimulate a T cell independent B cell activation. As can be seen in figure 4-11, both IAP inhibitor monomer and dimer enhanced CFSE dilution in CpG stimulated B cells, while having no effect on unstimulated cells.

Figure 4-11 (next page). IAP antagonism costimulates CpG stimulated CD19+ purified splenocytes as determined by CFSE dilution at day 4 (A) while having no effect on unstimulated B cells (B). Combined results of three independent experiments are shown for absolute percent of CFSE diluted gate (C, left) and percentage increase over unstimulated controls (C, right).
Since IAP inhibitor treatment, surprisingly, did not impact CD40L stimulation in our hands, and in an effort to examine more physiological conditions, we moved to an ex vivo coculture system where germinal center B and T follicular helper cells are isolated from an OVA vaccinated mice at the peak of T follicular helper cell abundance and restimulated in vitro with anti-CD3 and anti-IgM. In this system we expect a more physiological CD40 signal provided by cell-cell contact between the relevant helper T cell and B cell. This helper cell will provide any additional signals that may have been missing in our recombinant CD40L stimulation experiments. Interestingly, these conditions revealed, as seen in figure 4-12, the ability of IAP inhibitor monomer, but not dimer to enhance isotype class switching in germinal center B cells. As can be seen in the figure, when germinal center B cells are cocultured with non-follicular helper CD4+ cells, very little expression of IgG1 is seen in the CD19+ gate after 5 days of ex vivo stimulation. However, with the relevant T follicular helper cell added to culture, up to 13% of CD19+ cells express IgG1, and this is further enhanced with monomer treatment.
Figure 4-12. IAP inhibitor monomer, but not dimer costimulates T cell dependent antibody class switching as determined in ex vivo co-culture of T follicular helper cells and germinal center B cells isolated from a germinal center reaction and restimulated for 5 days with anti-IgM and anti-CD3. Representative dot plots (top) for IgG1 versus GL7 staining and quantified (bottom, left) for CD19+ cells and Ki67 expression quantified for CD4+ cells (bottom, right). Results are representative of three independent experiments. Experiments conducted by P. Sage.
It was interesting to see that IAP inhibitor dimer did not costimulate this reaction, given our results in our T-cell independent conditions and intrinsic effect of both monomer and dimer in B cell survival and differentiation. When the CD4+ gate was analyzed, it was revealed that dimer treated cells exhibited Ki67 expression similar to vehicle treatment while monomer CD4+ cells exhibited increased Ki67 expression, indicating more proliferation in these cultures. We investigated, therefore, ex vivo stimulated T cells to see if dimer and monomer treatment could costimulate a T cell reaction. Previous work in our lab had revealed the ability of monomer to costimulate T cells under these conditions, and this comparison is presented in figure 4-13. The well-known ability of freshly stimulated naive T cells to upregulate CD25, or interleukin-2 receptor alpha-chain, and subsequently downregulate CD62L, or L-selectin, follows the physiological activation of these naive T cells, where autocrine IL-2 is used as an important growth and proliferative factor and the downregulation of the cellular adhesion molecule indicates their ability to partition out of secondary lymphoid tissue and into the periphery.
Figure 4-13. IAP inhibitor monomer, but not dimer co-stimulates ex vivo T cell stimulation by anti-CD3/28 as determined by flow cytometry analysis of CD25 and CD62L expression. Representative dot plots for unstimulated and anti-CD3/28 conditions given (top) and quantified absolute increases in total CD25+ gate (bottom, left) and in CD25+ CD62L- gate (bottom, right) across three independent experiments.
Stimulating these cells concurrently with monomer treatment, but not dimer treatment, increased the total fraction of CD25+ cells and the absolute increase of the CD62L-, CD25+ fraction (over non-stimulated, but same treatment wells). This indicated the ability of monomer, but not dimer to co-stimulate these T cells. Furthermore, when supernatants were analyzed by ELISA for IL-2 and the effector cytokine IFN-gamma, as shown in figure 4-14, both monomer and dimer led to an increase in secretion of IL-2. Only monomer, however, led to increased secretion of IFN-gamma.
Figure 4-14. IAP antagonism leads to increased IL-2 secretion (left) in T cells stimulated ex vivo with anti-CD3/28 at 48 hours, whereas only IAP inhibitor monomer enhances IFN-gamma (right) secretion as determined by sandwich ELISA, combined results of three independent experiments.
Finally, in our ex vivo system, we assayed for TRAF3 expression as this is key second messenger molecule in coordinating alternative NFκB and canonical NFκB in lymphocytes through mechanisms that are under active investigation. As can be seen in figure 4-15, TRAF3 is upregulated in monomer treated and stimulated T cells compared to dimer treatment, while no change in TRAF3 expression is evident in unstimulated but IAP inhibitor treated conditions. Furthermore, the opposite trend is observed (dimer leads to higher expression than in monomer treated cells) in ex vivo cultured splenocytes. This expression follows the pattern of IAP antagonism leading to increased survival with both monomer and dimer treatment, but only monomer being able to costimulate a T cell response. It argues for a key role for TRAF3 in mediating a T cell costimulatory signal through alternative NFκB.
Figure 4-15. Ex vivo stimulated, monomer treated T cells show increased expression of TRAF3 when compared to dimer treated, or vehicle treated T cells at 24 hours, while unstimulated T cells show similar TRAF3 expression in all conditions as determined by western blot. In contrast TRAF3 in whole splenocyte culture shows increased TRAF3 expression with dimer treatment. All cytosolic lysate fractions were normalized by BCA prior to loading.
When we moved to our scaffold delivery system (discussed in chapter 5) and we discovered that scaffolds loaded with monomer showed an increased infiltration of B cells and, importantly, follicular dendritic cells (FDCs) as determined by flow cytometry analysis in time course experiments (reported in chapter 5), we conducted an exploratory RNAseq experiment on total cellular infiltration at day 3 and day 13 post implantation. Lymphotoxin beta (LTB) and lymphotoxin beta receptor (LTBR), molecules known to be important in lymphorganogenesis, were significantly enriched transcripts compared to those in blank scaffold infiltrates. This indicated a potential positive feedback in the alternative NFkB signalling pathway, as it is known IAP antagonism can stabilize NIK upon degradation of cIAP1 and cIAP2, which are targets of our inhibitors. The colocalization of B cells and FDCs in monomer loaded scaffolds suggests the formation of a tertiary lymphoid structure. It is known that lymphoid tissue inducer cells (LTi, also known as innate-like lymphocyte type 3, ILC3) mediate the early stages of lymphoid tissue structure formation, through IL-17 and IL-22 secretion. We returned to our ex-vivo splenocyte culture to look for these cells.
Figure 4-16. Gating strategy for day 3 ex vivo cultured splenocytes to analyze LTi subset, which fall in the mononuclear gate on FSC and SSC, are low for live/dead viability stain, double negative for CD3, B220 and CD11b, but can express CD11c. Data is representative of two independent experiments.
The gating strategy is given in figure 4-16, where these cells are mononuclear by forward and side scatter, lineage negative (CD3-, B220-), CD11b negative, but can express CD11c+. As shown in the figure, these cells represent less than 1% of splenocytes. We assayed their total fraction, as a percentage of the parent gate under vehicle, monomer or dimer treatment, and these data are shown in figure 4-17. As can be seen, monomer treated cells show almost double the frequency found in vehicle treated wells, while dimer treatment is similar to vehicle treatment. This concludes the summary of our ex vivo results.
Figure 4-17. IAP inhibitor monomer enriches day 3 ex vivo splenocytes for LTi subset, while dimer is no different from vehicle treatment. Representative dot plots for IL7R versus CD4 for lineage-gated cells are shown (top) and frequencies are quantified (bottom) for 3 mice. Results are representative of two independent experiments.
Discussion

