# In vitro conditioning of antigen-reactive CD8$^+$ T cells with toll-like receptor agonists enhances their expansion in vivo in an adoptive transfer mouse model

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**Research Article**

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# Abstract

**Background:** Anti-cancer adoptive T cell therapy has shown significant anti-tumor responses in cancer patients. This therapy is based on in vitro activation of autologous T cells harvested from a cancer patient and infusing them back through blood. The efficacy of adoptively transferred T cells depends on the in vitro conditioning regimen in particular the type of used cytokines.

**Aim:** This study aimed to use toll like receptors (TLRs) ligands (TLRLs) to condition T cells in vitro as a novel approach instead of cytokines.

**Methods:** Spleen cells were harvested from TCR transgenic C57BL/6 pmel-1 mice, in which $CD8^+$ T cells are engineered to recognize melanoma MHC class-I peptides.Unfractionated splenocytes were cultured for 24 hours in vitro with media or melaoma peptide plus or minus IL-12 (10ng/ml), poly(I:C) (TLR3L; 25ug/ml) or CpG (TLR9L; 10ug/ml). Then, activation, proliferation and cytokine production of the cultured cells were assessed. In another set of experiments, the cultured cells were harvested and infused into syngeneic B6 mice followed by vaccination to evaluate their antigen-specific expansion and contraction.

**Results**: Conditioning of donor splenocytes in vitro with the TLR3L or TLR9L during antigen stimulation increased the antigen specific activation, proliferation, and cytokine production. Interestingly, in vitro treatment of the unfractionated splenocytes with TLR9L enhanced the activation and proliferation of B cells regardless antigen stimulation. Adoptive transfer of TLRL-conditioned peptide stimulated $CD8^+$ T cells into naïve mice showed better survival and higher expansion in response to concomitant vaccination with peptide. Interestingly, in vitro conditioning of $CD8^+$ T cells with TLR9L and in vivo conditioning with TLR3L resulted in the best antigen specific expansion of these cells upon their adoptive transfer into recipient mice.

**Conclusion**: These results show that provision of $CD8^+$ T cells in vitro and in vivo with certain TLR agonists can markedly enhance their antigen specific responses upon their adoptive transfer, opening a potential application of this approach in anti-cancer adoptive immunotherapy.


# Introduction

Adoptive cell therapy (ACT) has recently emerged as one of the most successful immunotherapies. It encompasses a wide range of cell types, including stem cells, T cells are genetically modified to express a chimeric antigen receptor (CAR) [1], dendritic cells, and natural killer cells [2]. These cells from patients' blood can be modified using genetic engineering and modern cell culture techniques to train them to be more effective against certain tumour cells. However, with certain cancer cells types, only select subsets of cells have showed encouraging regression [3].

ACT of autologous tumor-infiltrating lymphocytes (TILs) has been used in humans for over 30 years to treat metastatic melanoma, and continued modifications are making it increasingly more effective

against other types of cancer [4]. TIL-based ACT is a distinctive cell therapy, in which tumor-derived immune cells are expanded in a multi-step process and infused back into the individual [5]. In this setting, T cells are harvested from the tumor-bearing host, stimulated in vitro with the cognate antigen in the presence of IL-2 and then infused back to the same host with the main goal to home to tumor site and attack tumor cells.

Indeed, efficacy of ATC has been found to result in efficacious anti-tumor immunity against several cancers [6,7]. However, ATC still needs to find a way to improve its ability to produce better functional and persistence of adoptively transferred T cells. Conditioning of tumor reactive T cells in vitro with IL-2, IL-12, and IL-15 has been found to enhance the persistence and function of T cells post transfer Additionally, we have reported in a series of our studies that conditioning of T cells with IL-12 during antigen-stimulation in vitro greatly increases the number, survival, and anti-tumor responses of CD8+ T cell after their adoptive transfer into recipient mice [8–12]. These effects of IL-12 were not mediated by the increased proliferation of the T cells in vitro but rather rescued their programmed cell death [12]. Therefore, exploring novel approaches that can more effectively enhance survival, activation and proliferation of T cells both in vitro and in vivo is of a paramount significance to the application of adoptive T cell therapy in the clinical settings.

