B CELL CARRIER FOR VIRAL DELIVERY

THE UTILIZATION OF ACTIVATED B CELL AS CELL CARRIER FOR VIRAL VECTORED ANTIGEN DELIVERY IN THE ACCELERATION OF CD8 T CELL RECALL RESPONSE

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Abstract

Cancer vaccine therapy aims at harnessing effective antigen specific immune responses to treat tumor. In particular, CD8+ T cells have the unique capacity to recognize and destroy tumor cell throughout the body. One potential approach to elicit high numbers of effector CD8+ T cells to control tumor growth is through repeated vaccination, a strategy called prime-boost vaccination. However, booster immunization is relatively inefficient during primary immune response because pre-activated effector T cells tend to impair robust antigen presentation. This phenomenon has been interpreted as a negative feedback mechanism where recently activated CD8+ T cells clear the antigen-bearing dendritic cells (DCs) and prevent memory T cells from the access of the boosting antigen. Interestingly, however, using in vitro activated B cell as a viral vector delivery system, we can boost T cell responses with the minimum viral input at a very short interval between immunizations. This B cell carrier is capable of delivering different viral vectors expressing different antigens, displaying a potential for broad application. The mechanisms behind B cell carrier-mediated efficient secondary responses are three fold: 1. Without the engagement of MHC molecules and antigen presentation, B cell carrying viral vector can bypass the killing by pre-existing effector T cells 2. B cells can delivery viruses to B cell follicles, a place separated from effector T cells, and mediate memory T cell expansion. 3. B cells can deliver antigen to both spleen and lymph node and induce antigen specific T cell expansion in multiple lymphoid organs. Our studies provide a novel boosting platform to accelerate CTL responses that has

important clinical implications.

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ABBREVIATIONS:

Ad: adenoviruses
ACM: Adipocytes conditional medium
ACT: Adoptive t cell transfer
APC: Antigen presenting cells
APC: allophycocyanin
BSA: bovine serum albumin
CFSE: 5 and 6- carboxyfluorescein succinimidyl ester
CLL: Chronic lymphocytic leukemia
CTL: Cytotoxic T lymphocyte
DC: Dendritic cell
DT: Diphtheria Toxin
FBS: fetal bovine serum
FITC: fluorescein isothiocyanate
GFP: green fluorescent protein
GM- CSF: granulocyte-macrophage colony–stimulating factor
IL: Interleukin
IFN: Interferon
im: Intramuscular
ip: Intraperitoneal
iv: Intravenous
LCMV: lymphocytic choriomeningitis virus
MOI: multiplicity of infection
NK: nature killer
PBS: phosphate buffered saline
PE: phycoerythrin
PE-CY5: PE conjugated with cyanine dye Cy5
PE-CY7: PE conjugated with cyanine dye Cy7
PerCP: peridinin chlorophyll protein complex
PE-Texas Red: PE conjugated with Texas Red
PFA: paraformaldehyde
PFU: plaque forming unit
RPMI: Roswell Park Memorial Institute
SA: streptavidin
TAA: Tumor associated antigens
TCR: T cell receptor
TNF: Tumor necrosis factor
Treg: Regulatory T cell
VSV: Vesicular stomatitis virus
Vac: Vaccinia virus

Introduction

With the discovery of tumor associated antigens (TAA), cancer immunotherapy has undergone significant development in the past 20-30 years. Although it has been shown that immunization against TAAs can induce CD8+ CTL responses and anti-tumor immunity in animal models, evidence of comparable outcomes in humans has been sparse. A major constraint on the success of cancer vaccines is the requirement for rapid generation of high numbers of effector and memory CD8+ T cells to control tumor growth. One potential approach to circumvent this problem is through repeated vaccination (conventionally known as *prime-boost immunization*). However, booster immunization is relatively inefficient during the primary immune response because pre-activated effector T cells tend to impair robust antigen presentation (Ludewig,2001, Guarda,2007, Medema,2001). This phenomenon has been interpreted as a negative feedback mechanism where recently-activated cytotoxic T lymphocytes (CTL) clear the antigen-bearing antigen presenting cell typically dendritic cells (DCs) and prevent naïve or memory T cells from the access the boosting antigen (Ronchese and Hermans,2001, Wong and Pamer,2003). Indeed, we and others provide evidence that antigen-bearing DCs are susceptible to CTL killing, and repeatedly-administered DC vaccines fail to engage naïve or memory T cells in the presence of vigorous CTL response (Yang,2006, Luketic,2007). Thus, the identification of boosting strategies that can bypass CTL-mediated negative feedback regulation is desirable.

Interestingly, the CTL-mediated barrier can be overcome with a sufficiently high dose of antigen (Wong,2004) but it is not clear whether this approach simply loads more DCs with antigen than the CTL can clear or whether the high dose actually delivers antigen to an APC. We have also recently reported that intravenous vaccination with recombinant vesicular stomatitis virus (rVSV) could provoke massive secondary expansion at the height of the primary $CD8^+$ T cell response when very few memory T cells are detectable (Bridle B, unpublished data). Our further studies indicate that rVSV-mediated boosting requires B cells but not DCs (Bridle B, unpublished data) suggesting that B cells may represent an ideal system for booster immunization to rapidly amplify the magnitude of effector and memory T cell responses and anti-tumor immunity. Understanding the mechanisms by which B cells mediate antigen presentation to facilitate rapid boosting will provide important information that can be used to design optimal vaccination strategies for rapid generation of protective responses.

Additionally, we have developed a novel culture method using conditioned media from adipocytes, which allow us to generate large numbers of activated B cells from normal donors (Tomic,2006). This will provide an additional advantage over DCs to meet certain therapeutic schedules where multiple vaccinations are required.

1. Specific Background

1.1 The development of cancer immunotherapy

Cancer, which consists of a large, heterogeneous class of diseases with great mortality, is a significant world health concern. According to the statistics provided by Canadian cancer society, as of 2007, cancer has surpassed cardiovascular disease (heart and cerebrovascular) as the leading cause of death in Canada. An estimated 177,800 new cases of cancer and 75,000 deaths will occur in Canada in 2011. Thus, novel, effective cancer treatment strategies are urgently needed.

Different approaches have been developed to fight cancer, among which, cancer immunotherapy represents a relative newly developed area. Cancer immunotherapy aims to utilize a patient's own immune system to generate anti-tumor immune responses to eliminate primary lesions and metastases. It has been extensively investigated in the past few decades with many accomplishments that can be categorized broadly into two groups: active immunotherapy and passive immunotherapy (Dillman,2011, Aldrich,2010). Active immunotherapy aims at priming immune responses against cancer and includes strategies such as peptide-based vaccines, DNA vaccines or viral vector based vaccines (Guo and Wang,2009). Passive immunotherapy represents a different approach to manipulate components of the host's immune system to target cancer. Antibodies or other immune system components that are made *ex vivo* (i.e. in the laboratory) and administered back to patients to provide immunity against cancer. These include the

adoptive T cell transfer (ACT) strategies (Aldrich,2010), in which clonally expanded tumor-specific T cells are generated and administered to back to patients. Besides ACT, therapeutic antibody treatment including monoclonal antibodies (MAbs) and immunoglobulin (Ig)-fusion proteins also holds great potential for effectively treating cancer (Chan and Carter,2010). In summary, during the past 25 years, 17 immunologic products have received regulatory approval based on anticancer activity as single agents and/or in combination with chemotherapy (Dillman,2011). These include the nonspecific inflammatory stimulants BCG and levamisole; the cytokines interferon-α and interleukin-2; the monoclonal antibody rituximab, and until very recently, the FDA proved the first DC based vaccine by Dendreon Corporation for prostate cancer (Kantoff,2010)

1.2 The advantages of cancer immunotherapy

Physicians and clinical scientists have developed different strategies for the treatment of cancer, including surgical removal, radiotherapy, chemotherapy or combinations of these approaches. However, it is hard to maintain a complete elimination of tumor, and its metastases, moreover, these approaches are associated with dangerous side effects (Hanahan and Weinberg,2011). As cancer is a chronic progressing disease and often comes with metastasis, if metastasis cannot be controlled, cancer will not be cured. Compared to these traditional therapies, cancer immunotherapy represents a powerful therapeutic tool. The reason why traditional treatments fail to kill metastasic tumors is because these treatments cannot generate

"tumor specific memory" which will provide long-term tumor protection, while cancer immunotherapies can. Immunotherapy generates mostly two types of immune memory: one is B cell memory which is basically based on neutralizing antibodies against pathogens, toxin or other "foreign invaders"(Amanna and Slifka,2011). The other type of immune memory involves T cell memory, which relies on the quick differentiation of memory T cells upon secondary encounter with the same antigen to produce effector T cells that will quickly clear the antigen. And different immunotherapeutic strategies can be combined to achieve a synergistic effect or to overcome the suppressive environment created by tumor cells.

1.3 $CD8^+$ T cells in anti-tumor immunity

$CD8^+$ T cell represents a critical part in adaptive immunity and has been well demonstrated can provide protection against intracellular pathogen, virus infection and cancer development. In cancer, activated $CD8^+$ T cells can directly recognize TAAs presented by the major histocompatibility complex (MHC) class I molecules on tumor cells and initiate cytotoxic effects through perforin, granzyme B, IFN-γ and production of other cytokines. Thus, inducing large number of tumor specific $CD8^+$ T cells holds great promise in cancer immunotherapy because of their potent ability to specifically target tumor cells. In the field of cancer immunotherapy, it has become more and more urgent to develop effective therapeutic model which mainly rely on the long lived T cell memory. So we are going to talk about memory T cell differentiation and function in anti-tumor immunity in the next few paragraphs.

1.3.1 Memory T cell generation

Memory T cells are derived from naïve T cell precursors through the signals received by T cell receptor (TCR) when they encounter self or foreign antigens presented by antigen presenting cells (APC) in draining lymph nodes of infection or disease (Obar and Lefrancois,2010, Sallusto,2010). Currently, there is still a debate going on regarding memory T cell differentiation. One model suggests, after being stimulated by APCs along with other co-stimulatory signals, naïve T cells differentiate into effector T cells within 7-9 days. Effector T cells gain the ability to migrate to peripheral tissues, secrete pro-inflammatory cytokines and lyse infected target cells. Following clearance of infected or damaged target cells, 90-95 percent of effector T cells will undergo apoptosis, leaving a small but stable population of antigen-specific memory T cells (Obar and Lefrancois,2010, Daniels and Teixeiro,2010). Another appealing model suggests, depending on the longevity of antigen MHC complex stimulation, sustained stimulatory signals drive cells into a short-lived effector pool, whereas more transient but sufficient signals favor generation of memory cells(Lanzavecchia and Sallusto,2002, Intlekofer,2006, Kaech,2002).

1.3.2 The advantages of memory cell in generating effective cellular immunity

The advantages of targeting memory T cell in mediated cellular immunity can be concluded as the following four aspects: better quality, better quantity, long live memory and expanded anatomical locations"(Zaiss,2010, DiSpirito and Shen,2010). Despite the diversity of memory T cell differentiation, it is widely accepted that an

effective immunization will exert high frequency of effector T cells and may also lead to a large number of memory T cells (Murali-Krishna,1998). As memory T cells are all antigen experienced, compared to naive T cell repertoire, they have a higher ratio of antigen specific T cell, and these memory T cells also have a much lower threshold for activation compared to naïve T cells, they can quickly respond to a small amount of antigen and undergo extensive proliferation to repopulate the effector T cell pool. Moreover, it has been shown that $CD8^+$ T cell memory differentiation does not require the continuous presence of antigen, which means practically, the first input of antigen stimulation by primary vaccination is sufficient to initiate the process of the memory differentiation (Jameson and Masopust,2009). This quality provides another advantage of $CD8^+$ memory T cell mediated immunity: long term protection. (Gerlach,2011). Another distinctive quality of CD8 memory T cell is that they tend to home to secondary lymphoid organs as well as circulating through the peripheral tissues, (Daniels and Teixeiro,2010) and therefore potentially increase the chance of encountering antigen and mediating protection.

1.3.3 How to induce ideal cellular immune responses

In the past decades, different groups have made great effort to improve cancer immunotherapy, and there are several key aspects to consider in designing a good cancer vaccine inducing $CD8^+$ T mediated immunity(Hoft,2011). First, choose the right antigen and vaccine vector for CD8 T cell response and memory generation. With the identification of tumor-associated antigens (TAA), allows for a targeted

immunotherapy, and it is critical to choose the right antigen: self-antigen or foreign antigen, single epitope or multiple epitopes to generate the right type of responses. Also with the accumulated understanding of different vaccine vectors, it is also of great importance to choose the right vector and the right administration route(Bachmann and Jennings,2010). For example, different routes (intramuscular, oral, intranasal, intrarectal) of adenoviral-vectored vaccine delivery can result in different phenotype and trafficking of vaccine-induced CD8+ T cells. (Rollier,2011)Secondly, it is important to determine the subset of CD4+ T cell to target which will assist in tumor clearance (Zhang,2009), along with assisting in generating effective $CD8^+$ cytotoxic T cell (CTL) responses and memory T cell formation(Janssen,2003), and thirdly choosing an effective, and relevant adjuvant that can help antigens uptaken by APCs or in other ways that can help to maximize the vaccine efficacy. In addition, effective strategies should aim at reducing tumor-induced immunosuppression, by reducing the numbers of regulatory T cell (Treg) and other suppressive cells which can hinder cancer vaccine efficacy, more combined immunotherapy strategies have been designed with this purpose. Moreover, with the development of modern molecular technology, the delivery system, route and schedule of the vaccine should be optimized to improve both efficacy and safety (Bachmann and Jennings,2010, Bolhassani,2011). I will discuss some of these questions related to our work in the next few paragraphs.