The results from the various ex vivo systems described above reveal an interesting and complex role of IAP antagonism in immune cells, particularly those of the adaptive arm of immunity. In erythrocyte-depleted splenocyte culture, IAP antagonism led to less cell death over time. This result was surprising because IAP antagonism is generally recognized to result in more apoptosis, through either autocrine secretion of TNF[103], or through the TNF-independent formation of a ripoptosome[104]. As previously mentioned TNF can be an activating cytokine in hematopoietic lineages (compared to a pro-death signal in other lineages), and this increased survival likely represents differential activity based on cell context. Importantly, several groups have shown the ability of IAP antagonism to activate alternative NFkB through NIK stabilization, and NFkB activation can lead to expression of pro-survival genes. Beyond the expression of these genes, it is also appreciated that alternative NFkB is a common signaling pathway initiated by TNFRSF signaling, and these pathways operate in costimulation and differentiation of lymphocytes (see chapter I).

When we analyzed immune subsets, we found preferential survival of B cells while T cell numbers were no different when compared to vehicle treatment by 7 days of ex vivo culture. We further showed in purified B cell culture that the increased survival is at least partially due to direct activity on B cells, although an additional effect through a bystander cell is possible in the splenocyte system. Given the intense study conducted on the BAFF-R pathway in B cells, the first explanation that presented itself was that IAP antagonism, through alternative NFkB signaling, is mimicking a BAFF-R survival signal. We are likely seeing increased survival, as CFSE dilution experiments showed IAP antagonism did not result in de novo proliferation. However, in addition
to increased persistence of total B cells, we observed differences in subset composition. There is a dramatic enrichment in the follicular B cell subset upon monomer and dimer treatment at 7 days ex vivo culture.

Our experiments comparing immature to mature B cells suggested that IAP antagonism is having a direct effect on treated cells, rather than effecting the differentiation of FO B cells (see appendix I). In addition, the ability of BAFF-R to regulate CD21 and CD23 independent of its peripheral survival function in B cells has been reported[105], despite the fact initial studies of the BAFF-R knockout mouse reported a lack of mature B cells. When we analyzed purified B cells for other differentiation markers, however, IAP antagonism dramatically enriched pre-plasmablast cells, an otherwise rare subset in spleen, and therefore IAP antagonism could impact differentiation under non-stimulatory and stimulatory conditions.

Under stimulatory conditions, we saw a meaningful costimulatory effect of T cell independent B cell stimulation (CpG), with enhanced CFSE dilution in the context of IAP inhibitors. Interestingly, when we stimulated B cells with recombinant CD40L, while we did not see an increase in cell numbers with IAP inhibitors compared to vehicle treatment, we also did not see an inhibition of stimulation. The latter case was predicted based on published results using IAP inhibitors in the context of CD40 signaling, where it was shown cIAP1 and cIAP2 were necessary for transmission of a CD40 signal[106]. However, in those studies, cells were pre-treated with IAP inhibitors for 4 hours, whereas in our study we delivered stimulatory agonist and inhibitors at the same time, at the onset of ex vivo culture. Pretreatment of IAP inhibitors will lead to stabilization of NIK, and possibly to an accumulation of TRAF3 as well that could later inhibit the propagation of the stimulatory signal. In our experiments, it is likely the alternative
NFkB activation is further enhancing the stimulatory signal with concurrent treatment of inhibitor and agonist.

Interestingly, in these experiments, there were no meaningful differences revealed between monomer and dimer treatment. While both inhibitors were added to culture at the same molar concentration, the dimer compound has twice the binding and inhibitory sites as the monomer compound. In our T cell dependent B cell reaction, however, we observed the ability of monomer, but not dimer to enhance isotype class switching to IgG1. Furthermore, proliferation in the CD4+ cells was enhanced in monomer, but not dimer treated cultures. Our ex vivo T cell stimulation experiments followed this pattern, where monomer, but not dimer was able to increase the CD25+ CD62L- fraction of cells in culture compared to vehicle treatment, the surface expression of which represents activated naïve T cells. While treatment of both compounds led to increased presence of IL-2 in culture, only monomer compound enhanced levels of IFN-gamma. This data taken together suggests that IAP monomer, but not dimer can costimulate T cell activation, which had been shown with monomer compound previously[75].

Regarding the activity on target proteins, it has been reported that dimer, but not monomer is able to degrade XIAP, the only IAP family member to be directly involved in inhibiting caspases[107], indicating that XIAP may play a role in T cell activation. The requirement for caspase 8 in T cell activation is well known[108-110], and the transcription of caspase 3 in T cell activation has been revealed[111], although its function in this context is currently under investigation. More relevantly perhaps, it has been shown that XIAP can bind caspase 3 fragments and prevent their full cleavage to their effector form in activated T cells, albeit this has only been
shown in human cells, not yet in mouse[112]. This suggests that XIAP could be playing a pro-
activation role in our system, and this role is abrogated by dimer inhibitor, but not monomer.

The final difference observed between monomer and dimer treatment in our ex vivo systems was the ability of monomer, but not dimer to enrich for LTi cells. Under the current model, these cells are known to be responsive to LT and begin the initial stages of secondary lymphoid tissue development[113]. There are also known to participate in homeostatic, suppressive as well as inflammatory roles in the gut[114, 115]. They are related to Th17 cells in that they share a similar “master” transcription factor, RORγt[116], but being innate immune cells do not express antigen-specific activating receptors. Since we see enrichment of these cells in our monomer, but not dimer treated culture, it may also suggest an unappreciated role for XIAP in the lineage differentiation of this subset, or it may be a difference yet to be discovered. It is of interest to determine if IAP antagonism can effect the functionality of these cells in addition to their enrichment in ex vivo culture.