Toll-like receptors (TLRs) are expressed in innate cells as well as in adaptive (T and B) immune cells [13]. TLRs belong to the family of innate immune receptors, which play an important role in the activation of innate immunity, regulation of cytokine expression, indirect activation of the adaptive immune system, and the recognition of pathogen-associated molecular patterns (PAMPs) [14, 15]. These receptors can be triggered upon binding to their specific agonists, mainly microbial products, resulting in stimulation of immune cells and production of not only one cytokine by a plethora of cytokines [16]. For instance, TLRLs, in particular TLR3 and TLR9Ls, have shown potent adjuvant effects to improve standard vaccination protocols in preclinical [17, 18] and clinical settings [19].

In this context, we have reported that the TLR3L poly(I:C) is a potent adjuvant for $CD8^+$ T cell responses, through increasing T cell number, function, cytokine production, and anti-tumor response [7, 20, 21]. These adjuvant effects were associated with increases in the by increasing the numbers of dendritic cells in mice [20, 22] and in pancreatic cancer patients as well as increases in the numbers of NK cells in mice [23]. In addition, in vivo administration of poly(I:C) at the peak of dendritic cell (DC) expansion aftercyclophosphamide treatment induces inflammatory cytokine production and increases in the number of activated DCs in lymph nodes[22]. It showed also that poly(I:C) target $CD8^+$ T cells directly and activate them in vitro, where adoptive transfer of these cells resulted in appreciated antigen-specific $CD8^+$ T cell response and greater expansion in vivo [20, 22].

Given these in vitro and in vivo adjuvant effects of TLRLs to T cells with the fact that TLRLs act on different populations to induce more than one cytokine, we hypothesized that triggering of TLR signaling in T cells themselves or in the accessory innate immune cells during antigen stimulation of T cells in vitro

pose an interesting alternative to cytokine conditioning of T cells. Under this setting, upon adoptive transfer of in vitro TLRL-conditioned T cells the same or different TLRLs can be administered to stimulate the adoptively transferred T cells as well as the host immune cells to enhance the overall immune responses. We tested this hypothesis in this study and we found that in vitro conditioning of bulk immune cells from spleen with TLRLs can enhance $CD8^+$ T cell expansion in vivo after adoptive transfer.

# Materials And Methods

### Mice

Pmel-1 TCR Tg (C57BL/6; Ly5.2) mice were bred with wild type Ly5.1 mice to generate Ly5.1 mice heterozygous for the pmel-1 TCR Vα1/Vβ13 transgene. The transgene expression was confirmed by PCR analysis. In pmel-1 Tg mice, $CD8^+$T cells expressing the Vα1/Vβ13 TCR specifically recognize the $H\text{-}2D^b$ restricted human $gp100_{25–33}$ epitope (KVPRNQDWL: $gp100_{25–33}$). This peptide represents an altered form of the murine $gp100_{25–33}$ (EGSRNQDWL) with improved binding to the MHC class-I. All animals were housed under specific pathogen-free conditions with institutional and federal guidelines. The research study was approved by the Ethical Committee, Faculty of Science, Tanta University, Egypt, before the commencement of the study. All methods were carried out in accordance with relevant guidelines and regulations.

### Cell lines, antibodies and reagents

Anti-CD16/CD32, fluorescein isothiocyanate, phycoerythrin, allophycocyanin, and cytochrome-conjugated mAbs, including anti-Ly5.1, anti-CD4, anti-CD8, anti-NK1.1, anti-CD62L, anti-CD11c, anti-CD25, anti-CD40, anti-MHC-I, anti-B220, anti-CD80, anti-CD69, and anti-CD86 were purchased from Pharmingen (San Diego, CA). Human $gp100_{25–33}$ EGSRNQDWLL melanoma peptide (American Peptide Company) was dissolved in 10% DMSO (Sigma) and diluted in phosphate buffer saline (PBS). The TLR3L, poly(I:C) and TLR9L, CpG ODN 1826 were purchased from InvivoGen. Poly(I:C), CpG, IL-12, and hgp100 were stored at -80°C, and reconstituted in PBS before use.

### Measurement of cytokines in vitro

Splenocytes from pmel-1 mice were prepared in complete RPMI 1640 medium, and $5x10^6$ spleen cells were cultured and pulsed with hgp100 peptide (5 mg/mL) with either IL-12 (10ng/mL), poly(I:C) (25ug/mL), or CpG ODN 1826 (5ug/mL). Supernatants were collected 24 hours later and the levels of IFN-γ and TNF-α were measured by cytokine bead array (Pharmingen).