1.4 Dendritic cell-based cancer vaccines

1.4.1 The development of DC based vaccines

DC based vaccines have been investigated for more than 30 years with great success in both animal models and human clinical trials with the first clinical result published in 1995(Van Tendeloo,2001). Past and ongoing clinical trials include using autologous DC loaded with tumor cell lysate, idiotype antibodies, antigenic peptides, DNA or total RNA extracted from tumor cells, or tumor cell–DC fusions in patients with melanoma, renal cell neoplasms, prostate cancer, B-cell lymphoma, and many other malignancies.(Gilboa,2007, Palucka,2011)

DCs are a heterogeneous population bridging innate and adaptive immunity. DCs are known as professional APCs, and play pivotal roles in T cell and B cell responses against pathogens and viral infection. The two major DC subsets are the myeloid DCs (mDCs) and the plasmacytoid DCs (pDCs). pDCs are considered the front line in anti-viral immunity owing to their capacity to rapidly produce high amounts of type I interferon in response to viruses (Van Tendeloo,2001) There are also other DC subsets with distinctive functions, such as CD8αDC, which are responsible cross priming of viral antigens(den Haan,2000).

As discussed above, CTL-mediated immunity has been widely studied to generate anti-tumor activity. As professional APCs, currently DC based vaccines are usually designed for T cell activation, to generate tumor specific T cell responses. The major functions of DCs in T cell priming include: efficient capture of antigens in peripheral tissues and transport antigens from the periphery to secondary lymphoid

organs. Their high levels of MHC-Ⅰ and MHC-Ⅱ molecules, along with co-stimulatory molecules (e.g., CD40, CD80, CD83, CD86) enable them to efficiently present antigens to, and prime T cells. In addition, DCs can synthesize and secret immune-stimulatory cytokines, such as IL-12 and IFN-α, which are also required to enhance antigen presentation and T cell priming(Palucka,2011, Kalinski,2009).

E*x vivo* expansion of DCs has been established to develop DC based cancer vaccine (Baar,1999). Basically, it is normally a 1-2 week process: CD34+ precursors cells are purified from blood and differentiated in the serum- free medium in the presence of granulocyte-macrophage colony–stimulating factor (GM-CSF) and interleukin-4, and subsequently matured with a combination of pro-inflammatory cytokines (Palucka,2011). However, the protocol for DC preparation from human is not consistent between different groups, especially the cytokines they used to mature DC. There is also no consensus on the route of DC delivery (Rolinski and Hus,2010). These technical issues remain a problem for pushing DC vaccines for further clinical translation.

How to load antigens onto DCs is another critical question in designing DC-based cancer vaccines.(Gilboa,2007). Considerations include the form and amount of antigen being loaded, the persistence of antigen presentation, and the efficacy of the antigen loading techniques. Among those questions, the primary concern is which forms of antigen-loading that can facilitate activation of $CD8^+$ or $CD4^+$ T cell responses. Exogenous peptides corresponding to the epitopes presented

by MHC-I or MHC-II molecules are the most favorable candidates for DC antigen loading. However, since peptide affinity varies by HLA, the identification of individual peptides for each HLA is logistically challenging. Alternatively, whole protein antigens have also been used by some groups to allow for organic antigen processing; these have shown promising results in eliciting T cell response. Other antigen forms such as nucleic acid transfer have also been reported to be effective for antigen loading. Our group has previously published a paper demonstrating that recombinant VSV expressing tumor associate antigen can be used to infect DC as a novel way of antigen loading onto DCs. (Boudreau,2009, Boudreau,2011) Using VSV-infected DC as a therapeutic vaccine not only attained the expected effect of stimulating $CD8^+$ T cell responses, more importantly, it also activated a DC- nature kill cell (NK) signaling pathway to gain a greater therapeutic effect than normal DC vaccines by activating innate immunity.

1.4.2 The limitations of DC vaccine in immunotherapy

However, there are also questions to be considered before the broad application of DC vaccines; one of the major obstacles is that it is always hard to generate enough DCs for treatment, and in order to reach good efficacy, the current treatment regimens often include several repeated injections of DC vaccines (Gilboa,2007, Baar,1999). Thus, DC treatments become a very expensive therapeutic strategy, which greatly hinders the application of DC-based cancer immunotherapy. Therefore, there is a need to find other candidates, such as B cells, to make this APC-based cancer

immunotherapy cost effective.

Another limitation for DC based vaccines is that the results in clinical trials do not mirror the efficacy observed in animal models. In a review by Engell-Noerregaard and his colleagues(Engell-Noerregaard,2009), only 57 of 626 melanoma patients (9%) showed objective responses (ORs) (20 complete response and 37 partial response) when treated with the DC-based vaccinations, but no significant correlations were noted between those ORs and the tested parameters. Tumor cells can create a suppressive microenvironment for cytotoxic immune responses, thereby escaping from the immune surveillance which normally eliminates nascent tumors. Tumor cells can also down-regulate their expression of MHC molecules, induce T cell anergy and secret suppressive cytokines, such as IL-10, VEGF and TGF-β which will impair DCs and T cell functions and limit the efficacy of DC-based vaccines. (Van Tendeloo,2001).

1. 4. 3 Future directions of DC based vaccines.

Because of the noticed insufficience of DC based vaccines in cancer immunotherapy, different approaches have been considered to further the increase the efficacy of DC based vaccines. For instance: DC can be genetically modified to promote cytokine production (*eg*. IL-12, TNF-α, type I interferon), or up-regulate co-stimulatory molecule expression to reach higher anti-tumor CTL responses (*eg*. CD86, CD83) or modify DC vaccine to stimulate other immune components such as NK cells (Boudreau,2011). In addition, the combination of DC vaccination with other

treatments such as GM-CSF cytokine treatment anti-CTLA4 antibody treatment or Treg depletion among others(Adema,2009), may help in promoting more successful outcomes for DC- or other cell-based vaccines.

1. 5 Use of B cells as an alternative platform for cell-based vaccines

As we have discussed above, there are practical difficulties for making DC based vaccines an affordable cancer treatment approach worldwide. B cells are another kind of APCs which can be easily isolated from peripheral blood at much higher numbers and therefore have come into view as a very attractive alternative to DCs.

B lymphocytes, as an important component in immune defense network, have a predominant role in antibody production against pathogen infection. Thus, cancer immune therapies using B cells have mostly focused on antibody production; their function as APC in cancer immunotherapy has been overlooked for a long time. As professional APCs, B cells can activate or tolerize T cells, thus promoting or regulating T cell responses. It has been reported that antigen presenting B cells (peptide pulsed) can stimulate T cell responses *in vitro* and *in vivo* when B cells are activated to up-regulate CD40 and other co-stimulatory molecules. (Vanden Bush,2009). It has been shown by different groups that resting B cells cannot function as APCs; only activated B cells have the ability to present MHC-I or MHC-II antigens to $CD4^+$ or $CD8^+$T cells(Zanetti,2004, Rodriguez-Pinto,2005). Thus, the use of B cells as a vaccine antigen carrier requires their activation, which can be accomplished by antigen delivery or CD40 cross-linking. Cross-linking of BCR stimulated by antigen,

induces B cell growth, proliferation and survival, and is necessary for the changes in the antigen processing machinery that also induce the CD86 expression. Alternatively, B cells can be activated by ligation of CD40 by CD154.(Yang,2006, Batista and Harwood,2009). CD40-derived signals sustain B cell activation and promote several changes related to their antigen-presenting function. These include enhancement of antigen processing (Rodriguez-Pinto,2005), increase in the expression of MHC class II molecules, stabilization of CD86 expression, and up-regulation of CD80. CD40 engagement also induces the redistribution of these molecules in the plasma membrane to favor antigen presentation. (Clatza,2003)

It is well defined that naïve B cells circulate in the peripheral blood and continuously home to secondary lymphoid organs, specifically in B cell follicles(Pereira,2010, Cyster,2010). Naïve B cells encounter antigen within the B cell follicle areas and become activated B cells, keynoted by the up-regulation of CCR7 ligands: CCL19 and CCL21(Cyster,2010). CXCL19 and CXCL21 will attract activated B cell to T cell zone for antigen presentation after activated B cells meet cognate antigen. B cell APC function is well established for MHC-II presentation of antigens internalized via surface Ig for stimulation of $CD4^+$ T cells, but the overall role of B cells in MHC-I Ag presentation has been controversial.

After years of effort made by clinical scientists, different ways of generating CD40-activated B cells with effective antigen presenting ability or using TLR ligands agonist for stimulation *ex vivo* have been developed, even from peripheral blood of

cancer patients.(Liebig,2010, Tobian,2005). Different antigen loading methods have been tried for the *ex vivo* manipulation of B cells. It has been shown that activated B cells incubated with tumor cell lysates or relevant peptide can stimulate antigen-specific $CD4^+$ and $CD8^+$ T cell proliferation *ex vivo* (Rodriguez-Pinto,2005). Other methods like mRNA electroporation (Van den Bosch,2005), *ex vivo* transduction with non-viral DNA (Gerloni,2004), and targeting with anti-CD19 antibodies have also been shown as effective ways of loading antigens onto B cells for activation of T cell antigen-specific responses. These altogether lay great clinical foundation for developing B cell-based cancer vaccines.

1. 6 Prime and boost regimen in cancer immunotherapy and negative immune regulation.

With the discovery of TAA and the development of DC based vaccines, many groups including our own have successful experiences in developing prophylactic vaccines with good tumor protection in animal models. However, the major problem remains in this field is that it is difficult to generate cellular or humoral immunity which is sufficient to treat existing cancer. In the past few years, great effort has been made to develop vaccine strategies to induce potent immunity; one of the most successful ones is the prime and boost strategy. Prime and boost refers to repeated immunization which can be homologous or heterologous. Homologous prime and boost refers to repeated delivery of the same vaccine, it is very effective in generating strong humoral response but fails to boost cellular responses. Heterologous prime and

boost uses repeated immunizations with different vectors carrying the same antigen. The heterologous approach is efficient in generating high-level cellular immunity and has been shown in many models to control pathogenic infection and ven tumor growth in some models(Woodland,2004). With increasing understanding of T cell immunity, the mechanisms through which heterologous prime and boost are effective are becoming clearer. The primary immunization selectively activates some T cells with certain epitopes of pathogens or cancer cells, and the boosting immunization reinforces the activated memory T cell population by delivering the antigens a second time. Moreover, compared to naïve T cells, memory T cells have a much lower threshold to be activated and differentiate into effector T cells. The key question that remains in prime and boost strategy is to choose the proper boosting time. The widely accepted optimal prime and boost interval is 2-3 months based on T cell differentiation theory(Jameson and Masopust,2009). As discussed above, after primary vaccination, naïve T cells differentiate into effector T cells and most of these effector T cell undergo apoptosis in the contraction phase. Thus, only a small number of T cells further differentiate and form the pool of long lived memory T cells. Therefore, it is thought that the optimal boosting time should be the late stage of effector to memory transition (Sallusto,2010). Another reason why it is difficult to attain a short prime and boost interval is the negative regulation presented by the effector T cells during secondary vaccination, which eliminates APCs presenting their target antigen. (Wong and Pamer,2003). However, it is of great importance to quickly

boost antigen specific responses in developing cancer therapeutic vaccines because the time allowed for treatment is always stringent, and developing cancers establish immunosuppressive conditions, which may dampen the T cell responses generated by immunization.. It is always difficult to boost high T cell responses within a short time. It has also been shown by Phillip Wong and his colleagues that antigen specific memory T cells can be generated as soon as 5-7 days after primary immunization(Wong,2004). Based on these findings, there is a possibility to rapidly boost memory T cell responses shortly after primary immunization, if we can bypass neutralization by the pre-existing immunity generated by the primary vaccine.

Another consideration for priming and boosting is to choose a good boosting vaccine. Different groups have shown that many viral vectors can be used as booster vaccine to induce effective CTL responses and $CD8^+$ memory despite the strong T cell response raises against the viral vectors themselves, such as vaccinia virus (Vac) (Young,2006), and VSV(Bridle,2009) among others. The major concern in the choice of viral vectors as booster vaccines is they also induce strong T cell responses against viral antigens. DC-based vaccines are very potent in priming T cell responses as discussed above, however, it has been shown by different groups that DCs as antigen presenting cells are very sensitive to perforin-mediated CTL killing and it greatly impairs the efficiency of secondary antigen presentation. As a consequence, DC-based vaccines may not be good candidates for a booster vaccine.

1.7 Viral vectors in cancer vaccines

1.7.1 Use of viral vectors in immunotherapy

In 1982, Vac was described as the first viral vector for immunotherapy, and since then, viral vectors have been broadly investigated to develop cancer therapeutic vaccines by expressing a variety of genes *in vivo*, such as TAAs, cytokines and accessory molecules (Bonnet,2000). As we have discussed above, the success of inducing a tumor specific T cell response needs at least three signals: the target antigen, processed by antigen presenting cells and presented in the context of MHC molecules, danger signals, including cytokines, and co-stimulatory molecules being expressed on the APC. The very attractive advantage of using viral vectors to elicit antigen-specific immune response is that viral vector delivery mimics the natural viral infection and inducing potent danger signals and induces up-regulation of co-stimulatory molecules. (Harrop,2006). There are some requirements of a good viral vector system. First, it should be compatible with existing standard current cancer vaccines and proven to be safe for clinical use. Second, a good vaccine virus should induce long lasting antibody responses or CTL responses, but be independent of pre-existing immunity and unable to productively replicate in human cells. In addition, certain practical considerations are necessary: it should be capable of delivering a large therapeutic payload, easy and inexpensive to manufacture and requires only simple storage requirements (Young,2006, Liu,2010).

1.7.2 Effects of pre-existing immunity to viral vectors

Adenovirus (Ad), vaccinia virus (Vac) and most of the commonly used viral vectors are sensitive to pre-existing immunity and most of the human population has pre-existing immunity to the viral vectors currently developed in cancer immunotherapies. Similarly, the presence of the neutralizing antibodies and activated immune cells such as antigen specific effector T cells which were raised in response to the primary viral infection or vaccination will greatly limit the efficacy of the viral vectors. (Bonnet,2000). However, in many cases, repeated virus delivery is needed, in order to get higher T cell responses against recombinant antigens. Similarly, for oncolytic viruses, repeated virus injections can help reach a better oncolytic effect (Pandha,2009). To circumvent the problems associated with pre-exisiting immunity to virus vectors, strategies have been developed in order to bypass the effect of pre-existing immunity to virus vaccination. For the effect of pre-existing cellular immunity, one of the commonly used strategies is the heterologous prime and boost as mentioned above. Using two different viral vectors to deliver the same antigen was can indeed designed to solve the problem of pre-existing immunity to induce high-level T cell responses (Woodland,2004). Different successful heterologous prime and boost strategies have been developed by different groups, *eg* Lentivirus priming and rVac boosting can generate high quality $CD8^+$ memory T cell with good protection of autochthonous melanoma (Xiao,2011). We ourselves also have successful experience of using Ad priming followed by VSV boosting, which can

reach a five-fold increase in antigen specific T cell responses and therapeutic tumor protection(Bridle,2010). For overcoming the pre-existing neutralizing antibody responses, A new strategy of using cell carrier to deliver viral vectors has been suggested by some virologists (Willmon,2009). Cell carriers including T cells(Qiao,2008), macrophages and even tumors cells(Power,2007) have been tested carrying multiple viruses including retrovirus, VSV, and vaccinia virus because of their tropism to the tumor microenvironment, or for anatomic locations where tumors are known to be resident. The mechanisms of how the cell carriers can help to overcome the effect of pre-existing antibody responses needs to be further investigated and further improvements to guarantee the targeting and efficacy of cell carriers need to be made (Willmon,2009).