**Conclusion**

In our ex vivo experiments, we uncovered the novel ability of IAP inhibitors to increase survival of B cells and enhance B cell proliferation in a T cell independent reaction. Furthermore, both compounds lead to the persistence of the follicular B cell subset, in contrast to no effect in the marginal zone compartment. In addition, IAP antagonism was able to enrich for cells of a pre-plasmablast phenotype. IAP inhibitor monomer, but not dimer, was able to enhance a T cell dependent reaction and lead to more isotype class switching ex vivo. It was possible this was due to differences we uncovered in T cell costimulation experiments, where monomer, but not dimer
was able to enhance CD25+ and CD25+ CD62L- subset frequencies. While both compounds increased IL-2 production in stimulated T cell cultures, only monomer enhanced IFN-gamma production, an important effector cytokine. Finally, we saw enrichment of LTi cells with monomer, but not dimer treatment in ex vivo cultured splenocytes. These results are important as dimer compounds are being pursued as next generation cancer therapies. Monomer inhibitors, however, may prove to be important immunotherapies given in concert with conventional chemotherapy and radiation, or in concert with primary immunotherapies, at least based on these ex vivo results.

The results described above opened two interesting paths to pursue in vivo. In the next chapter, I will describe experiments that used our bioabsorbable scaffold system to treat tumors with peritumor delivery of compound and, in a second effort, our ability to enhance an in vivo antibody response.
Chapter 5. Modulation of an in-vivo immune response with local delivery of IAP inhibitors

Introduction

Given our ex vivo results in B and T cells we pursued local delivery of monomer and dimer in peritumor therapeutic vaccination, both in WT and RAG deficient mice. Along with these studies, I will describe results showing local delivery of inhibitor monomer in a naïve subcutaneous pocket, analyzing both lymphocyte and stromal subsets in cellular infiltrates over a time course. We also pursued vaccination with B16 lysate in the context of monomer to assay an in vivo antibody response. We further expanded these antibody response experiments, loading OVA protein in scaffolds, revealing interesting effects of IAP antagonism in vivo.

Results

Having established the costimulatory ability of our IAP inhibitor in our ex vivo system, we were interested in improving our anti-tumor immune response in our B16 model by delivering our IAP inhibitors in a time dependent fashion in the peritumor space. We selected a scaffold delivery system that had shown meaningful efficacy in delivery GM-CSF containing vaccine in the pre-clinical B16 model and was being pursued for a first-in-humans trial. These scaffolds are composed of poly-lactide-co-glycolide (PLG) polymer that has been manufactured to accept both hydrophobic and hydrophillic agents. We first optimized the manufacture in our laboratory (discussed in chapter 3), and then confirmed the time-dependent release of our monomer
compound (figure 5-1) as it was the compound we could load into the material at the highest concentration due to solubility limits of the dimer compound.
Figure 5-1. IAP inhibitor monomer loaded into the organic phase releases over time as measured by mass spectrometry of supernatants of in vitro incubated scaffolds. Concentration calculated by reference to a prepared standard curve on the same instrument. Signal is over 2 orders of magnitude greater than noise and signal from blank scaffolds incubated under the same conditions.
Scaffolds were placed in PBS containing BSA carrier and incubated at 37 C for 72 hours. At each 12 hour time point, supernatant was collected and fresh PBS plus carrier was added to wells. Supernatants were analyzed by LC/MS, and total amount of analyte was calculated by reference to a standard curve analyzed on the same instrument under the same conditions. Blank scaffolds were used as controls, showed response indistinguishable from instrument noise, exhibiting a response at the expected ion peak over 2 orders of magnitude less than monomer loaded scaffolds. As can be seen in figure 5-1, monomer releases from scaffolds within 72 hours to an accumulated total between 3-4 mg/scaffold, which represents 50-60% of the total added to scaffolds at the outset of manufacture (6 mg/scaffold). It is also evident in the figure that some scaffold to scaffold variability exists as these three scaffolds belonged to the same batch of manufacture.