### Culture of CFSE-labeled cells

Spleen cells from Pmel-1 mice were harvested, homogenized, and washed in Hank's balanced salt solution (HBSS). Pooled cells were labeled with 0.5uM CFSE and cultured in 6-well plate at $1x10^6$ cells/ml

for 72 hours in culture media alone (1mL), hgp100 (1ug/mL), poly(I:C) (50ng/mL), imiquimod (10ng/mL), or CpG ODN 1826 (5ng/mL). Cells were analyzed for phenotypic analysis by flow cytometry.

### Flow cytometry

Fresh single-cell suspensions were prepared and $1x10^6$ cells were incubated with anti-CD16/CD32 for 5 minutes on ice to block non-specific binding. Cells were then stained with the indicated conjugated mAb for phenotypic analysis, and incubated for 30 minutes on ice. The cells were then harvested, washed twice, and resuspended in 0.3mL of 0.5% bovine serum albumin, 0.02% sodium azide solution. Cells were analyzed by flow cytometry using the Cell Quest software package (Becton Dickinson, San Jose, CA).

### Lymphodepletion, adoptive cell transfer and vaccination

Spleen from Pmel-1 TCR transgenic mice were harvested, homogenized, and washed in HBSS (Cellgro, Herndon, VA). Pooled cells were cultured for 72 hours in culture media with peptide (1ug/mL hgp100) plus or minus poly(I:C) (50ng/mL), CpG ODN 1826 (5ng/mL), or IL-12 (10ng/mL) for 3 days at $37^0$C and 5% $CO_2$. Pmel-1 cells were also cultured for 3 days in the presence of the same doses of poly(I:C), CpG, or IL-12 but in absence of peptide stimulation. In vitro Pmel-1 splenocytes ($1 \times 10^6$/300μL) conditioned as above were adoptively transferred into Ly5.2 B6 recipient mice via tail vein injection. Twenty four hours before adoptive cell transfer, these recipient mice were treated via intraperitoneal (i.p.) injection with with 4ug/mouse CTX. In some experiments, mice were also treated with 100μg $hgp100_{25\text{-}33}$ in 100mL PBS by subcutaneous (s.c.) injection with or without concomitant intrapeitoneal (i.p.) administration of 200 mg poly (I:C) in 300 mL PBS 1 day 7days after adoptive transfer. Pmel-1 $CD8^+$ T cells were monitored at the indicated time points post cell transfer by flow cytometry after staining cells with anti-Ly5.1 and anti-CD8 mAb.

### Statistics

Statistical analyses were performed using the Student's t-test. Log-rank nonparametric analysis using GraphPad Prism (GraphPad Software, Inc.) was used to graph and analyze the survival data. All P values were two sided, with $p<0.05$ considered significant.

## Results

### TLR3L and TLR9L enhance proliferation and cytokine production of T cells during antigen stimulation in vitro

Given our recent studies in which we reported the beneficial effects of IL-12 to enhance CD8+ T cell responses *in vitro* [24], we wanted first to test if conditioning unfractionated Pmel-1 splenocytes with TLRLs can induce greater cell expansion and cytokine production in vitro as compared to IL-12. To this end, un-fractionated Pmel-1 splenocytes were cultured with either IL-12, the TLR3L poly(I:C), or the TLR9L CpG in the presence of peptide stimulation for 72 hrs. we found that both Poly(I:C) and CpG enhanced the

proliferation of Pmel-1 cells (Fig. 1A) as well as their function measured by IFN-γ (Fig. 1B), TNF-α (Fig. 1C), and IL-2 (Fig. 1D). Of note, the cells conditioned with CpG elicited higher effects than those of poly(I:C) and IL-12. This supports the role of TLRLs in general and TLR9 in particular in augmenting cytokine production. Together, these data indicate that conditioning T cells with TLRLs during antigen presentation can result in comparable if not better production of inflammatory cytokines as compared to IL-12.