1.7.3 The safety issue of using viral vectors

Another key property of an applicable viral vector delivery system for clinical use is their safety especially when viral vectors need to be repeated delivered. Because anti-viral response will be generated after primary viral delivery, or because there is pre-existing immunity against the viral vector been used, it is important to guarantee the safety while retaining vaccine efficacy. In our experiments we have been using replication-deficient Adenovirus, Vaccinia Virus and VSV as viral vectors to deliver antigen and mediate anti-tumor immune responses. The hu-Ad5 vector has been administrated in different clinical trials and shown to be safe. (Rollier,2011, Young,2006).The attenuated strains of Vaccinia virus have been reported to be

avirulent and safe among humans (Rollier,2011, Young,2006). Although there are some preclinical study regarding VSV toxicity in both mice and rat model (Jenks,2010), most studies have focused on VSV's potent oncolytic properties, however, its ability to generate efficient T cell responses and definitely needs to be evaluated clinically.

1.8 Initial hypothesis

In this thesis, we attempt to develop a B cell based cancer vaccine in generating tumor specific T cell responses as an alternative to DC based vaccines.

1.9 Specific Aims:

1. To establish methods for the *ex vivo* activation and expansion of B cells.
2. To investigate B cells' function in both primary T cell activation and memory T cell expansion in comparison with DC-based vaccine thus the possibility of using B cell as vaccine vector.
3. To investigate the role of B cells as vaccine vectors in T cell priming and memory T cell expansion (if B cells can be proven to be alternative candidate to DC in vaccine development in aim 2).

2. **Materials and Methods**

2. 1 Animals

Female 6-8 week old C57BL/6 mice were purchased from Charles River Laboratory (Wilmington, MA) and housed in a specific pathogen-free facility. Female B6/JIK <BTML>D<BTML>N12 (MHC K^bD^b knockout) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). P14, a TCR transgenic strain whose CD8+ T cell can only recognize a lymphocytic choriomeningitis virus (LCMV) glycoprotein 33-41 (LCMV-GP33) epitope, and CD11c-DTR transgenic mice were bred in the central animal Facility in McMaster University. Mice were given food and water *ad libitum* during experiments. All animal studies complied with Canadian Council on Animal Care guidelines and were approved by McMaster University's Animal Research Ethics Board.

2. 2 Recombinant Viruses

A recombinant vesicular stomatitis virus (rVSV) of the Indiana serotype rVSV-ΔM51 possesses a deletion mutation in the coding region for the matrix protein (Bridle,2010, Stojdl,2003). rVSV-GP33 was the rVSV-ΔM51 vector engineered to express the D^b-restricted immunodominant CD8 T cell epitope of LCMV GP33-41; rVSV-GFP was the same VSV vector engineered to express GFP and used as an indicator for virus infection. rVSV-MT was VSV vector contains no transgene and was used as a control vector in these experiment. Vaccinia Virus-GP33 (rVac) was a recombinant vaccine vector carrying LCMV GP_{33-41} epitope. The recombinant

rAd5-GP33-ER is an E1/E3-deleted human type 5 Adenovirus vector which was engineered to express the immunodominant $CD8^+$ T cell epitope of LCMV-GP_{33-41}, it has been described elsewhere.(Bassett,2011).

2. 3 Peptides

The H-$2K^b$ restricted OVA peptide, ($OVA_{257-264}$; SIINFEKEL) was synthesized by Pepscan Systems (Lelystad, Netherland). The H-$2K^b$- restricted epitope from the N protein of VSV (RGYVYQGL) was purchased from Biomer Techbologies (Hayward, CA). The H-$2D^b$ restricted peptide of LCMV glycoprotein ($GP33_{33-41}$; KAVYNFATM) was purchased from the Dalton Chemical Laboratory (Toronto, Canada). Peptides were dissolved in distilled water and stored at -20°C.

2. 4 B cell and Dendritic cell culture

B cells were purified from the spleen of wild-type C57BL/6 mice using negative selection kits (Miltenyi Biotechnology, Germany). Purified naïve B cells were then cultured with adipocyte-conditioned medium (ACM) (provided by Dr. David Spaner) as described (Tomic,2006), supplemented with 20ng/ml IL-2, Imiquimod (IQ), and Phorbol 12, 13-dibutyrate (PDB) at the concentration of 2 x 10^6 B cells / ml.

Bone marrow-derived dendritic cell precursors were harvested from mouse femurs and tibias as described before(Boudreau,2008). Dendritic cell precursors were then seeded at 2 x 10^5 cells/mL in DC culture medium (RPMI 1640, 10% FBS, 2mM L-glutamine, 100U/ml penicillin, 100ng/ml streptomycin, 50uM β-mecaptoethanol, 1Mm sodium pyruvate and 0.01Mm non-essential amino acids) supplemented with

40ng/ ml recombinant murine GM-CSF (Peprotech, USA) for 7 days. On Day 3, 50% fresh DC cultured medium plus GM-CSF was added to the culture dishes; on Day 6, one third of the culture medium was replenished fresh DC culture medium plus GM-CSF as described (Boudreau,2008).

2. 5 B cell and DC VSV infection

B cells were harvested after 3 days of culture as described above by pipetting up and down from the bottom of the culture dish and then "infected" with rVSV-GP33 or rVSV-GFP to measure the infection rate at a multiplicity of infection (MOI) of 25 on a rotator in the cell culture incubator at 37 °C, 5% CO_2 for 2 hours. B cells were washed three times with PBS before they were used in experiments.

DCs were harvested after 7 days of culture by scraping the bottom of the culture dish and infected with rVSV-GP33 or rVSV-GFP at MOI 25 (Boudreau,2009) in a 50 mL BD falcon tube and mixed by inversion. DCs were then plated back to the culture for 2 hours to complete the infection.

2. 6 Viral titration in culture supernatant

B cells and DCs were prepared and infected with rVSV as described above. Supernatants were collected post-infection by centrifugation, and rVSV-GP33 was used to infect Vero cells as the positive control. Vero cells were then infected with collected supernatant or rVSV-GP33 for 1 hour in 37 °C, 5% CO_2 and 1% agarose gel was subsequently overlaid onto the culture dish. The gel was allowed to solidify for 30 minutes before culture dishes were returned to the incubator. Vero cells were then

cultured at 37°C, 5% CO_2 for another 24-48 hours to form the viral plaques. Viral titers were quantified by the plaque assay formed on vero cell monolayer. Each sample was diluted and assessed in triplicate.

2. 7 Virus challenge and prime - boost immunization strategy

rAd-GP33 and rAd-SIIN was injected at 1×10^8 pfu intramuscularly (im), rVac-GP33 and rVac-SIIN was delivered at 2×10^7 pfu intraperitoneally (ip). Mice were either primed with rAd-GP33 or rVac-GP33, 10-14 days after primary immunization, 3 million rVSV-exposed or peptide-pulsed B cells or rVSV-exposed or peptide-pulsed DCs were given by intravenously (iv) injection as a secondary vaccine. rVSV exposed or peptide-pulsed DCs were also given by footpad (fp) injection as control groups. Pure rVSV-GP33 was given by iv injection and used as control secondary vaccine.

2. 8 Lymphocytes preparation

Blood (200 μL) was collected from the peri-orbital sinus for peripheral blood mononuclear cells (PBMC), and red blood cells were lysed with 2 ml ACK lysis buffer (0.15 mol/L NH_4Cl, 10.0 m mol/L $KHCO_3$, 0.1 m mol/L Na_2EDTA, PH 7.2-7.4). Spleens or lymph nodes were gently ground between microscope slides then filtered through a 0.22 μm filter to create a single cell suspension. Red blood cells were then lysed with lysis buffer as described above. After lysing (same with PBMC, spleen samples and lymph node samples), remaining cells were washed with PBS by centrifugation at 1500 rpm for 5 minutes and repeated twice. After centrifugation,

cells were then re-suspended at 2 million cells/ml in complete RPMI 1640 (Invitrogen, Grand Island, NY) as described before for next step experiment.

2. 9 Peptide stimulation, surface and intracellular staining

Flow cytometry was used to quantify antigen specific T cell immune response 7 or 6 days after primary or secondary vaccination, respectively. Single cell suspensions were prepared from blood or spleen as described before in 96 well plates, and stimulated with peptide (1 μg/ml). Golgi Plug (BD Biosciences; 1 μg/ml) was added after 1 hour of incubation to prevent release of cytokines. After 5 hours of total incubation time, cells were treated with Fc block (anti CD16/32 antibody, BD Biosciences) for 10 minutes and stained with anti-CD4 and CD8 antibodies for 20 minutes. Cells were subsequently permeabilized with Cytofix/Cytoperm (BD Pharmingen) following the manufacturer's protocol, and stained for intracellular IFN-γ and TNF- α with antibodies for 20 minutes. All antibodies used for flow cytometry were described in Appendix Table 1. Flow cytometry data were acquired using a FACS Canto with FACS Diva 5.0.2 software (BD Biosciences) and analyzed with Flow Jo Macintosh Version 9.0.

2. 10 Administration of FTY720

FTY720 (Cayman Chemical, Ann Arbor, Michigan) was injected intraperitoneally to inhibit lymphocyte egress from peripheral lymphoid organs two hours before secondary vaccination (Jeyanathan,2010). FTY720 drug powder was first diluted in 100% ethanol according to the manufacturer's recommendations to make

the stock solution, which was further diluted with PBS to the proper concentration before use. Four mg/kg body weight of FTY 720 was administered to vaccinated mice in 200 μL total volume.

2. 11 BrdU incorporation assay

Immunized mice received an ip. injection of 1 mg BrdU and BrdU-containing drinking water (0.8 mg/ml) 24 hrs prior to tissue harvesting. Lymphocytes from spleen and lymph nodes were prepared as described previously. After Fc blocking, lymphocytes were stained with antibodies against CD8 and BrdU (BrdU staining kit, as per manufacturer's instructions; BD Biosciences), and H-2D^{b}-GP33 tetramer for antigen specific T cell proliferation.

2. 12 Recovery of CFSE labeling B cells and DCs

B cells and DCs were cultured as described above, and cells were then washed with PBS and re-suspended at 2× 10^{7}/ml in RPMI containing 10 μM CFSE. After incubation at 37°C water bath for 15 minutes, cells were washed with PBS containing 5 % FBS to get rid of the excessive CFSE. Cells were washed thoroughly with PBS before use. 3 million CFSE labeled virus exposed B cells or DCs were given by iv delivery to pre-immunized mice. 12 and 24 hours post injection, spleen and lymph nodes were harvested, digested and create single cell suspension. Cells were then stained with B220 for B cell or CD11c for DC and analyzed by flow cytometry to quantitate how many CFSE labeled B cells or DCs can be recovered in secondary lymphoid organs.

2. 13 CD11c DTR chimeras

In order to distinguish whether the endogenous $CD11c^+$ APC are involved in B/VSV mediated secondary T cell expansion, CD11c-DTR chimeric mice were generated. Wild-type C57BL/6 recipient mice were irradiated (2x550 Rads; 48 h interval) and then received $5x10^6$ CD11c-DTR transgenic mouse-derived bone marrow cells (CD11c DTR→WT)1 hour after the second irradiation, $5x10^6$ wide type C57BL/6 bone marrow cells were also injected to irradiated mice (WT→WT) as control . BM was depleted of T cells using a cocktail of anti-CD4 (clone GK1.5), anti-CD8 (clone 2.43) and anti-Thy-1.2 (clone 30-H12) followed by treatment with low-toxicity guinea pig complement (Cedarlane Laboratories Ltd, Burlington, ON). The purpose of T cell depletion is to avoid the major histocompatibility mismatches between donors and recipients. Mice were given three months for immune reconstitution prior to experimentation.

2. 14 Confocal Microscopy

We used confocal microscopy to visualize the locations where antigen was delivered to secondary lymphoid organs by different vaccine strategies. Briefly, spleen and lymph node samples were collected from immunized animals then sectioned into 8 μm frozen slides and stored at -70 °C before use. Frozen slides were warmed up to room temperature before use, and fixed with 1% paraformaldehyde (PFA) for 5 min and then washed with PBS thoroughly. Samples were first incubated with Fc block (1:200, clone 2.4 G2, BD Biosciences) in dark at room temperature for 20 minutes to

block the non-specific binding sites, followed by the primary rabbit anti-VSV antibody (1: 3000) and PE-texas Red conjugated rat anti-murine B220 (1:800) in the dark at room temperature for 1 hour. Secondary FITC-labeled goat anti-rabbit antibody (1:750) was used to visualize the VSV protein, and incubated in the dark at room temperature for 1 hour. Slides were then washed with PBS followed by PBS-Tween to wash away excessive antibodies. Mounting medium was at last added on top of the slides to prevent the fading of florescent signals. The prepared slides were subjected to the investigation using a confocal laser scanning microscope (LSM 510 Meta imaging system, Carl Zeiss). Excitation of fluorescent dye was at 590 nm for PE-Texas Red, and 488 nm for FITC.

2. 15 Statistic analysis

GraphPad Prism version 5 for Windows (GraphPad Software, San Diego, CA, USA) was used for graphing. For statistical analyses, GraphPad Prism and Minitab Statistical Software (Minitab Inc., State College, PA, USA) were used. Student's t-test, one-way ANOVA were used to analyze the data as appropriate. All data were presented as means ± SE and differences between means were considered significant at $P<0.05$.