Once we confirmed that compound released from scaffolds in a time-dependent fashion, we implanted scaffolds in the flanks of naive B16 mice and analyzed total cellular infiltrate after 9 days of implantation, by counting Trypan blue negative cells with a hemacytometer. As can be seen in figure 5-2, scaffolds loaded with monomer in the organic phase, but not the aqueous phase showed total cellular infiltrate similar to GM-CSF loaded scaffolds, used as a positive control. Neither dimer incorporated into the organic, or aqueous phase resulted in robust infiltration compared to blank scaffolds. It is important to note, that in these experiments, scaffolds were loaded with the maximum amount of compound for each condition, given solubility limits of the compounds in the various phases. Therefore, we are not comparing equal molar amounts of each compound in each phase. Given this data, we selected monomer loaded scaffolds to treat growing B16 tumors in a peritumor implantation site. It is important to note
that, while we are not adding anything to the scaffolds in addition to compound to modulate immunity, we are generating a surgical wound through the implantation process.
Figure 5-2. IAP inhibitor monomer as loaded into the organic phase results in total cellular infiltrates similar to that found in GM-CSF loaded scaffolds after 9 days of implantation as determined by hemacytometer counts of trypan negative cells, while other loading conditions and dimer compound do not. Scaffolds were loaded to the maximum allowed given the solubility of the two compounds in the two phases. Experiment was repeated at least once with similar results.
Since it was apparent that local delivery of IAP inhibitor could have novel immuno-stimulatory effects (to include the potential of costimulating a tumor-specific adaptive response, given our ex vivo data), we implanted blank, monomer (loaded into the organic phase), or dimer (loaded as admixed powder) scaffolds directly adjacent to growing B16 tumor, 6 days post challenge. At this time point, tumors have emerged and are generally a few millimeters in diameter. It was important that tumors were large enough that the implantation procedure itself would not grossly disrupt tumor growth, but not so large as to make overcoming tumor burden and potentially engendering regression unattainable. As can be seen in figure 5-3, mice treated with monomer as delivered by a peritumor scaffold implant exhibited slower tumor growth and significant reduction in tumor size by day 6 post implantation, while dimer treated and blank scaffold treated mice showed similar tumor sizes.
Figure 5-3. B16 tumors grow slower in animals receiving peritumor implants of monomer, but not dimer loaded scaffolds which exhibit growth no different from blank scaffold implanted mice. Tumor growth is shown over time (A) and all replicates across at least three independent experiments shown (B, left and right). No treatment group removed in B, right side for clarity.
Figure 5-4. B16 tumor bearing C57BL/6J mice treated peritumorally with monomer scaffolds as loaded into the organic phase, but not dimer as admixed powder survive long term. Additional controls include blank scaffold treated and non-treated mice as indicated. Data is the combination of two independent experiments.
We conducted long-term survival experiments, and the combined results are shown in figure 5-4. As can be seen, monomer treated mice (by peritumor scaffold implant) show long term survival of 20%, whereas other conditions show no survival. This result was interesting as previous activity of monomer in our B16 model was only seen in the context of GVAX immunotherapy, where inhibitor was given systemically. In systemic treatment, IAP inhibitor monomer alone had no effect on tumor growth, or on survival of mice. This indicated that perhaps monomer was able to reactivate lymphocytes located in the peritumor margin but otherwise suppressed by the tumor microenvironment, currently an intense field of investigation. We isolated cellular infiltrates in peritumor implanted scaffolds and stained for lymphocyte markers. Interestingly, as shown in figure 5-5, with gating strategy, monomer loaded scaffolds showed increased frequency of CD8 T cells. This is quantified across all mouse replicates in figure 5-6. While CD4+ fraction and absolute number did not differ significantly between blank and monomer loaded scaffolds, both frequency and absolute number of CD8+ T cells was greater in monomer loaded scaffolds compared to blank.
Figure 5-5. Peritumor implanted scaffolds loaded with monomer in the organic phase show enrichment over blank scaffolds of CD8+ T cells, as determined by flow cytometry analysis of scaffold cellular infiltrates. Gating strategy is shown at top, while representative CD4 versus CD8 staining is shown at bottom.
Figure 5-6. Quantification of T cell subsets in peritumor scaffold infiltrates. PLG scaffolds loaded with monomer and implanted peritumorally to growing B16 tumor (day 3 post challenge) exhibit a higher percentage and higher absolute number of CD8+ T cells, compared to blank scaffolds implanted in the same location. All scaffolds were explanted when tumors reached 17-20 mm in diameter and cellular infiltrates were analyzed by flow cytometry.
This suggested the efficacy of monomer in our system was dependent on T cell activity. In addition, given that dimer did not show survival, and an important difference revealed in our ex vivo studies indicated that dimer could not costimulate a T cell response, it remained possible that the anti-tumor activity seen with monomer treatment was dependent on T cells. We, therefore, investigated tumor protection in RAG deficient mice, who lack mature lymphocytes. This result is shown in figure 5-7. In this experiment, and in an effort to increase therapeutic activity, as well as to enable direct comparison with dimer admixed in powder, we admixed monomer at the same molar concentration and treated mice as before. Interestingly, monomer treated mice, whether they were RAG deficient, or wild type showed increased survival compared to dimer loaded, or blank scaffold treated mice. Mice treated with admixed monomer had increased toxicity, as three mice in the cohort died shortly after implantation with small tumor burden. This potentially reveals a toxicity in our model that is dependent on the presence of lymphocytes. However, given the robust survival of RAG KO mice, it is possible that T cell activity is not crucial for the anti-tumor activity in our system. Another possibility remains that for long term protection the T cells are necessary in our model, and the surviving mice will have to be monitored for continuation of tumor growth.
Figure 5-7. RAG deficient mice show long-term survival similar to WT C57BL/6J mice when treated peritumorally with monomer scaffolds, loaded as admixed powder, while dimer-containing and blank scaffolds impart no long-term survival. Data represents one preliminary experiment.
We designed a series of timecourse experiments, where naive mice were implanted with blank, or monomer (in the organic phase) loaded scaffolds. At days 3, 13, 22 and 30 post implantation, scaffolds were removed and digested. Resultant single cell suspensions were stained for a panel of hematopoietic markers and analyzed by flow cytometry. Interestingly, we observed an enrichment of B cells at day 13 post implantation. This results are given in figure 5-8.
Figure 5-8. Scaffolds loaded with IAP inhibitor monomer show an enrichment in B cells over time compared to blank scaffold controls when implanted subcutaneously in wild-type B6 mice. Data is combination of at least three independent experiments.
We followed up this observation with staining of stromal cells, and this data is presented in figure 5-9. Interestingly, in comparison to naive lymph nodes, scaffolds exhibited a significantly higher fraction of CD45- cells (1% versus 60%), and this was commensurate with what was observed in our initial panel, where a majority of cells were negative for all our hematopoietic markers, especially at later time points. Also, scaffolds included the presence of blood endothelial cells (CD31+, PDPN-), something than had been observed by macroscopic observation during explant, as well as fibroblastic reticular cells (CD31-, PDPN+), considered to be important for certain lymph node functions. Scaffolds did not show evidence of lymphatic endothelial cells (LECs, CD31+, PDPN+). There were no differences observed in these subsets comparing blank scaffolds to monomer loaded scaffolds.
Figure 5-9. PLG scaffolds exhibit FRCs and BECs, but not LECs compared to naive lymphnode stromal cell populations, and presence of monomer compound in the organic phase is no different from blank scaffolds as determined by flow cytometry analysis of scaffold infiltrates after 13 days of subcutaneous implantation.
When we looked at CD45- CD35+ subsets, however, a dramatic enrichment of these cells was seen at 13 days post implant, consistent with surface staining of follicular dendritic cells (FDCs), as shown in figure 5-10. As both FDCs and B cells were enriched in monomer loaded scaffolds at this time point, it suggested the formation of a B cell follicle. We performed RNAseq analysis on day 3 and day 13 infiltrates as an exploratory experiment. Interesting, in addition to B cell markers and CXCL13 being upregulated in monomer loaded scaffolds at day 13, confirming our flow analysis, we saw fold increase of LTB and LTBR, molecules known to be important in the formation of lymph node structure. Based on this finding, we looked for LTi cells in our ex vivo system as discussed in chapter 4.
Figure 5-10. PLG scaffolds loaded with monomer IAP inhibitor showed a dramatic increase in FDCs when compared to blank scaffolds implanted subcutaneously in the flanks of B6 mice and explanted after 13 days. Represented dot plots (top) shown for n=5 mice per experiment. Percentage of gated cells (bottom left) and absolute number of gated cells (bottom right) are shown. Scaffold infiltrate was analyzed by flow cytometry and hemacytometer counts of trypan negative cells. Results are representative of three independent experiments.
Given the ability of monomer to costimulate B cells and effect their differentiation in our ex vivo system, we endeavored to explore antibody response in vivo. In our first set of experiments, we loaded scaffolds with B16 cell lysate in addition to CpG, CpG with monomer or with GM-CSF. Twenty-one days after implantation sera was harvest from mice and analyzed by direct ELISA with B16 lysate as target. Figure 5-11 shows these results. While both monomer loaded conditions showed an apparent, although not statistically significant increase in total anti-B16 IgG titer (as measured by optical density of HRP conjugated secondary antibody), the titer was not to the level induced by conventional GVAX vaccine. In addition, the background in this system (with whole cell lysate as target) was significant.
Figure 5-11. Scaffold vaccination with B16 lysate and adjuvant as indicated shows an apparent increase over response of naive sera, but not as great as that provided by GVAX vaccination. Sera was harvested at day 21 post vaccination and analyzed by direct ELISA using B16 lysate as target for total IgG in sera. Sera samples were diluted in dilution buffer at a ratio of 1:50.
In order to better address the specific activity of IAP antagonism, we moved to a defined antigen system, loading ovalbumin (OVA) in our scaffolds. Scaffolds were loaded with OVA in addition to adjuvant as indicated in figure 5-12. As expected CpG loaded scaffolds resulted in robust anti-OVA antibody after 21 days implantation. However, when IAP inhibitor was added to these scaffold, the antibody response was significantly ablated. In contrast, monomer inhibitor in combination with OVA resulted in a response similar to OVA alone (which was significantly above response of naive sera). This indicated that in the presence of CpG, IAP monomer was reducing total IgG, but did not effect antibody response in the absence of CpG.