### Treatment of splenocytes in vitro with TLR3 or TLR9 agonists induced proliferation of non-$CD8^+$ cells with a $CD62L^{low}$ phenotype

Because we used unfractionated splenocytes in Figure 1 above, we next determined whether the fraction of non-Pmel-1 T cells (i.e. antigen non-specific $CD8^+$ cells) that exists in the culture contributed to the enhanced overall proliferation shown in Figure 1. To this end, unfractionated splenocytes from Pmel-1 mice were labeled with CFSE and cultured in media alone, poly(I:C), or CpG in the presence or absence of antigen.Then, the cell proliferation was measured after 72 hrs by flow cytometry after gating on $CD8^+$ T cells and non-CD8+ T cells (i.e. other cells in the culture such as $CD4^+$ T cells, B cells, macrophages and dendritic cells). Under this setting, most of the $CD8^+$ T cells are reactive to peptide.

We found that in absence of peptide stimulation, TLR3L and TLR9L induced proliferation of only non-$CD8^+$ T cells (Fig. 2A, upper panel). As expected, antigen stimulation alone resulted in greater proliferation of Pmel-1 $CD8^+$ T-cells (antigen-reactive) than co-stimulation with either TLRL (Fig. 1A, **lower panel**). However, concomitant stimulation with peptide and each of TLRL increased the proliferation of both non-$CD8^+$ and $CD8^+$ T cell levels (Fig. 1A, **lower panel**). Down regulation of the homing molecule CD62L on immune cells indicates to their activation. Indeed, treatment with either of TLRL alone induced a $CD62L^{low}$ phenotype on non-$CD8^+$ T-cells, with CpG inducing a vastly larger population than poly(I:C) (Fig. 2B **upper panel**), indicating that non-$CD8^+$ T cells also expressed the activation phenotype. These cells were still increased in number in the presence of peptide stimulation (Fig. 2B, **lower panel**), suggesting that conditioning cells with TLRLs can induce activation and proliferation of non-$CD8^+$ T cells even in the absence of antigen-stimulation.

### TLR3L and TLR9L induced activation and proliferation of B cells

Noting the effects of TLR3L and TLR9L on non-CD8 cells above, we sought to identify the non-$CD8^+$ T cell population responsible for this marked proliferation. To this end, we cultured CFSE-labelled Pmel-1 cells as above with poly(I:C) or CpG. Cells were then harvested and stained for specific immune cells, including dendritic cells ($CD11c^+$), $CD4^+$ T cells, NK cells (NK1.1), and B cells. Interestingly, both TLR3L and TLR9L induced expansion of B cells in absence of antigen stimulation (Fig. 3A). Notably, the TLR9L CpG induced higher proliferation (> 90%) of B cells as compared to poly(I:C) as well as the TLR7L imiquimode, which we usd here as another control for TLR9L.

Given this unique higher effect of CpG, we repaeated the same experiments but with only CpG in the presence or absence of antigen stimulation. Interestingly, CpG also induced B cell proliferation (Fig. 3B **lower panel**) but with less effects than in the absence of antien stimulation (Fig. 3B, **upper panel**), demonstrating that triggering of TLR9 signaling pathway during antigen-specific activation of $CD8^+$ T cells can yield concomitant expansion of B (($B220^+$) cells and $CD8^+$ T cells in vitro. Accordingly, these data indicate that the TLR-induced enhancement in cell proliferation in Fig. 1A is mediated in part by the increased non-specific proliferation of B cells.

### CpG-induced proliferation of B cells associates with their activation

We tested whether the TLR9L-induced proliferation of $B220^+$ cells paralleled the reported up-regulation of co-stimulatory molecules. To this end, we cultured unfractionated splenocytes from pmel-1 mice in vitro with or without peptide stimulation and treated these cells with media alone or with CpG for 24 hours. We found that both in the presence (Fig. 4A) and in the absence (Fig. 4B) of peptide stimulation, $B220^+$ cells showed expression of CD25, CD69, CD40, CD80, and CD86. CD80 and MHC-I were only marginally affected (data not shown). Treatment of pmel-1 cells with with CpGin the presence of antigen stimulation increased the percentage of B cells ($B220^+$) expressing CD25, CD69 and CD40 as compared to with no CpG (Fig. 4A, **upper panel**). Interestingly, treatment of pmel-1 cells with CpG in the absence of antigen stimulation also increased the pmel-1 cells expressing these CD25, CD69 and CD40 molecules as well as CD86 as compared to cells treated with media alone (Fig. 4B, **lower panel**). Of note, the effects of CpG on the activation of B cells was higher in the abence of antigen stimulation as compared with the presence of antigen.