3 Results

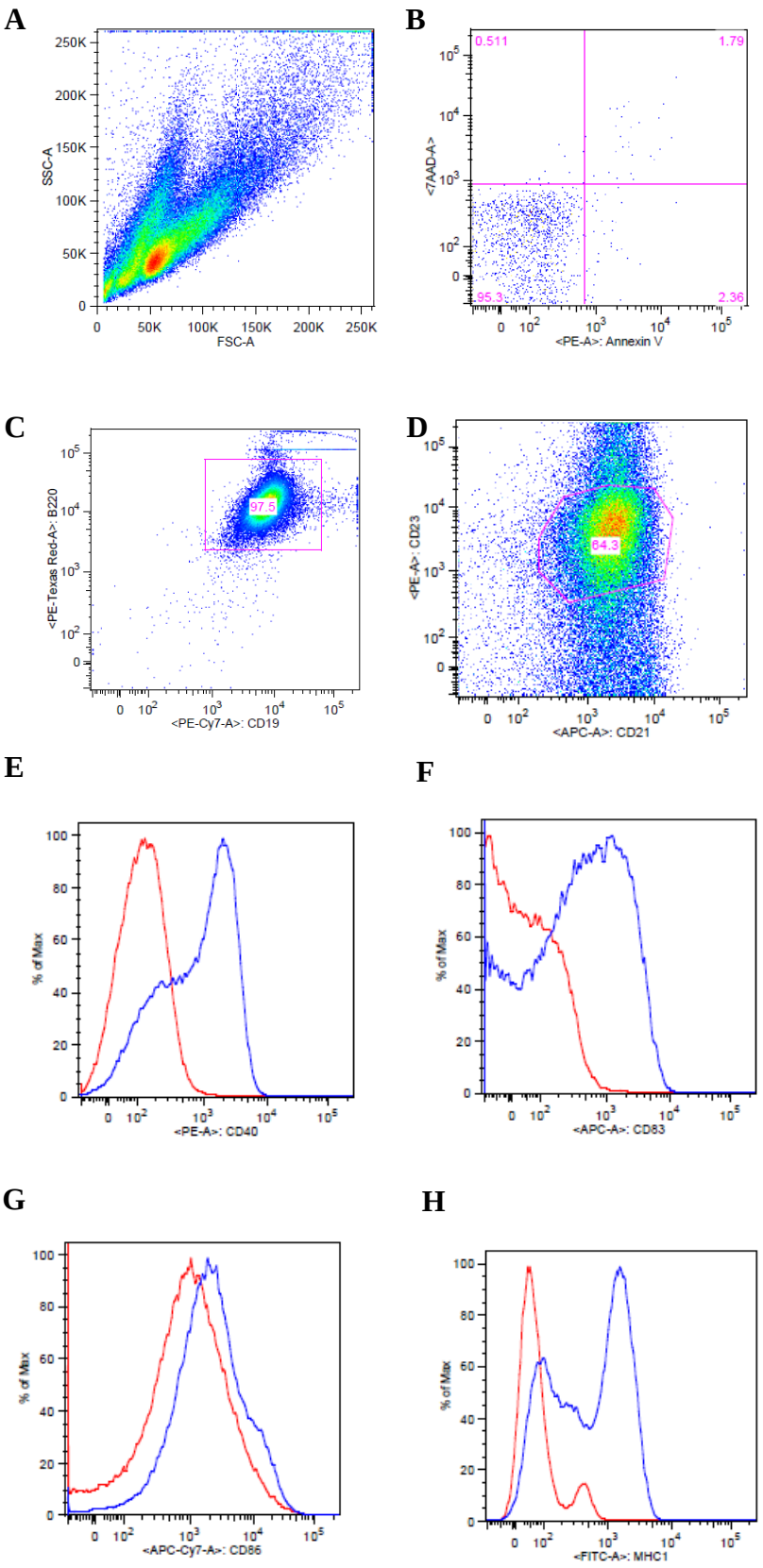
3.1 Characterization of *ex vivo*-cultured B cells

To generate a large number of and activated B cells *in vitro*, we adopted a B cell culture method from Dr. David Spaner (Sunnybrook Health Science Center, CA), which has been shown to enhance the immunogenicity of normal and malignant human B cells (Tomic,2006). The basic components in this culture are adipocyte-conditioned medium (ACM) supplemented with IL-2, IQ, a TLR7 agonist, and PDB, a phorbol ester. Naïve B cells were negatively purified using magnetic kits (Miltenyi, Germany) from mouse spleen and cultured with combined stimulation of three factors mentioned above for 72 hours. After 72 hours, Cultured B cells are enlarged when observed under microscope as well as detected by flow cytometry(Figure 1A). B cells maintain high viability according to Annexin V and 7-AAD staining (Figure 1B) while B cell cultured with normal culture medium (cRPMI+IL2) showed a high percentage of cell death (data not shown). As shown in Figure 1C, B cells maintained more than 95% purity of the canonical surface markers CD19 and B220. Interestingly, the proportion of different B cell subsets changed during culture. Specifically, this culture method selectively increased the number of follicular B cells (Figure 1D) compared with other B cell subsets including marginal zone B cell and memory B cells. In order to get a conclusive view of the B cells we used, we also detected activation surface marker expression on B cells by flow

cytometry. As shown in Figure 1E, the expression of CD40, CD83 (Figure 1F) and MHC I (Figure 1H) are obviously up-regulated in the cultured B cells, and there is slight increases in the expression of CD86 (Figure 1G) compared to freshly isolated B cells. Overall, our results recapitulated those reported (Tomic,2006), and demonstrate that expanded B cells indeed possess the machinery for T cell activation, including both antigen presentation and co-stimulation.

3.2 B cells are superior to DCs for boosting immune responses when "pulsed" with VSV.

We have previously published that rVSV-infected DC can provide substantial anti-tumor protection and therapy through the activation of both innate and adaptive immune responses (Boudreau,2009). However, the use of DCs as a booster vaccine is limited by their sensitivity to killing by recently-activated effector T cells, the negative regulation of pre-existing immunity (Ludewig,2001, Luketic,2007). Interestingly, however, our recent studies indicate that direct injection with a high dose of rVSV vaccine could boost massive secondary expansion at the height of the primary $CD8^+$ T cell responses, suggesting that the negative regulation can be bypassed under certain circumstances. More strikingly, our further studies demonstrated that B cells, but not DCs, were required for antigen presentation during secondary T cell expansion. These results prompted us to ask the first question: can B cells carrying antigen provide any benefit over DCs in secondary T cell response? To address this question, cultured B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SSC-A
FSC-A
B
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1.79
95.3
2.36
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F
% of Max
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G
% of Max
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H
% of Max
<FITC-A>: MHC1

Figure1. Phenotypic analysis of *ex vivo* generated B cell. B cells were purified from spleen and cultured as described in materials and methods. After 72 hours of culture, cells were washed, and stained with fluorescently-labeled antibodies to detect the expression of variety of B cell surface markers. (A) Representative flow cytometry dot plots show cultured B cell gained a larger cell size. (B) Annexin V and 7-AAD double staining show cultured B cell viability. (C) Canonical B cell surface marker: CD19, B220. (D) B cell surface marker CD21 and CD23 show the proportion of the Follicular B cell in cultured B cell population. Representative histogram plots show the up-regulation of cultured B cell (gated $CD19^{+}B220^{+}$ cells) expressing (C) CD40, (D) CD83, (E) CD86, (F) MHC class Ⅰ compared to freshly isolated B cells. *Red is fresh isolated B cell, blue is activated B cell.*

cells or DCs were infected with rVSV-GP33 (MOI of 25, a dose optimized previously that gave maximal infection of DCs with minimal toxicity) and used as booster agents in mice that were primed with rVac-GP33. Data in Figure 2 show that i.v. delivery of 3 x 10^6 B cells infected with rVSV-GP33 (B/VSV-GP33) could boost a significant CTL response 7-14 days after priming with rVac-GP33, whereas the same number of rVSV-GP33-infected DCs (DC/VSV-GP33) failed to provoke secondary T cell responses, suggesting that B cells are indeed superior to DCs for eliciting a rapid recall T cell response. We excluded the possibility that i.v. injection represents a suboptimal route for DC vaccination because footpad injection with the DCs also failed to generate a boosting effect (Figure 2). To determine whether the inability of DCs to boost secondary response at the early stage was due to their sensitivity to effector T cell-mediated elimination, we increased the interval between priming and boosting to allow for the primary immune response to wane. Mice were primed by inoculation of rVac-GP33 and boosted with B/VSV-GP33 or DC/VSV-GP33 as described above after 30 days. As expected, DC/VSV-GP33 were able to boost antigen-specific CD8+ T cell responses at this later point, consistent with the notion that pre-existing effector T cells prevent excessive antigen presentation by DCs (Figure 3). Interestingly, B/VSV-GP33 still achieved a better boosting response compared to DC/VSV-GP33, suggesting that virus-infected B cells were superior for generating secondary antigen specific T cell responses with or without the presence of pre-existing CTLs.

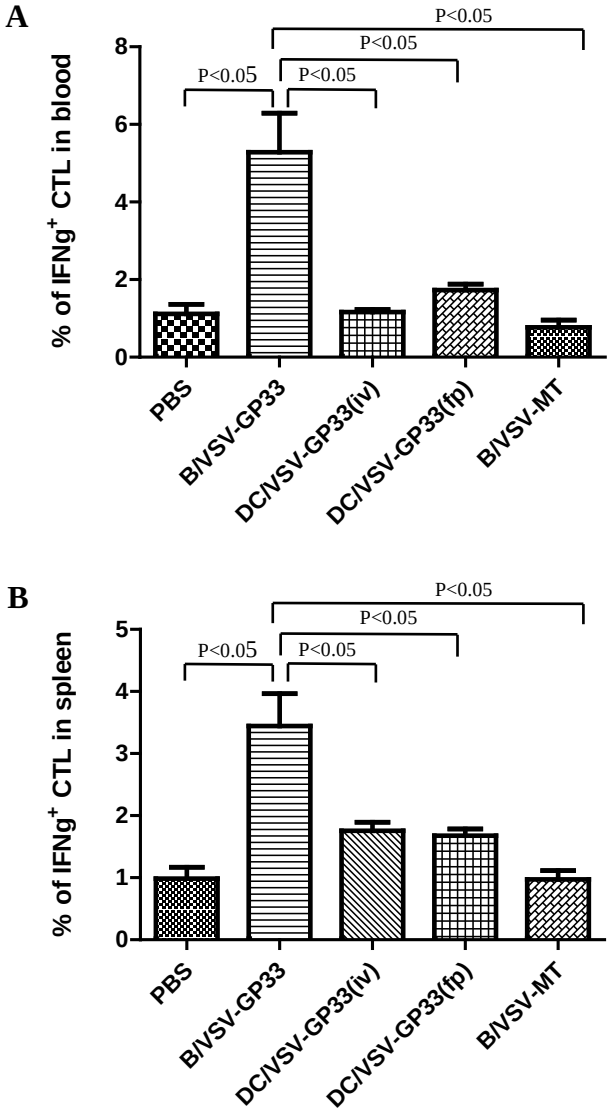


Figure2. B cells are more potent than DCs for booster immunization in the presence of CTL. C57BL/6 mice were primed with Vaccina-GP33, 10-14 days after primary vaccination, mice were boosted with DC/VSV-GP33, B/VSV-GP33, B/VSV-MT or PBS as control. Secondary responses in blood (A) and spleen (B) were measured 5 days after boosting. B/VSV-GP33 group is significantly different from all other groups. Data were generated from 3 independent experiments. ($p<0.05$) (n=5)

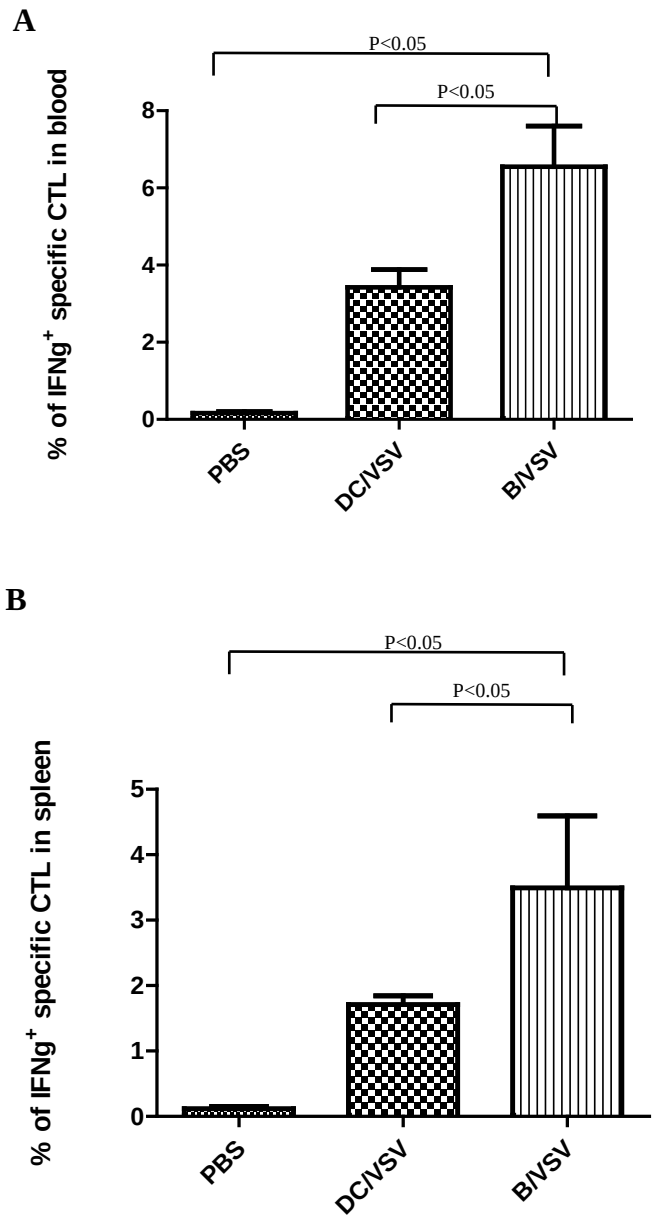


Figure3. B cells are more potent than DCs for booster immunization in the absence of CTL. C57BL/6 mice were primed with rVac-GP33, 28 days after primary vaccination, mice were boosted with DC/VSV-GP33, B/VSV-GP33 or PBS as control. Secondary responses in blood (A) and spleen (B) were measured 5 days after boosting. B/VSV-GP33 boosting response is significantly higher than DC/VSV group and PBS boosting group. Data were generated from 3 independent experiments. ($p<0.05$)($n=5$)

3.3 Virus-pulsed B cells do not present the target antigen

Given that virus-exposed B cells are able to boost T cell responses, even during the effector phase of the primary T cell response, we speculated that B cells, but not DCs, may be resistant to CTL killing. Interestingly, however, this notion is not supported by the fact that only rVSV-GP33-exposed B cells but not GP33 peptide-pulsed B cells could promote secondary CTL expansion (Figure 4). To determine whether viral infection may alter the susceptibility of DCs and B cells to CTL killing, we first quantitated transgene expression in B cells and DCs following infection with rVSV-GFP. To our surprise, while >70% DCs were GFP positive, very few B cells were directly infected by the virus (Figure 5). This observation prompted us to hypothesize that rVSV-pulsed B cells may function as a carrier to deliver viral vectors instead of mediating direct antigen presentation. To address this hypothesis, we exposed B cells from K^bD^b (MHC I)-deficient mice to rVSV-GP33 and inoculated them into mice that had been primed with rVac-GP33. As shown in Figure 6, both class I-deficient and -sufficient B cells boosted the same level of the secondary T cell responses, suggesting that direct antigen presentation by rVSV-pulsed B is not required for boosting T cell responses. Taken together, these findings support the notion that B cells act as virus carriers, rather than direct APCs. These results imply that B/rVSV could bypass CTL-mediated elimination because, in contrast to DCs that were efficiently infected by rVSV vector to present recombinant antigen and thus become targets for CTL, B cells do not express the target antigen and therefore remain

immune to T cell killing.