We boosted these mice with the same scaffold they received for the priming vaccination. Interestingly, as can be seen in the figure, all groups increased in antibody titer, particularly those conditions containing monomer. This can be seen in the apparent greater increase in both monomer conditions.
Figure 5-12. Antibody response from OVA containing scaffold vaccination. C57BL/6J mice were vaccinated and boosted with scaffolds loaded as indicated to determine sera IgG antibody response to OVA antigen by direct ELISA. Sera was collected at day 21 post-priming and 21 days post boost (or 42 days post priming) and all samples were analyzed on the same ELISA plate. Sera was diluted at a ratio of 1:50 in dilution buffer (PBS with 0.05% tween).
We wondered whether IAP monomer could have a specific activity during the boost phase that might be confounded by its presence during the priming vaccination. Accordingly, we designed an experiment where all mice received OVA only scaffolds during the priming vaccination. For the boost vaccination, one group received OVA only, one group received OVA plus monomer in the organic phase and the final group received no boost vaccination. The IgG antibody response as measured by direct ELISA with OVA protein target is given in figure 5-13. Interestingly, when monomer compound was only present in the boost vaccination mice had increased titers of anti-IgG1, but not anti-IgG3 serum antibody against OVA. This in vivo result is in accordance with the enhanced isotype class switching we observed in our ex vivo germinal center reaction, thus showing that IAP antagonism can costimulate a B cell response.
Figure 5-13. IAP monomer enhances OVA antibody response when delivered in boost vaccine.

C57BL/6J mice were primed with OVA loaded scaffolds and boosted with OVA loaded scaffolds, or OVA loaded scaffolds containing monomer IAP inhibitor, or left without boost at 21 days after initial implantation. Sera was harvested prior to boost and 21 days after boost and assayed by direct ELISA for anti-OVA IgG as indicated. Results are representative of two independent experiments.
Discussion

Our initial \textit{in vivo} result showed that IAP monomer loaded scaffolds exhibited increased cellular infiltration over blank scaffolds after 9 days of implantation within a subcutaneous pocket, and this infiltration was similar to that found in GM-CSF loaded scaffolds the increased infiltration of which had been reported previously [117]. Implanting these scaffolds in a peritumor location showed a dramatic reduction in tumor growth for monomer scaffold, but not dimer scaffold treated mice. Given that a major difference revealed in our \textit{ex vivo} systems was that monomer, but not dimer could costimulate a T cell response, this suggested a T cell dependent anti-tumor effect. In addition, we observed increased CD8+ T cells in scaffolds implanted in a peritumor location, suggesting again our anti-tumor effect might be dependent on T cells. When we treated RAG deficient mice, lacking recombination sufficient lymphocytes, however, monomer scaffold treated mice exhibited increased survival as did wildtype mice when compared to blank scaffold treated controls. It is important to note that, in order to achieve the same amount of compound per scaffold, dimer (as its solubility in the emulsion organic phase was significantly less) was admixed with PLG microspheres prior to foaming. This incorporation method would be expected to result in similar, albeit slightly accelerated release. In the experiment treating RAG deficient mice with scaffold, we admixed both monomer and dimer to enable a direct comparison, even though this represented a different incorporation method from our initial peritumor delivery experiments. Nevertheless, while there may be a partial T cell dependent anti-tumor effect, the reduction in tumor growth and increased survival might be due to alternative mechanisms which differ between monomer and dimer treatment, at least at the concentrations tested.
An interesting observation in the experiment with RAG deficient mice was that wildtype mice treated with monomer resulted in toxicity, where 3 mice died with little, or regressed tumor burden, and this was not observed in RAG deficient mice. This indicated a vigorous lymphocyte-dependent reaction that was happening upon monomer treatment, and it suggests further optimization might be necessary in this peritumor system to reveal the full immune characteristics. One possibility would be decreasing the amount of compound to lower the toxicity and improve the therapeutic window. It might even be possible to reveal a T cell dependent effect once the amount of compound is titrated down. If this is the case, it would indicate that at the higher effective dose in the “burst” release of powder incorporated drug, an innate mechanism might initially be limiting tumor growth. It has been reported that IAP inhibitor treatment (in this case, dimer, but not monomer) can lead to NLRP3 inflammasome activation and increased secretion of IL-1 in phagocytic cells. In our laboratory, we revealed homotypic aggregation (typically an indication of activation) of human dendritic cells when treated with IAP inhibitors, or with TLR agonist, and this effect was produced by both monomer and dimer compounds. Since these studies (those in our laboratory and those reported in literature) use compounds from different companies, it is possible some of the reported differences are a result of different chemistries specific to the various sources. The anti-tumor effect underlying our observations, however, might be more complicated still.

In our expanded, time course experiments, it was revealed that some differences existed in total numbers (while percentage of viable cells did not differ) of myeloid subsets in monomer loaded scaffolds compared to blank scaffolds. In particular, CD11c- CD11b+ cells (a phenotype consistent with macrophages) were slightly increased at day 3, and CD11c+ CD11b+ cells
(inflammatory monocytes) were present in greater numbers at days 13 and 22 post implantation. And this might be a manifestation of the effects of IAP antagonism on innate subsets as described in a previous chapter. At this time, since our infiltration experiments were conducted with monomer and blank scaffolds, we do not have the ability for a direct comparison to infiltration in dimer loaded scaffolds. More dramatically still, though, we observed an enrichment of B cells after 13 days of implantation, comparing monomer loaded (in the organic phase) with blank scaffolds. Further experiments revealed an enrichment of FDCs at this timepoint as well (while other stromal sets were no different between monomer loaded scaffolds and blank). The presence of these two cell subsets in the subcutaneous pocket suggested the presence of a B cell follicle, a type of tertiary lymphoid structure. While we have not, as yet, revealed the three dimensional association of these cells, FDCs are exceedingly rare at this anatomical location. Considering our ex vivo results, the ability of monomer to enrich for LTi cells, but not dimer suggests the potential to form such structures. Since we see a majority of compound has been released from scaffolds by day 3 in our in vitro release assay, it seems likely inhibitor is having an effect at an early timepoint which leads to the evolution of the later enrichment of B cells and FDCs. Furthermore, RNAseq analysis of total cellular infiltrates at day 3 and day 13 revealed enrichment (again comparing monomer loaded scaffolds to blank scaffolds) of LTB and LTBR.