### Triggering of TLR3 and TLR9 in naïve $CD8^+$ T cells in vitro enhanced their homeostatic expansion expansion in vivo.

The data shown above conclude that conditioning of pmel-1 $CD8^+$ T cells with TLRLs, namely CpG, elicited cytokine production comparable to IL-12 as well as superior expansion and activation of a mature population of B cells with up-regulation of co-stimulatory molecules. Given these data, we hypothesized that upon injection of cells conditioned with TLRLs during antigen stimulation in vitro, these cells could affect the number of antigen-specific $CD8^+$ T cells post adoptive transfer. To address this hypothesis, we conditioned unfractionated splenocytes from Pmel-1 mice with IL-12, TLR3L, or TLR9L in the presence of peptide and then adoptively transferred cells into naïve mice on day 0. The percentage of transgenic pmel-1 $CD8^+$ T cells was analyzed in the peripheral blood on day 5 after adoptive transfer to measure the homeostatic-driven proliferation of transgenic $CD8^+$ T cells. Overall, we found that the percentage of these cells was low (<1%) in the blood of all groups (Fig. 5A). However the cells that were conditioned in vitro with CpG showed relatively higher numbers (0.6%) as compared to cells conditioned with PPS (<0.1%), IL-12 (<0.3%) and TLR3L (<0.1%) (Fig. 5A).

### Triggering TLR3 and TLR9 in $CD8^+$ T cells during antigen presentation in vitro enhanced antigen-specific $CD8^+$ T cell expansion in vivo.

In addition, our group established poly(I:C) as a potent adjuvant in vaccination regimens (Salem, 2005; Salem, 2006; Salem, 2009; Salem, 2007; Salem et al., 2020). We then conducted the same experiment except this time administering vaccination with or without poly(I:C) on day 7 after adoptive cell transfer and analyzed the cell numbers in the peripheral blood on day 12. Adoptive transfer of T cells was preceded 24 hour with preconditioning of the host with CTX. Vaccination with peptide alone enhanced the expansion of $CD8^+$ cells by about 10-fold (Fig. 5B). The expansion of the cells was further increased by about 10-fold when the adjuvant poly(I:C) was mixed with the peptide during vaccination (Fig. 5C). Comparable to the in vitro experiments (Fig. 1A), conditioning with CpG elicited superior expansion compared to poly(I:C) and IL-12 (Fig. 5C). These data indicate that the enhanced proliferation and activation of B cells during conditioning un-fractionated pmel-1 cells in vitro with TLR9L may explain the enhanced survival and antigen-specific expansion of the pmel-1 cells in vivo. Furthermore, these data indicate that condition of $CD8^+$ T cells in vitro with CpG enhances their homeostatic-driven expansion in vivo.

**Brief conditioning of $CD8^+$ T cells** in vitro **with TLR9L enhances the responses of these cells to vaccination and TLR3L** in vivo

The data described above showed that conditioning of unfractionated cells with TLR9L during peptide presentation in vitro can enhance $CD8^+$ T cell expansion in vivo after vaccination with peptide and poly(I:C). To understand whether this beneficial effect of TLR9L to $CD8^+$ T cells depends on the antigen presentation microenvironment in vitro, we conditioned unfractionated Pmel-1 splenocytes with TLR3L or TLR9L for 72 hours and then adoptively transferred them on day 0 into recipient mice pretreated 24 hours before with CTX. Mice transferred with TLR3L-conditioned or TLR9L-conditioned T cells were then vaccinated with peptide mixed with either the TLR3L poly(I:C) or the TLR9L CpG. Under this setting, TLR-conditioned cells were challenged in vivo with the same TLRLs used in vitro or with a different TLRL. The mice were then re-challenged after 30 days, the time point when all cells are contracted and become resting memory cells. As shown in Figure 6, the cells conditioned either in the presence of TLRL showed similar expansion to vaccination in vivo regardless whether the vaccination was mixed with relevant or irrelevant TLRL. Of note, the CpG-conditioned cells showed the highest expansion when measured on day 5 post adoptive transfer and peptide vaccination. When the cells were contracted by day 30 and then challenged with peptide mixed with relevant or irrelevant TLRL, the in vitro TLR9-conditioned T cells showed the highest expansion in vivo but only when TLR3L was added to the vaccination. These data are consistent with those in Figure 5, showing high expansion of cells that was conditioned in vitro with TLR9L and in vivo with peptide + poly(I:C). Of note, when the expansion levels of the adoptively transferred T cells in Figures 5 and 6 are compared, it appears that cells conditioned in vitro with TLR9L in absence of peptide stimulation expand in vivo with a higher rate than those conditioned in vitro in the presence of antigen presentation. Taken the results in Figures 5 and 6 together, it can be suggested that just brief conditioning of T cells in vitro with TLR9L alone can enhance the responses of these cells to vaccination and poly(I:C) in vivo upon adoptive transfer.