3.4 B/VSV boosting is neither limited by a specific primary vaccine vector nor the antigens it expresses

Our results demonstrate that B/VSV is effective for boosting even after a short interval following primary vaccination using rVac. To determine whether B/VSV was efficient for boosting T cell responses in general or in the presence of higher CTL frequencies, rather than simply those primed by rVac, we employed rAd as the primary vaccine. We chose rAd because it has been shown to induce strong primary immune responses. Also, we included another antigen SIIN, the $CD8^+$ H-$2K^b$-restricted epitope from chicken ovalbumin (OVA), to see if we could widen the use of B/VSV as a secondary vaccination platform using different antigens. Consistent with previous results, B/VSV still functioned as an effective booster vaccine for T cell responses primed with rAd5-GP33 or rVac-SIIN (Figure 7). These results showed promising potential for B/VSV as a booster vaccine because they indicate that it can be applied to different antigens and primary vaccine platforms.

3.5 Cultured B cells' ability to target secondary immune responses is not restricted to VSV.

Our data demonstrated that B/VSV functions well for boosting primary immune responses, regardless of how they are primed or the antigen that they target. Next, we investigated whether this function was intrinsic to B cells or specific to rVSV. Thus, we pulsed B cells with a vaccinia vaccine (rVac-GP33) for use as a booster vaccine.

Wild-type mice were primed with rVSV-GP33, and then boosted with B/rVac-GP33, as shown in Figure 8. B/rVac boosted mice achieved a 10-fold increase in CTL response, suggesting the strength virus-pulsed B cells for boosting immune responses is not limited to those exposed to rVSV.

3.6 B cells are necessary for efficient delivery of vaccine vectors to engage secondary T cell responses

To this point, our results demonstrate that the role of B cells during boosting is not via direct presentation of virally encoded antigens. Instead, B cells carry and deliver virus, presumably for presentation by a host cell population. To firmly establish the necessity of B cells as a carrier to deliver boosting virus, we wanted to rule out the possibility that the same amount of rVSV can elicit secondary T cell responses independently of B cell carriers when directly injected i.v. To address this possibility, we titrated rVSV released from infected B cells prior to i.v. injection. As detailed in Materials and Methods, we infected B cells with rVSV at 25 pfu/cell for two hours prior to injection. The supernatant was collected at the time when rVSV infection started and at the time when the infection was finished. We then titrated the virus particles from the supernatants, and considered the difference between virus titres before and after virus infection to be the amount of virus that was carried by B cells. As shown in Figure 9A, 3×10^6 B cells therefore carry 2×10^6 pfu rVSV in total after 2 hours' infection at a MOI of 25. However, unlike B cells carrying virus, when inoculated 2×10^6 naked rVSV-GP33 in the absence of B cells, could not boost

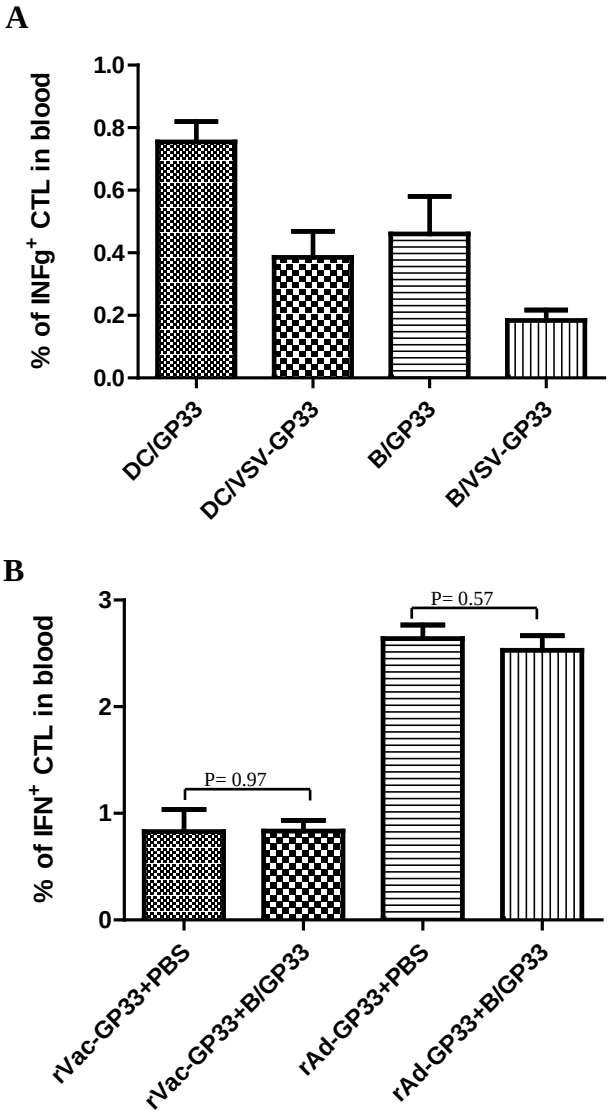


Figure4. B cell pulsed with peptide can generate antigen specific response as a primary vaccine, but fail to generate boosting response as a booster vaccine. A. C57/BL6 mice were primed 3 x 10^6 B cells or DCs pulsed with GP33 peptide or infected with VSV-GP33; nine days after immunization, primary immune responses against GP33 were measured by intracellular staining. B. C57/BL6 mice were primed with rVac-GP33 or rAd-GP33, 14 days after priming, mice were boosted B cell pulsed with GP33 peptide. Boosting responses were measured 5 days post boosting by intracellular staining. There is no significant difference between B/peptide boosted groups and PBS control group. Data were generated from 2 independent experiments. ($P>0.05$)($n=5$)

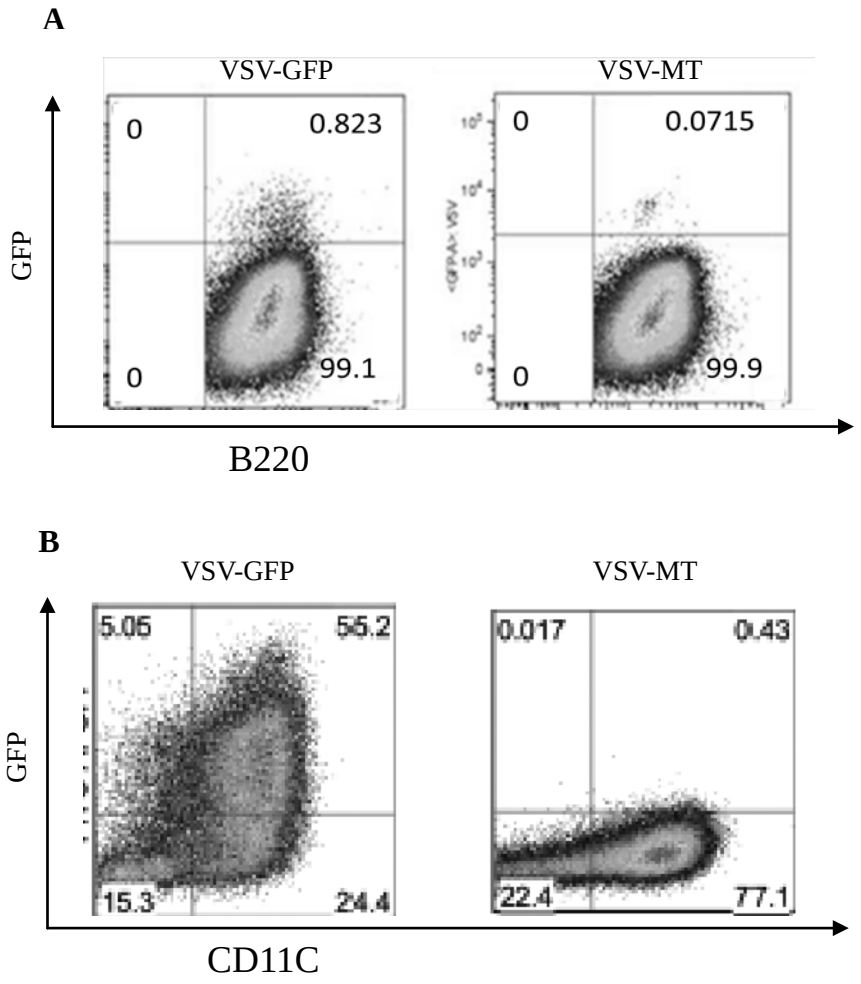


Figure5. VSV transduction efficiency in cultured B cells and DCs. B cells and DCs were prepared as described in materials and methods. After 3 days of culture of B cells and 7 days of culture of DCs, cells were harvested and infected with 25 pfu /cell VSV-GFP or VSV-MT as described. Infected B cells and DCs were cultured for another 24 hours to check GFP expression. GFP expression was used as indicator for rVSV transduction efficiency. Representative flow cytometry dot plot were shown depicting B cells stained with B220 (A) and DCs stained with CD11c (B).

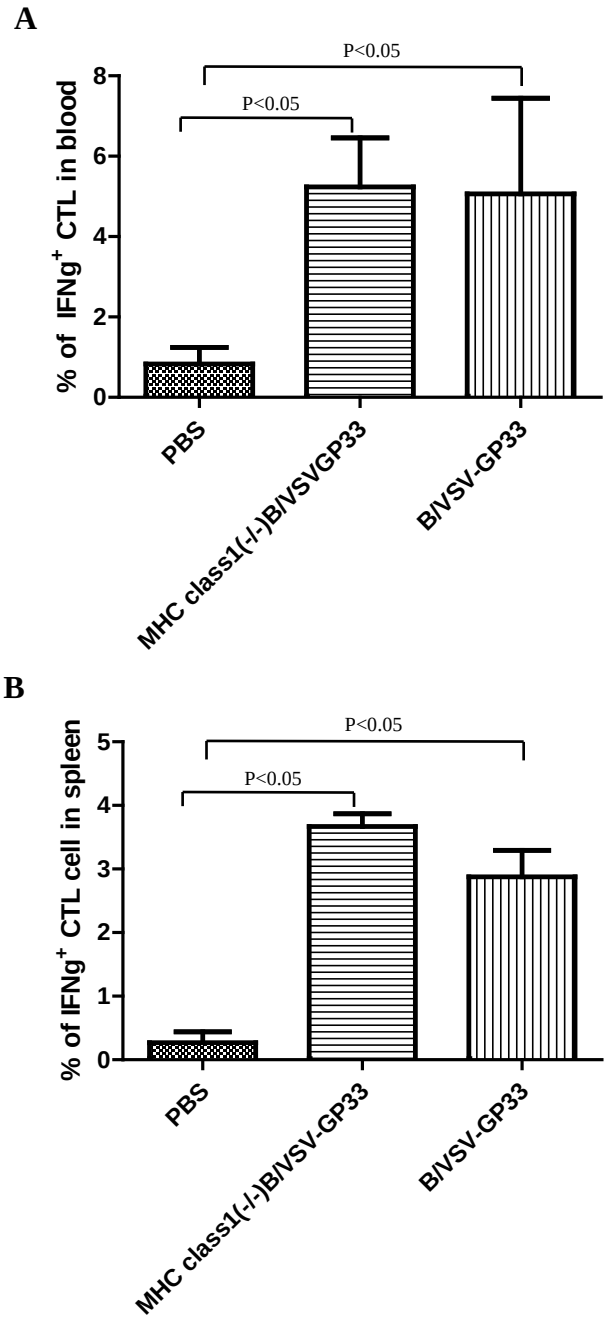


Figure6. Virus pulsed B cells do not directly present target antigen. C57BL/6 mice were primed rVac- GP33. Fourteen days after priming, mice were boosted with wild type or MHC-Ⅰ K^bD^b(-/-) B cells infected with VSV-GP33, or PBS as control. Boosting responses in blood (A) and spleen (B) were measured 5 days post boosting by intracellular staining. (P<0.05)(n=5)