LTBR-/ mice have no lymph nodes, colon-associated lymphoid tissue, or Peyer’s patches while thymus tissue is unaffected [118]. LTB-/- and LTa-/- mice have similar phenotypes[59, 119], showing that the LTB/LTBR pathway is critical in secondary lymphoid tissue development. Importantly, these mice also show defects in presence of FDCs and in B cell affinity maturation. In addition, the alymphoplastic (aly/aly) mouse has been identified as harboring a point mutation
in NIK that inactivates it and leads to the lack of development of lymph nodes[120] and splenic marginal zone[121]. Since we know that LTB and LTBR are responsible for lymphoid tissue development and that they signal through alternative NFkB, our infiltration analysis, including RNAseq and flow cytometry data argue for a local impact of IAP inhibitors in addition to what is occurring as a result of implantation alone. Ectopic lymph node tissue has been generated in pancreas by engineered expression of LTa and LTb by islet cells[122]. Our local delivery of a small molecule which mimics this pathway adds new possibilities in treatment for human disease.

Due to the costimulatory effects we observed with monomer in our ex vivo assays, we conducted a series of antibody response experiments. In our first experiment, we included relevant antigens of interest, incorporating B16 lysate into scaffolds. Vaccination with monomer plus CpG revealed no difference in anti-B16 IgG after 21 days. However, this direct ELISA exhibited a high level of background in part due to the heterogeneous nature of antigens present in the tumor lysate. We moved to a defined antigen system to reduce the amount of noise. Interestingly, we observed an ablation of anti-OVA antibody response when monomer was delivered in the context of CpG adjuvant and antigen, but not when administered in addition to antigen. We boosted these mice with the same scaffold with which they were primed, and noticed that in both conditions (one with CpG and one without) that contained monomer, the change in antibody from priming to boost was higher than in the other conditions. When we only included monomer compound in our boost vaccine, we revealed a difference in anti-OVA IgG1, but not IgG3 isotype in the presence of monomer. This result follows nicely from our ex vivo system, where cells were re-stimulated as well. Why would monomer compound only have an effect during the boost reaction? Given that we observed conversion to a pre-plasmablast
phenotype in our *ex vivo* B cell stimulations, it might be that presence of monomer has a more potent effect during the affinity maturation, once a broad pool of antigen reactive cells have been evolved by a priming reaction. Upon re-stimulation more antibody reactive cells are present. We plan to conduct an affinity maturation experiment to see if, in addition to increasing the titer of antibody, IAP antagonism can impact the affinity of the resulting antibodies. It is intriguing to speculate whether, IAP antagonism would allow the survival of B cell clones that would otherwise die through a lack of positive selection, a direct effect on B cells, or if this effect is mediated through an ectopic tertiary structure.

**Conclusion**

Our *in vivo* results expand upon our *ex vivo* results in surprising ways. While we have evidence an *in vivo* isotype class switching can be enhanced by local delivery of IAP inhibitor in conjunction with antigen, it was only revealed in the boost vaccine, rather than in the primary vaccination. If the adjuvant effect of IAP antagonism depends on the presence of a substantial compartment of antigen experienced lymphocytes, then an enhanced antibody response might still be possible in a patient, as numerous tumor-antigen experienced lymphocytes would likely be present. Furthermore, in local peritumor delivery we see meaningful reduction in tumor growth with monomer, but not dimer treatment. However, our experiment using RAG deficient mice did not reveal the requirement of T cells in our anti-tumor effect. Nevertheless it seems evident that IAP inhibitors can behave as a novel adjuvant compound. Both monomer and dimer versions can co-stimulate a T cell independent antibody response, while monomer compound is able to enrich for lymphoid tissue inducer cells, an important precursor to secondary lymphoid tissue formation. In addition, in naïve mice, local delivery of monomer led to the enriched local
infiltration of B cells and FDCs, further indicating the potential to evolve higher order immune interactions. Using a compound that can specifically impact an important late stage differentiation transcriptional pathway in lymphocytes and marrying it with a local delivery vehicle where the persistence of drug can be controlled opens the way for a new generation of immunomodulatory vaccines. Much attention is focused currently on the immune contexture of cancer patients. The ability of small molecules to generate, or influence ectopic lymphoid structures is of great relevance not just in cancer immunotherapy, but in related fields such as autoimmunity.
References


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Figure A.1. Immature, or mature B cells isolated from spleen survive to the same extent in ex vivo culture when treated with IAP inhibitors. Data representative of two independent experiments, error bars indicate standard deviation among three mice. Cells were isolated from spleens of wildtype B6 mice and sorted according to the gates indicated in the top panel and then cultured ex vivo as indicated for 7 days.
Figure A-2. Draining lymph node cellularity from OVA vaccinated mice are similar in pattern to anti-OVA IgG titer as determined at 21 days post vaccination. Error bars represent standard deviation among three mice.
Figure A-3. Confirmation of the absence of B and T cells in RAG deficient mice. Data are representative of multiple mice in two independent experiments. Splenoctyes were isolated and stained for CD3 and CD19 and analyzed by flow cytometry.
Figure A-4. Surface pores are reduced in size as sucrose porogen size is reduced as indicated.

Scaffolds were manufactured according to standard procedure including sucrose at the different sizes; otherwise the scaffold manufacture was the same. Completed scaffolds were sputter coated with an Au/Pt source and imaged by SEM. Images are representative of 3 scaffolds per group in at least two independent experiments.
Figure A-5. Crystal structures for BIR3 domain of cIAP1 bound to monomer inhibitor[123] (left) and dimer inhibitor[124] (right) as determined by X-ray diffraction to a 1.79 and 2.60 angstrom resolution respectively. Dimer compound is shown to bind multiple BIR3 domains intramolecularly, thereby inhibiting XIAP binding caspases 3, 7 and 8. Images were downloaded from RCSB Protein Data Bank (www.rcsb.org) on 5 May 2015.