# Discussion

Although recent studies, including ours, have been dedicated to evaluate and dissect the adjuvant effects of different TLRLs in vitro, no studies addressed the possible use of TLRLs to condition T cells in vitro to enhance its application in adoptive T cell therapy. This study demonstrated that conditioning of naïve or antigen-stimulated $CD8^+$ T cells with the TLR3L poly(I:C) and the TLR9L CpG induced activation and proliferation of cells expressing $B220^+$, the typical marker for B lymphocytes. These effects did not prevent antigen-stimulated $CD8^+$ T cells from responding to antigen and the resultant proliferation. Interestingly, our data show for the first time that TLR conditioning of $CD8^+$ T cells in vitro in bulk population of immune cells (i.e. un-fractionated population containing all immune cells including B cells) resulted in enhancement of the antigen-specific expansion of $CD8^+$ T cells upon their adoptive transfer into a recipient host followed by peptide vaccination and concomitant administration of a TLRL as an adjuvant. The antigen-driven expansion of the adoptively transferred TLRL-conditioned $CD8^+$ T cells was greatly enhanced without in vitro antigen stimulation and when the TLRLs used in vitro and in vivo are different. These data allow us to suggest that provision of heterologous TLRLs during antigen priming and boosting of $CD8^+$ T cells can markedly enhance the antigen specific responses than the use of homologous TLRLs. These data have a potential application in anti-cancer adoptive immunotherapy. The success of anti-tumor immunity depends on the generation of effector T cells that can differentiate into functional long-lived memory cells [25, 26]

Adoptive cell therapy (ACT) has slowly been expanded to other cancer types using new approaches such as genetically engineered T-cells and other methods of antigen targeting. It now appears that immune targeting of mutated “neoantigens” plays a major role in successful ACT, as well as in other immunotherapies such as checkpoint inhibitors[27]. This modality consists of in vitro stimulation of T cells from the patient own peripheral blood or tumor with a specific tumor antigen and cytokines and then infusing these cells back to the patient blood in combination with stimulatory cytokines such as IL-2. In adoptive T cell therapy, T cells are often stimulated with antigen in vitro in the presence of certain cytokines known to enhance the growth and survival of T cells during antigen stimulation in which Tcells can recognize unaltered tissue-differentiation antigens on tumors[28]. Conlon et al., 2019 and Dwyer et al., 2019 reported that IL-2, IL-7, IL-12, IL-15, and IL-21 are among the candidate cytokines that improve the survival and proliferation of T cells in vitro.

Indeed, this study has been focusing on improving the efficacy of adoptive T cell therapy approach by enhancing the functions of T cells before the adoptive transfer by treating T cells with IL-12 (Redeker and Arens, 2016) or by transducing them with a T cell receptor of high tumor-reactivity (Ping et al., 2018). Among the immunostimulatory cytokines that potentiate immune responses against cancer, such as IFN-α, the IL-2 family, IL-12, granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-10 to enhance the responses of the adoptively transferred T cells to peptide-based vaccination [29]. Furthermore, we have used the TLR3L poly(I:C) as an adjuvant in vivo to induce rapid induction of inflammatory cytokines and the activation of NK cells and dendritic cells [30]. We have extended our

studies and showed here that subsequent conditioning of bulk immune cells in vitro with TLR3 or TLR9L regardless concomitant activation with cognate antigen can greatly enhance the antigen-driven expansion of $CD8^+$ T cells upon their adoptive transfer in a recipient host.