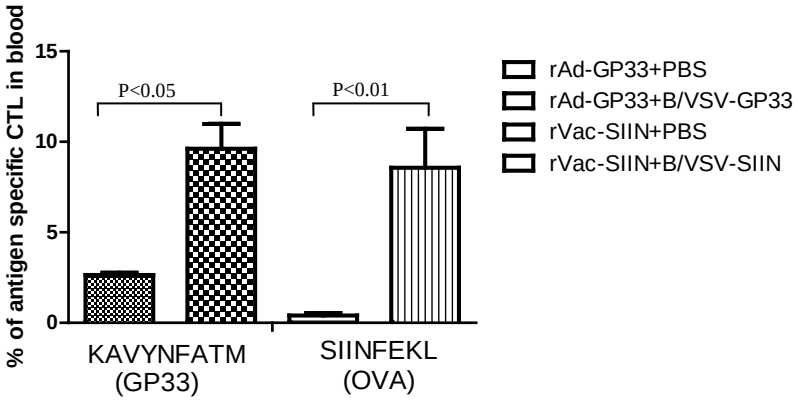


Figure7. B cell-mediated strong boosting effect is not limited by specific antigens or priming vaccines. C57BL/6 mice were primed with rAd-GP33 or rVac-SIIN. Fourteen days after primary vaccination, mice were boosted with B/VSV-GP33 or B/VSV-SIIN. Secondary T cell responses were measured 5 days after boosting. Data were generated from 2 independent experiments. (P<0.05)(n=5)

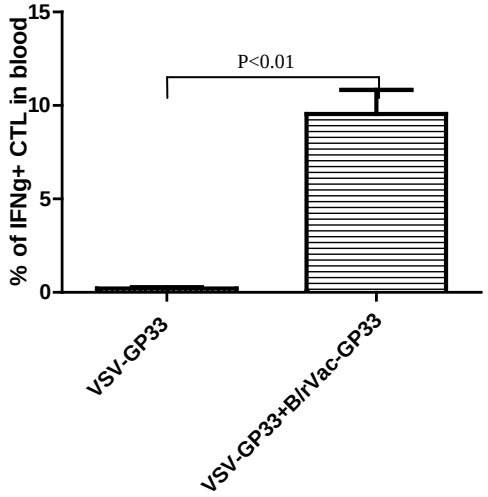


Figure8. B cell-mediated strong boosting effect is not specific to a certain booster vector; B cells can be loaded with other viruses. C57/BL6 mice were primed with $1x10^9$ pfu rVSV-GP33 and boosted with B/rVac – GP33 14 days post-priming. Secondary responses were measured 5 days post secondary immunization. Data were generated from 2 independent experiments. (P<0.01) (n=5)

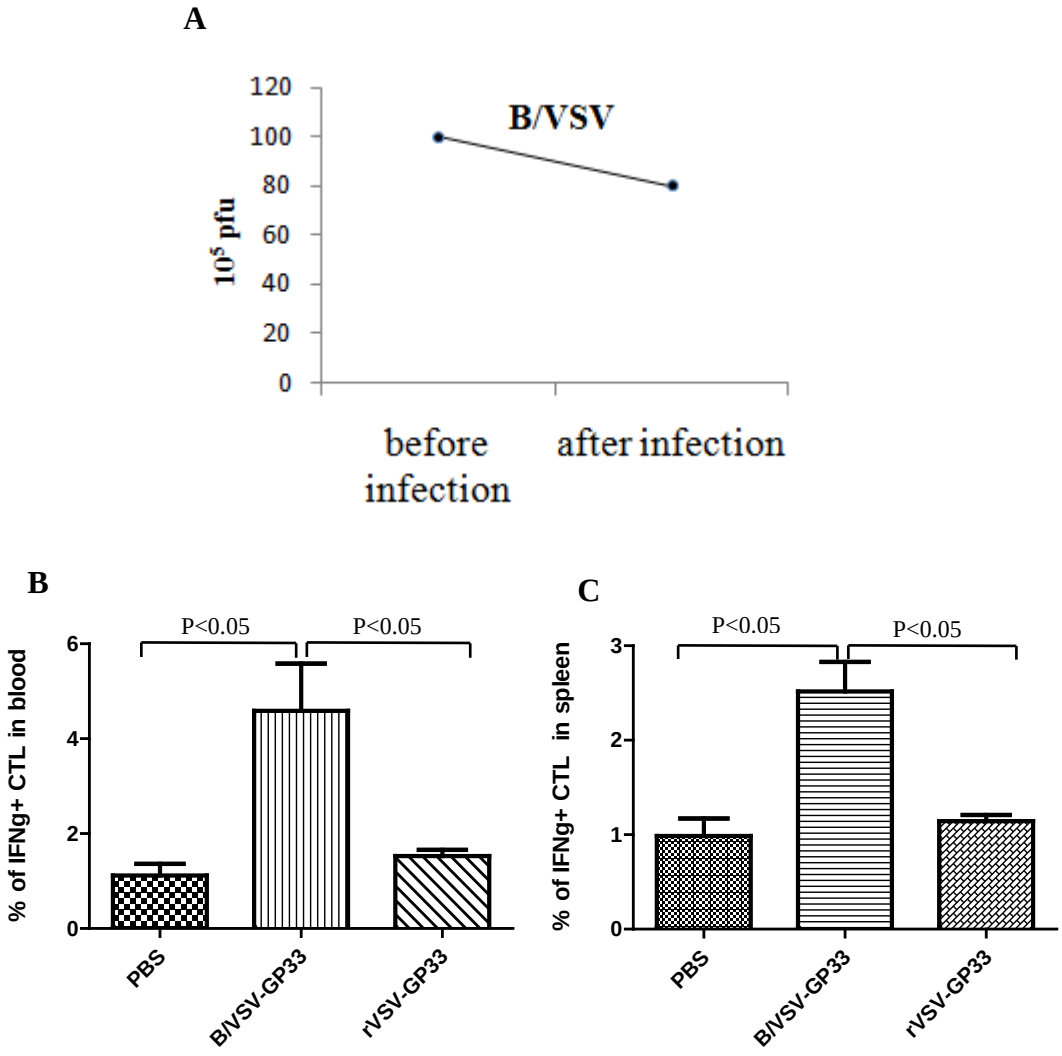


Figure9. B/VSV is a more potent booster than rVSV alone carrying the same amount of virus. A. Samples of B cell infection supernatant were collected just before the infection and after 2 hour infection. Viral progeny were quantified by plaque assay. C57/BL6 mice were primed with rVac-GP33, 14 days post priming, mice were boosted with B/VSV – GP33 or 2×10^6 pfu r VSV-GP33 or PBS as control. Secondary responses in blood (B) and spleen (C) were measured 5 days post secondary immunization. Data were generated from 2 independent experiments. ($P<0.05$) (n=5)

memory T cell responses (Figure 9B, 9C), suggesting that B cells are critically required to deliver and/or amplify rVSV-mediated antigen presentation by host APCs.

Combined with previous data showing B/rVac-GP33 but not the vector alone could boost $CD8^+$ T cell responses (Figure 8), our results clearly suggest that *ex vivo*-cultured B cells may function as an effective carrier for efficient viral vector delivery to amplify secondary T cell responses.

3.7 B cells, not other lymphocytes can carry viral vector for recall T cell responses

Until this stage, we have demonstrated B cells as carrying viral vectors are more efficient than virus alone in generating recall T cell responses. However, Richard Vile and his group have previously published a paper suggesting that naïve T cells can be exploited to chaperone VSV to lymphoid organs to purge metastatic tumors. This prompted us to consider whether our findings are unique to B cells or whether it is also common to other lymphocytes. Thus we "pulsed" both T cells and B cells with virus, and used as booster vaccine, to see if we can also observe a boosting benefit by using T lymphocytes as viral carrier. As the results in Figure 10 show, when we used the same amount of VSV-GP33 pulsed T cell and B cells as boosting vaccine in rVac-GP33 primed mice, T/VSV-GP33 group could not boost any secondary T cell responses while B/VSV-GP33 group could. This result tells us that only B cells, and not other lymphocytes, are capable of viral delivery to induce antigen-specific memory T cell expansion.

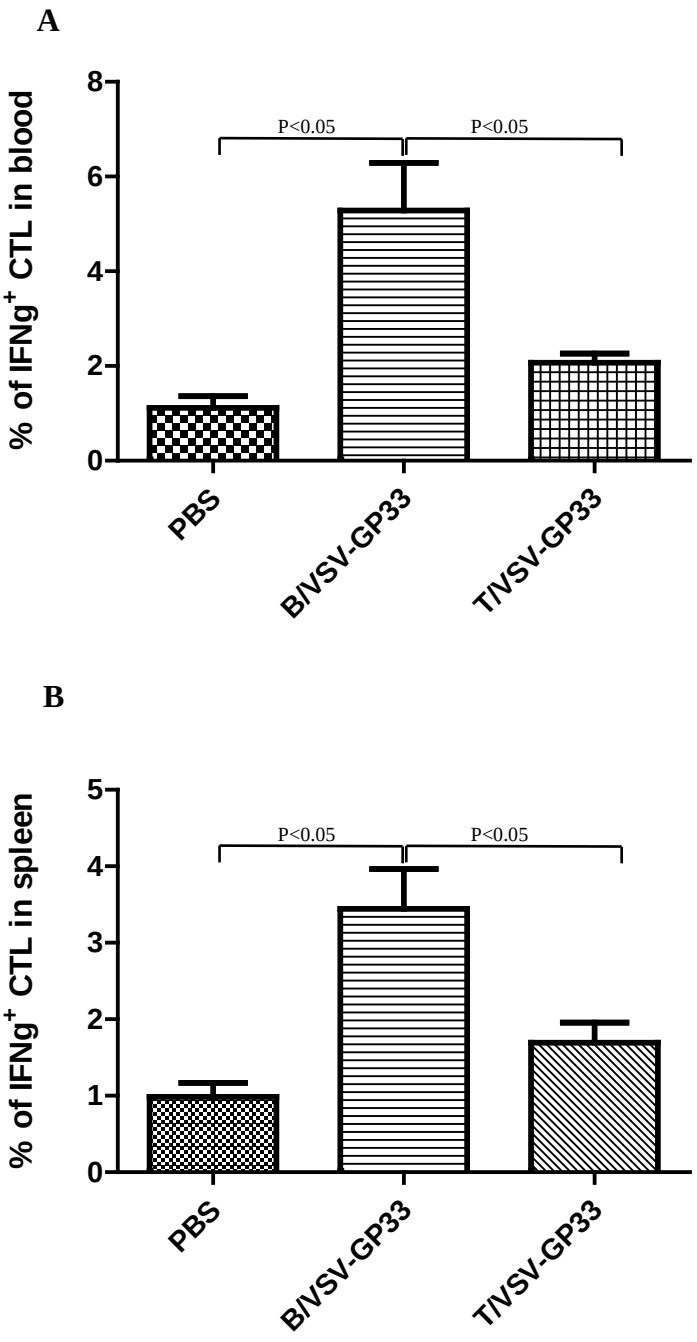


Figure10. B cell can deliver viral vector for secondary T cell responses, however, T cells cannot. C57BL/6 mice were primed with rVac-GP33, and, 10-14 days after primary vaccination, mice were boosted with T cells pulsed with VSV-GP33 (T/VSV-GP33) or B/GP33. Secondary responses were measured in blood (A) and spleen (B) 5 days post-secondary immunization by intracellular staining. Data were generated from 2 independent experiments. ($P<0.05$)($n=5$)

3.8 Injected B cells can be recovered in the secondary lymphoid organs and are primarily localized to the follicular regions

Having established a role of virus-exposed B cells as carriers, we wondered where antigens were delivered. Based on current understanding and our own data which shown that central memory T cells are the primary responders to booster immunization (Byram Bridle, Derek Clouthier, data not shown). Central memory T cells express both CD62L and CCR7 and therefore tend to home to secondary lymphoid organs such as spleen and lymph nodes(Dauner,2008). Thus, we sought to detect whether B cells could effectively migrate to these organs following i.v. injection, especially in mice with pre-existing CTL. Figure 11A and 11B show that CFSE-labeled, rVSV-exposed B cells could be recovered from both spleen and lymph nodes 24 hours after i.v. delivery during the peak CTL response induced by Ad-GP33primary immunization. In contrast, DCs infected with rVSV-GP33 were rarely recovered from either lymphoid organ.

To further visualize the localization of injected B cells within secondary lymphoid organs, spleen and lymph node sections were examined using confocal microscopy. Interestingly, the majority of injected B cells were located in the follicular regions (Figure 12), co-residing with central memory T cells (Dauner, Williams, et al. 2008). It is likely that B cells may deliver viral vectors to this particular area where follicular APCs present antigens to memory T cells. This finding is especially noteworthy, given that our B cell characterization experiments (Figure 1) demonstrated that the majority

of cultured B cells possess a follicular B cell surface phenotype. This may also be the reason why B cells home to B cell follicles after i.v. delivery. Since effector T cells do not cross into the marginal zone, antigen presentation by follicular APCs will not be affected by pre-existing CTL. Moreover, this may also explain how such a low dose of viral particles carried by B cells can stimulate such strong secondary T cell responses.

3.9 Early proliferation of antigen-specific $CD8^+$ T cells occurs in spleen and lymph nodes

Having determined that injected B cells localize to the follicular zones, we sought to determine whether this was also the location of antigen presentation and T cell expansion. Since we have shown that the B/VSV-mediated boosting response is not limited to a certain primary vaccine, we primed the mice with rAd-hDCT. Fourteen days after primary vaccination, mice were boosted with B/VSV-hDCT or B/VSV-MT as controls. Prior to boosting with a B/VSV vaccine, mice were treated with FTY720, a drug that inhibits lymphocyte egress from central lymphoid organs (Jeyanathan,2010). To monitor T cell proliferation in the spleen and LN, we employed the BrdU incorporation assay. BrdU is a synthetic nucleoside that can be incorporated into newly synthesized DNA of replicating cells and it is widely used to detect proliferation (see materials & methods). Spleen and lymph nodes were harvested at 24 or 48 hours post B/VSV delivery. BrdU was injected 24 hours before lymphoid organs were harvested to allow us to capture the proliferation during the final 24 hours.