Figure A-6. Proposed model for role of cIAPs in regulating canonical NFkB. Under binding of receptor to ligand RIPK1 is brought into proximity of other receptor components to include cIAPs bound to TRAF2. cIAP serve as E3 ubiquitin ligase for degradative ubiquitination of RIPK1 inhibiting the formation of the death complex including caspase 8. Under conditions where cIAPs are pharmacologically inhibited, or degraded complex II can form. However, in immune cells TNFR1 signaling is biased towards activation and NFkB is active as a survival pathway.
Figure A-7. Proposed model for regulation of alternative NFkB signaling by cIAPs. Under steady state conditions, NIK protein is constitutively degraded by cIAPs complexed to TRAF proteins. Upon receptor ligation (TNFRII TRAF-binding molecules), this complex is recruited to the receptor and undergoes autoubiquitination and degradation. NIK is subsequently stabilized, builds up in the cytosol to a threshold and activates alternative NFkB. Pharmacologic inhibition, or degradation of cIAPs mimics this receptor event in that NIK is stabilized.
Appendix B: Experimental Materials and Methods

Tumor challenge with peritumor delivery of scaffold vaccine. Wildtype C57BL/6J, or RAG KO (B6.129S7-Rag1<sup>tm1Mom</sup>/J) female mice, 5-8 weeks in age, were purchased from Jackson Laboratory (Bar Harbor, ME) and used for all experiments as indicated. Tumor cells (B16-F10) were prepared in HBSS at a concentration of 3-5 million cells/mL, and 100 μL was injected into the left flank of animals, yielding a total dose of 300,000 to 500,000 tumor cells per challenge. Injections were conducted while mice were under anesthesia to limit animal movements and ensure reproducibility of injections. Scaffolds were manufactured as described and implanted directly underneath a growing tumor at day 3, or day 6 post tumor challenge as indicated, or tumors were allowed to grow in untreated conditions. All animal experiments were conducted under an IRB approved protocol.

PLG scaffold manufacture. Scaffolds were manufactured by preparing PLG microspheres containing 10% aqueous phase by the solvent evaporation method. Microspheres were lyophilized and combined with porogen at a 1:9 (microsphere:porogen) ratio by weight, and powder was mixed for 1 minute in a test tube with a stainless steel spatula to ensure homogenous distribution of components. Mixtures of microspheres were loaded into a stainless steel die shaped like a disk, 8 mm in diameter and 2 mm in height, and compressed in a hydraulic press for 1 minute at a pressure of 1500 psi. Compressed scaffolds were loaded into a pressure chamber that was subsequently charged with 800 psi carbon dioxide and allowed to equilibrate for 18 hours. Carbon dioxide was then released from the chamber until equilibration with atmosphere was achieved and the rate of release was controlled so that this process completed within 1 to 1.5 minutes. Scaffolds were leached for 12 hours in ddH₂O at a volume of 10 mL/scaffold. Scaffolds were washed in 70% ethanol and then twice with PBS prior to implantation in animals.

Surgical implantation and scaffold digestion. Mice were anesthetized with isofluorane. A surgical wound was created, centered on the back, and a pocket was tunneled under a tumor
(peritumor implantation), or in the subcutaneous tissue of the flank (naïve mouse implantation). A scaffold was implanted and the surgical wound was sealed by staple. Analgesic was administered directly following implantation and at 12 hours post implantation. Blank scaffolds were utilized in all implantation experiments to analyze the effects of implantation and of the material delivery system in isolation. For digestion, collagenase type IV (Worthington Chemical, NJ), or a mixture of β collagenase, δ collagenase, dispase II, and DNAse I (Roche) were used in PBS with 2% FCS was added to minced scaffolds explanted from mice. These scaffold pieces were incubated for approximately 45 minutes with increasingly frequent refreshing of the digestion media (15, 10, 10, 5, 2 minutes). Cells and supernatant were removed at these times and quenched in RPMI media with 10% FCS. Single cell suspensions were washed filtered and analyzed by flow cytometry on a Fortessa (BD Biosciences) using the following clones as indicated: B220: RA3-6B2, CD4: GK1.5, CD11c: N418, CD8a: 53-6.7, CD11b: M1/70, NK1.1: PK136 (BD Biosciences, or BioLegend). All animal experiments were conducted under an IRB approved protocol.

**Ex vivo T cell stimulation.** Spleens were harvested from wildtype, female C57BL/6J mice (stock number 664, Jackson Laboratories, Bar Harbor, ME) 5-8 weeks in age and mechanically disrupted using a 70 um mesh strainer. Erythrocytes were lysed in ACK lysis buffer and quenched in RPMI with 10% FCS. Cells were magnetically selected through positive selection of CD3-bead labeled cells (130-094-973, Miltenyi Biotec, Gladbach, Germany), and plated on 96-well round bottom plates precoated with anti-CD3 and anti-CD28 (100 uL per well, 10 ug/mL aCD3, 2 ug/mL aCD28, overnight at 4 °C) at a concentration of 250,000 cells per well in 100 uL RPMI with 10% FCS. IAP inhibitor monomer, dimer or DMSO vehicle was added at 200 nM at the outset of culture. Cells were harvested at 48, or 72 hours as indicated and analyzed by flow cytometry following standard protocol using anti-CD25 and anti-CD62L (BD Biosciences, clone PC61 and MEL-14 respectively) antibodies. All experiments included 3 mice per condition plated in three wells per condition per mouse.
**Ex vivo splenocyte culture.** Spleens were harvested from wildtype, female C57BL/6J mice (stock number 664, Jackson Laboratories, Bar Harbor, ME) 5-8 weeks in age and mechanically disrupted using a 70 um mesh strainer. Erythrocytes were lysed in ACK lysis buffer and quenched in RPMI with 10% FCS. Remaining splenocytes were plated in 96 well round bottom plates at a concentration of 1 million cells/well and a volume of 200 uL media. Cells were treated with IAP inhibitor monomer, or dimer at a concentration of 200 nM, or DMSO vehicle at the same dilution. At indicated time points, a small amount of cells was stained with Trypan blue and counted via hemacytometer, while the majority of cells were prepared for flow cytometry analysis. CD19 and CD3 antibodies were purchased from BD Biosciences (clones 1D3 and 17A2 respectively) and used in conjunction with Fc block for flow cytometry analysis. All experiments included 3 mice per condition plated in three wells per condition per mouse.