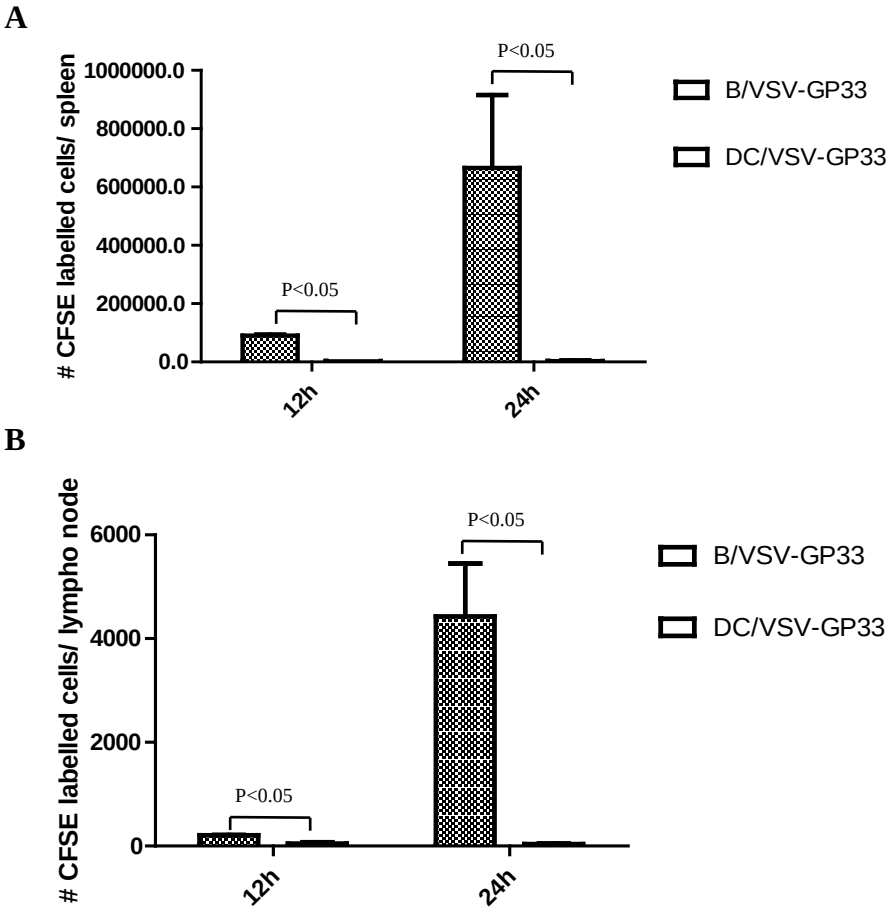


Figure 11. B cells delivered i.v. can be recovered from both spleen and lymph nodes in pre-immunized mice. C57BL/6 mice were primed with rAd-GP33, and B cells and DCs were prepared and infected with VSV-GP33 as described in materials and methods. Fourteen days after priming, mice were given 3 million CFSE-labeled B/VSV-GP33 or DC/VSV-GP33 i.v. Twenty-four hours after B cells or DC injection, spleen(A) and lymph nodes(B) were harvested (as described in materials and methods) and the number of recovered $CFSE^+$ cells was recorded. ($P< 0.05$)(n=3)

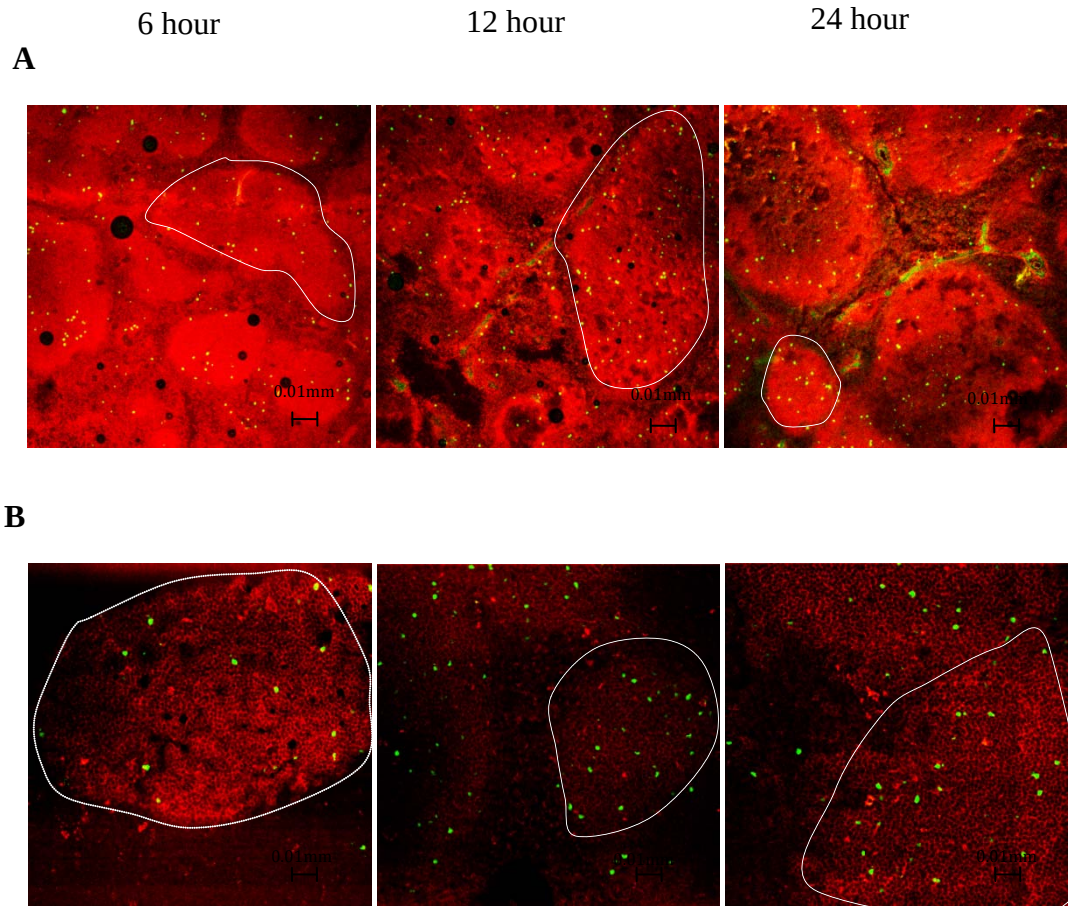


Figure12. Injected B cells are primarily localized in the follicular regions.
C57BL/6 mice were immunized with rAd-GP33, 14 days post primary immunization, mice were boosted with CFSE labeled B/VSV-GP33, 6, 12 and 24 hours post B cell delivery, spleen (A) and lymph nodes (B) were harvested, made into frozen slides, then stained with B220 (Red) to determine the localization of CFSE (Green) labeled cells .B cell follicle area was cycled with write lines.

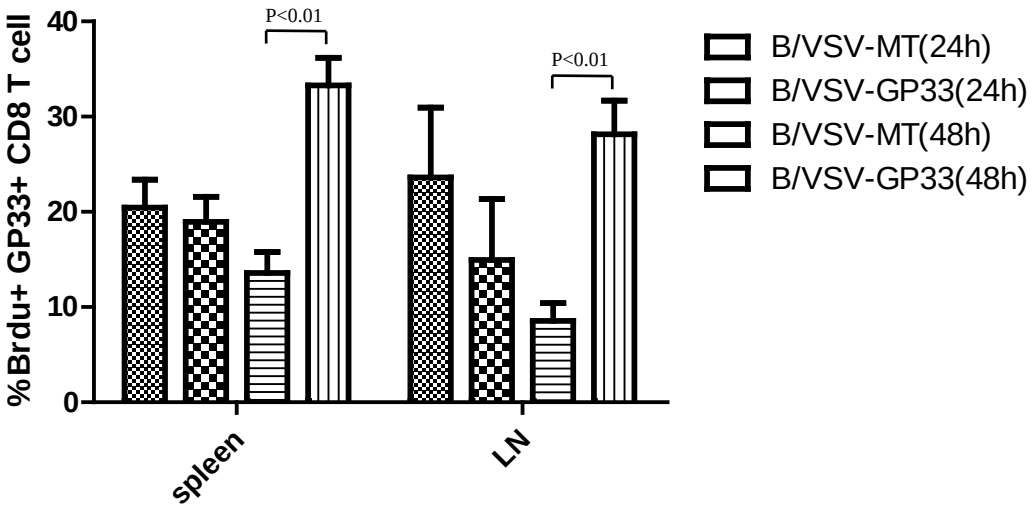


Figure13. Early proliferation of antigen-specific CD8+ T cells indeed occurs in spleen and lymph nodes in B/VSV mediated secondary T cell responses. C57BL/6 mice were immunized with Ad-GP33, and 14 days post primary immunization, mice were boosted with B/VSV-GP33 or B/VSV-MT as control. FTY 720 was given 2 hours before B/VSV delivery to block lymphocyte circulation. BrdU drug was delivered 24 hours before spleen and lymph nodes were harvested (materials and methods). Spleen and lymph nodes were harvested 24 hours or 48 hours post B/VSV boosting to measure antigen specific T cell proliferation by GP33 tetramer staining and BrdU staining. ($P < 0.01$)($n=5$)

Proliferation of antigen-specific T cells was determined by co-staining using anti-BrdU antibody and K^b-GP33 tetramer staining for antigen-specific cells. Antigen-specific $CD8^+$ T cell proliferation was observed in both spleen and lymph nodes after B/VSV-hDCT boosting compared to the B/VSV-MT group (Figure 12). Our previous studies demonstrated that after rVSV iv delivery, the earliest proliferation of antigen-specific T cells could only be detected in spleen, not in lymph nodes or bone marrow (Bridle B, unpublished data). This experiment extended these findings, demonstrating that *ex vivo*-derived B cells could not only recapitulate the boosting which occurs in the spleen following rVSV delivery, but additionally allowed this boosting to occur within the LNs.

3.10 Host $CD11c^+$ APCs are required to present the booster antigen

Finally, to identify the phenotype of host APCs that presented the booster antigen to expand memory T cells, we generated a chimeric strain of mice that allows for the pharmacological depletion of $CD11c^+$ APCs, primarily DCs. CD11c-DTR transgenic mice have been widely used for studies in which DCs are depleted . Normally, DT injection could maintain the depletion of $CD11c^+$ cells for 2 days. However, CD11c-DTR transgenic mice could not tolerate repeated DT injections to maintain depletion of these cell populations over long time periods. In order to maintain the absence of $CD11c^+$ cells throughout the boosting stage, we generated CD11c-DTR → WT chimeric mice, which could tolerate multiple DT injections. We used bone marrow cells from CD11c-DTR transgenic mice to reconstitute lethally irradiated

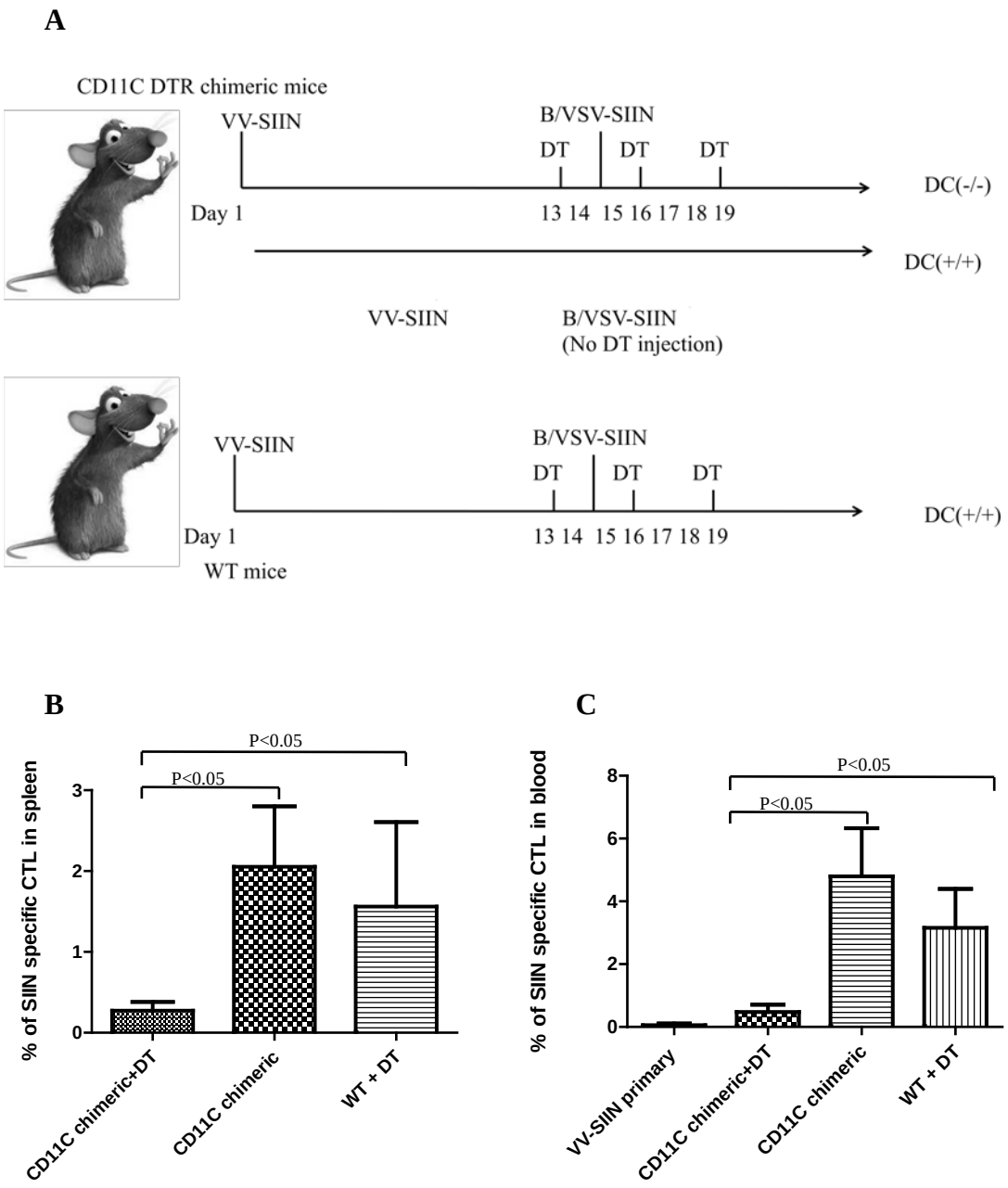


Figure14. Host CD11c+ APCs are required to present the booster antigen. A. Schematic figure showing the DC depletion process. CD11c DTR mice were made as described in materials and methods. Both wild type mice or CD11c-DTR mice were primed with rVac-SIIN, 13 days post boosting, DT were given by i.p delivery to wild type mice or CD11c DTR chimeric mice to deplete DCs, other, primed CD11c DTR mice did not receive DT as control for the effects of DT alone. On day 14, all mice were boosted with B/VSV-SIIN. DT was given every other day. Secondary antigen specific responses were measured 5 days post booting in both blood (B) and spleen (C).($P<0.05$)($n=5$)

wild-type mice (Figure 14A). In these mice, all $CD11c^+$ cells will be removed after diphtheria toxin (DT) injection. As shown in figure 14B and 14C, depletion of $CD11c^+$ cells abrogated the boosting effect of B/VSV, suggesting that host DCs may function as the third-party APC that presents antigen to trigger memory T cell proliferation.

4. Discussion

Although cancer vaccine therapy has demonstrated great progress in animal models and human clinical trials, further refinement of vaccination protocols is needed to increase therapeutic efficacy. Our group has focused on generating antigen specific T cell responses to control tumor development. We have previously showed that the Ad priming and VSV heterologous boosting regimen can generate strong anti-tumor immune responses and confer good therapeutic efficacy against established tumors. In this thesis, I report our recent finding, using activated B cells as viral vector delivery carriers to achieve an effective boosting response with the minimum viral load at a short prime and boost interval.

4.1 Finding an alternative to DC-based vaccines

The major limitation for DC vaccines' clinical application is that it is hard to generate enough DCs from peripheral CD14+ precursors, especially when the treatment requires multiple injections of DCs, making this treatment approach difficult to be widely applied. We moved our sights onto B cells because as another major subset of APCs. B cells share many biological features with DCs, such as a high level of MHC expression and the capacity to produce cytokines which enable them to regulate antigen-specific T cell responses. Furthermore, large numbers of autologous B cells can be readily prepared from the blood of patients and further expanded *ex vivo*(Zanetti,2004), thus providing an additional advantage over DCs and facilitate thus providing an additional advantage over DCs and facilitating vaccination protocols

utilizing repeated inoculation. However, like immature DCs, resting B cells preferentially induce tolerance instead of immunogenic T cell responses (ref) and thus the optimal method of activating B cells for vaccines remains to be determined.

We have tested several approaches for expansion of B cells *ex vivo*; among them, adipocyte-conditioned medium (ACM) culture was the only protocol that, in our hands, yielded significant B cell expansion. Dr. Spaner's group used this culture medium in combination with IL-2, IQ and PDB for expansion of B cells from CLL patients and demonstrated markedly enhanced immunogenicity(Tomic,2006). In our own experiments, the ACM culture method significantly increased B cell viability and expansion compared to cells cultured with the normal lymphocyte culture medium widely used by others (cRPMI +IL-2). After three days of culture, cells are enlarged while maintaining the standard B cell markers: CD19 and B220. When we looked into several surface markers including CD21, CD23, CD27 and IgM to distinguish the subsets within the cultured cells, we were surprised to find that this culture method specifically expands follicular B cells compared to other B cell subsets such as marginal zone B cells. Given that we have previously determined that follicular zone B cells are important for VSV-mediated boosting T cell responses (Byram Bridle, unpublished data), this observation led us to speculate that these B cells may have a better capacity to home to the B cell follicle areas in the secondary lymphoid organs where the antigens carried by them can be presented to memory T cells. We also noticed cultured B cells are more activated in the expression of MHC I and

co-stimulatory molecules, such as CD40 and CD83, compared with freshly isolated B cells. Although cultured B cells are indeed more effective than naïve B cells in boosting T cell responses in our system, the relevance of B cell activation remains to be determined because B cells only function as carriers for viral vector delivery, not antigen presentation. We found that both naïve and cultured B cells were equally capable of homing to secondary lymphoid organs (data not shown), the question whether activated B cells are "stickier" (i.e., carrying more viral particles on a single cell basis) or are able to provide bystander help (e.g., secreting stimulatory cytokines or co-stimulation) remains to be answered.

4.2 Virally loaded B cells represent a novel and effective booster vaccine platform

The prime-boost regimen has been recognized as the most effective vaccination approach to increase T cell responses but a rapid boosting is difficult to achieve because pre-existing effector T cells rapidly clear antigen-loaded cells, thus preventing adequate antigen presentation to memory T cells. Thus, prime-boost immunizations in a prophylactic setting are typically spread across a period of weeks to months to allow time for the memory T cells to dominate the available T cell pool and enable maximal secondary expansion. In the case of therapeutic vaccination for patients with chronic infection or cancer, where the effector T cells are needed for immune protection, boosting strategies that can bypass CTL-mediated negative feedback regulation and rapidly boost T cell responses would be desirable. Thus, although our initial intention was to develop B cell-based vaccine platforms as an alternative to DC vaccines, the

surprising finding that viral vector-pulsed B cells can bypass CTL-mediated negative regulation opens a new direction for cell/viral vector-based vaccine design. It is known that DCs are highly effective in T cell priming but their function in recall T cell responses is limited due to their sensitivity to CTL killing (Ludewig,2001, Luketic,2007). As a result, the efficacy of repeated DC immunization may be compromised, especially within short intervals. In the context of viral vector-based vaccines, our B cell approach may offer a novel and effective solution for rapid amplification of CTL responses. It must be noted that we and others have shown that direct injection with high doses of viral or bacterial vectors can also overcome CTL-mediated regulation to achieve a rapid boosting(Wong,2004, Bridle,2009), however, these vectors are associated with certain side effects even they are selected or engineered to attenuate the toxicity (ref). By direct comparison with a low dose of VSV vaccine, we demonstrate that B cell-based delivery approach is more efficient and thus potentially safer.

4.3 B cells are not just another cell carrier

Interestingly, the ability of B cells to overcome CTL-mediated killing is not due to their intrinsic properties. We provide evidence that B cells were not actually infected by our viral vectors but instead they only carried the vectors and delivered them into secondary lymphoid organs. The idea of cell carrier for virus delivery has been widely studied, especially in the field of oncolytic virotherpy (Willmon,2009). One of the reasons for such efforts is to bypass the effect of pre-existing neutralizing

antibody in order for repeated delivery of the virus to achieve better oncolytic effect. Another intention is based on the trafficking property of a cell type that can deliver the virus to certain locations. Thus, different groups of oncologists have designed various cell carriers for this purpose (Willmon,2009, Power,2007). In particular, Vile *et al* have previously reported that naïve T cells can carry and deliver oncolytic VSV to eliminate metastatic tumors from lymph nodes (Qiao,2008). This study made us wonder whether carrying viruses to lymphoid organs is a shared property of all lymphocytes. However, we found that the same number of naïve T cells pulsed with a VSV vector failed to boost secondary T cell responses suggesting that delivering vaccine vectors to lymphoid organs *per se* is insufficient to engage memory T cells for amplification. The difference between our novel B cell carrier and other cell carriers for oncolytic viruses is that B cells help to bypass the pre-existing cellular immunity for CTL boosting while other cell carriers help to bypass antibody responses for oncolysis. They share the similar concept of being cell carriers but function through different mechanisms and for different purposes. We report for the first time that B cells can be used to deliver viral antigen in the secondary T cell response boosting phase with the benefit of bypassing pre-existing T cell responses.

4.4 The old adage is still true – location is key

All of our data point to a possibility that the efficiency of B cell carriers must be due to their ability to deliver viral vectors into a right place. Indeed, our imaging data clearly indicate that injected B cells are exclusively located in the B cell follicular

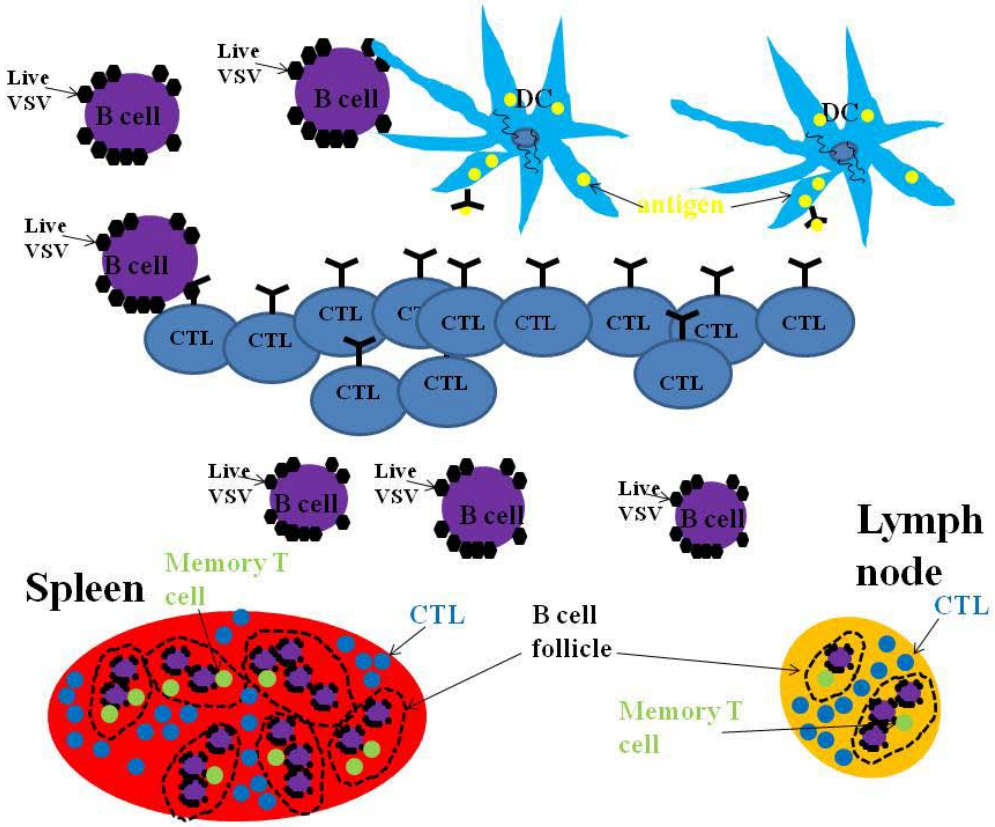
region both the spleen and lymph nodes. In the spleen, $CD8^+$ memory T cells are located in the T cell zones of the white pulp surrounded by follicular B cells. Using TCR-transgenic $CD8^+$ T cells, several groups have shown that after adoptive transfer to naïve recipients, effector $CD8^+$ T cells were only found in the red pulp, while memory $CD8^+$ T cells homed to the B cell follicles (Potsch,1999, Weninger,2001). Subsequent studies confirmed that this localization pattern held true for endogenously activated $CD8^+$ T cells. Lefrançois and Jacob showed that, upon secondary exposure to lymphocytic choriomeningitis virus or *Listeria monocytogenes*, memory $CD8^+$ T cells rapidly expanded and mobilized from the B cell follicles to the red pulp via bridging channels(Dauner,2008). After the immune response subsided, memory $CD8^+$ T cells homed back to the B cell follicles while effector cells remained in the splenic red pulp (Dauner,2008). These results point to a possibility that follicular APCs were responsible for memory $CD8^+$ T cell activation. The anatomical advantages of this co-localization of follicular APCs and memory $CD8^+$ T cells allow their intimate interactions while avoiding effector T cell-mediated killing.

In addition to spleen, we also demonstrated the existence of injected B cells in the lymph nodes where they were associated the B cell follicles. Furthermore, early antigen specific T cell expansion after secondary vaccination occurs in both spleen and lymph nodes by BrdU incorporation assay, suggesting that B/VSV platform can deliver antigen and mediated T cell expansion in multiple lymphoid organs.

4.5 The identification of endogenous APC

The demonstration of the requirement of endogenous CD11c+ APCs, presumably DCs, using CD11c-DTR chimeric mice appears to be logical as they are the most effective APCs. However, the complete phenotype of these DCs remains to be determined. As discussed above, $CD8^+$ DCs rarely circulate through the follicles and there is no report that follicular DCs are capable of antigen presentation to memory T cells. Furthermore, it has been previously reported that activated $CD8^+$ T cells also exp surface marker, so it is possible to that DT injection may not only deplete $CD11c^+$ DCs but also remove some $CD11c^+$ $CD8^+$T cells(Vinay and Kwon,2010), which complicates our interpretation at this point. To address this issue, I have recently established a method to address this issue, by adoptively transferring Thy1.1 splenocytes into Thy1.2 recipients, which allows simultaneous observation of both exogenous and endogenous T cell responses to prime and boost immunization (data not shown). I will use the same method to reevaluate the requirement of CD11c+ DCs in CD11c-DTR chimeras upon adoptive transfer of WT splenocytes. I also plan to visualize virus infected host cells within the follicular region by confocal analysis to attempt to identify their phenotypes.

In summary, we have demonstrated that virally pulsed B cells are very efficient booster vaccine which can generate strong T cell responses with the minimum viral load. We thus propose the following scheme to illustrate our novel findings:



As shown above, upon intravenous delivery, B/VSV will not be recognized by effector T cells because they do not present antigen. Thus, B/VSV can bypass effector T cell surveillance, while rVSV-infected DC will present antigen and therefore be killed by effector CTL. Once in the B cell follicular region, viruses will be passed to resident $CD11c^{+}$ cells for antigen presentation. Memory T cells are located in the follicular region and separated from effector T cells. In this scenario, memory T cell expansion can be achieved with the minimum amount of antigen stimulation.

Appendix

Table1. Flow cytometry antibodies

Antibody name	Fluorochrome	Dilution	Clone	Company
B220	PE-TAXAS-RED	1:400	RA4-6B2	BD*
CD4	Per-cp-cy5.5	1:800	RM4-5	BD*
CD8	PE-cy7	1:100	53-6.7	BD*
CD19	PE-CY7	1:500	1D3	BD*
CD21	APC	1:800	7G6	BD*
CD23	PE	1:400	B3B4	BD*
CD27	Biotin	1:200	LG3A10	BD*
CD40	PE	1:100	3/23	BD*
CD83	APC	1:50	Michel-19	BD*
CD86	Biotin	1:50	PO3	BD*
MHC class Ⅱ	FITC	1;50	10-3.6	BD*
IgM	Biotin	1:400	G155-228	BD*
IFN-γ	APC	1:100	XMG1.2	BD*
TNF- α	FITC	1:300	MP6-XT22	BD*

Notes: Cells were stained at 1×10^6/ ml in staining buffer (PBS+0.5% (w/v) bovine serum albumin, BSA) with antibody at the concentration indicated above for 20 minutes.
* BD Biosciences, (San Diego, CA)

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