**Ex vivo B cell stimulation.** Spleens were harvested from wildtype, female C57BL/6J mice (stock number 664, Jackson Laboratories, Bar Harbor, ME) 5-8 weeks in age and mechanically disrupted using a 70 um mesh strainer. Erythrocytes were lysed in ACK lysis buffer and quenched in RPMI with 10% FCS. Cell suspensions were stained with anti-CD19 antibody (BD Biosciences, clone 1D3) and sorted gating on lymphocytes by FSC and SSC and CD19+ cells on a FACS Aria (BD Biosciences). Sorted cells were stained with CFSE (Life Technologies, C34554) at a 1:2000 dilution from manufacturer’s stock concentration. Stained and washed cells were plated at a concentration of 400,000 cells/well in a volume of 200 uL in 96-well round bottom plates in phenol red free RPMI with 10% FCS added. Monomer and dimer IAP inhibitor were added at 400 nM, while DMSO vehicle was added a same dilution from stock. In stimulation conditions, CpG (Invivogen, tlr1-1826) was added at 1 uM.

**Ex vivo germinal center re-stimulations.** C57BL6/J mice were vaccinated with OVA/CFA emulsion in both flanks. After 7-8 days, single cell suspensions were prepared from both draining lymph nodes and sorted for GL7+ CD19+ cells (GC B cell) and CD4+ ICOS+ CXCR5+ GITR- (Tfh cell) cells on an Aria sorter (BD Biosciences) and plated in 96-well round bottom plates, at the following concentrations: 30,000 Tfh plus 50,000 CD19+ in 200 uL volume. Cells were re-stimulated with
anti-CD3 (2 ug/mL) and anti-IgM (5 ug/mL) antibody. IAP inhibitors were added at 200 nM and DMSO vehicle was diluted to a similar amount. After 5 days, cells were analyzed by indicated markers for proliferation and IgG isotype class switching by flow cytometry. All animal experiments were conducted under an IRB approved protocol. Experiments conducted by Peter Sage.

**TRAF3 western blot.** Splenocytes, or T cells were isolated and plated as described above. Lysate was prepared at 18 hours post initiation of culture. Membrane, cytosolic, and nuclear fractions were prepared according to manufacturer’s instructions by cell fractionation kit (9038, Cell Signaling Technologies). Lysate was reduced with DTT in loading buffer at 90 °C and loaded into a premade 10% Bis-Tris gel (NuPage, Life Technology) and ran at 100 V constant voltage for 1 hour. Gel was transferred overnight to a PDMS membrane (GE Healthcare) at 15 V constant voltage at 4 °C. Blot was blocked with 5% milk in TBST and then incubated overnight with Rabbit anti-mouse TRAF3 (4729, Cell Signaling Technology) at manufacturer’s recommended dilution at 4 °C. Blot was washed 3X in TBST for 5 minutes per wash on a nutating mixer. Blots were developed with TMB substrate (Pierce) and imaged on a LAS-4000 (GE Healthcare) using auto-exposure settings.

**OVA and B16 antibody response.** Scaffolds were manufactured as described with the addition of OVA being loaded into the aqueous phase at a final concentration of 50 ug/scaffold, IAP inhibitor monomer loaded into the organic phase at a final concentration of 6 mg/scaffold, CpG condensate (CpG:PEI precipitate) added into the aqueous phase at a concentration of 20 ug/scaffold, or GM-CSF added into the aqueous phase at a final concentration of 3 ug/scaffold, as indicated in the individual results, and implanted into the flank of mice. After 21 days, blood was harvested by submandibular bleed, and sera was prepared by allowing the blood to clot for 3-4 hours at room temperature, after which time samples were spun at 5,000 rpm (7.5cm diameter rotor) two times in succession. Supernatant was collected and after the final spin stored. For mice receiving a booster vaccination, scaffolds were prepared as indicated and
implanted into the opposite flank. Another 21 days were allowed to lapse, and sera was collected as before. All animal experiments were conducted under an IRB approved protocol.

**Direct ELISA for OVA, or B16 lysate target.** B16 tumor lysate was prepared by explanted tumors grown to 1-1.5 cm in diameter, which were then mechanically disrupted, filtered through 70 um mesh filters and incubated in PBS with collagenase type IV (255 units/mL) added for 45 minutes at 37 °C. Single cell suspensions were freeze/thawed for a total of 4 times by alternately immersing suspension in liquid nitrogen for 1 minute, or until entire volume was frozen solid and then for 5-8 minutes in a 37 °C bath until it was completely thawed. Lysate was added to the aqueous phase during microsphere manufacture and fabricated into scaffolds as indicated. For ELISA, plates (NUNC Immunon) were coated with OVA (or B16 lysate) at 2 ug/mL in PBS, 100 uL per well (Invivogen) which were incubated overnight at 4 °C. Plates were washed 3 times for all washes with PBS with 0.05% tween added. Blocking was done with 25 ng/uL BSA added to PBST for one hour at room temperature. Sera was added at 1:50-1:400 dilution in PBS with 0.05% tween added. Secondary antibody Rabbit anti-mouse IgG1/G3-HRP was added at a dilution of 1:2000 (Southern Biotech) and incubated for 1 hour. Plates were developed with TMB substrate for 15-30 minutes, arrested with 0.2 M HCl and analyzed on a SpectraMax plate reader (Molecular Devices) for absorbance at 450 nm.
Collaborations

1) Reduced anti-tumor lysate responses in GVAX treated LysM-Cre; PPAR-g fl mice" unpublished data (James Akin, Girija Goyal, Glenn Dranoff). I performed numerous B16 ELISAs to measure antibody response in WT mice receiving GVAX immunotherapy compared to conditional PPARg KO mice in support of a study elucidating the function of this transcription factor in myeloid cells participating in anti-tumor immunity, work led by Girija Goyal.

2) I trained Karrie Wong and Joanne Lim and assisted on the manufacture and implantation of PLG scaffolds and in the design of experiments in support of their projects investigating the immune-modulatory effects of JAK2 and mTOR inhibitors and in the anti-PRGN response, respectively, in GVAX immunotherapy.

3) I conducted hMDDC isolation and ex vivo culture in support of Matthias Piesche in his investigations of L1-CAM and its role in anti-tumor immunity. In particular, I conducted stimulations of hMDDC with TLR ligands in the presence or absence of L1-CAM recombinant protein and measured IL-6 in culture post stimulation.

4) I performed manufacture and implantation of PGRN containing scaffolds for vaccination in B6 wild type mice in support of work by Yuko Nakazaki investigating the role of PGRN antibody response in GVAX immunotherapy, particular in experimental human patients.

5) Work contained in this dissertation was selected for a talk entitled, “Local, time-dependent delivery of IAP inhibitor as a novel costimulatory adjuvant,” at the 2014 New England Immunology Conference at Woods Hole, MA, and as a result I received the AAI
Young Investigator of the Year Award. This was a fantastic experience, and I am grateful to Thorsten Mempel and Mark Exley for organizing such an outstanding conference.