Although both the TLR3L poly(I:C) and the TLR9L CpG enhanced the proliferation and activation of B cells among the other immune cells in vitro, our studies clearly showed that the nature of TLR conditioning in vitro and in vivo is critical for the optimal $CD8^+$ T cell expansion [31, 32]. Specifically, our data showed that conditioning of un-fractionated immune cells in vitro with CpG followed by conditioning with poly(I:C) upon adoptive transfer and vaccination induced higher expansion of $CD8^+$ T cells than conditioning with CpG both in vitro and in vivo or poly(I:C) in vitro and in vivo or poly(I:C) in vitro and CpG in vivo[20]. These studies open new avenue for the use of TLRLs not only as adjuvants to T cells in vivo, but also as a conditioning factor in vitro during adoptive T cell transfer. This hypothesis is supported by the results of a recent study showing that adoptive co-transfer of the TLRL2 BLP-pretreated but not untreated cytotoxic T-lymphocytes (CTL) and regulatory T-cells ( $T_{reg}$) from wild-type but not from TLR2 knockout mice was sufficient to restore antitumor immunity in SCID mice[33]. Further, another study showed that co-adoptive transfer of activated transgenic OT-1 $CD8^+$ T cells in vitro with peptide-pulsed B cells previously conditioned with or without CpG induced upregulation of co-stimulatory molecules on B cells, proliferation of $CD8^+$ antigen-specific T cells in vivo, and significant protection against tumor growth [34] [35].

Although we have not investigated in the present study the mechanisms behind this beneficial effects of conditioning with heterologous TLRLs as compared to those of homologous TLRLs, it could be suggested that conditioning with the TLR9L CpG induces upregulation of the TLR3 in T cells and as a consequences increases the responses of the TLR3L poly(I:C) on the T cells during antigen encounter in vivo. Indeed, our studies demonstrated high level of TLR3 expression in T cells stimulated with TLR3L poly(I:C), the T cell mitogen concavalin A or the cognate antigen, indicating that mono- or polyclonal stimulation of T cells can upregulate their expression level of TLR3. On the other hand, the beneficial effects of conditioning with TLRLs, in particular CpG, to the expansion of T cells in vivo could be explained at least in part by the enhanced proliferation and activation of B cells by CpG in vitro. These effects would instruct B cells to acquire better antigen presenting function in vivo upon adoptive transfer and allow them to cross prime antigen peptide to Pmel-1 cells and thus contribute to the enhanced expansion of the co-transferred $CD8^+$ T cells. Indeed, class-B CpG ODNs, like 1826, primarily target B cells causing them to differentiate into plasma cells.

TLR9 activation has been seen to dramatically increase hybridoma generation of purified B cells from 1-2% to 30-100%, supporting the high proliferative effect on B cells seen in our work [36]. The pursuing events include stimulation of antigen-specific B cells [37], inhibition of B cell apoptosis [38], enhancement of IgG class switch DNA recombination [39], and DC maturation and differentiation, resulting in increased activation of Th1 cells and strong CTL generation even in the absence of $CD4^+$T cell help [40]. CpG has also been found to upregulate the expression of CD40, CD80, CD86, and CD54 co-stimulatory molecules

on primary human and malignant B cells without altering the B cell phenotype. MHC class I and II was also increased in most samples [41], although in our experiments the effect on MHC I expression was minimal (data not shown).

Our work supports the effect of CpG class-B ODNs on upregulating co-stimulatory molecules on primary B cells. These effects of poly(I:C) and CpG on B cells might result in indirect activation of $CD8^+$ T cells and enhance their responses to in vivo vaccination upon adoptive transfer. These TLRLs might also impact $CD8^+$ T cell function by eliciting a milieu of cytokine by B cell themselves or other cells such as dendritic cells and NK cells. Consistent with this notion, bone marrow derived dendritic cells conditioned with poly(I:C) produced inflammatory cytokines like IFN-γ [20] and plasmacytoid dendritic cells activated by CpG secreted several cytokines and chemokine [42] [43]. In addition, NK cells are activated by CpG ODNs to become cytotoxic and secrete high levels of IFN-γ, but the same NK cell may not necessarily engage in both cytokine secretion and cytotoxicity [44].

The beneficial effects of the combinatorial treatment with CpG in vitro and poly (I:C) in vivo to augment $CD8^+$ T cell responses in vivo is consistent with previous studies showing that combination of these TLRLs in vivo resulted in enhancement of $CD8^+$ T cell response in a DC-receptor targeted DNA vaccine. The value of combination therapy was initially examined under conditions where a single TLR agonist only delayed tumor growth. Intra-tumoral delivery of CpG ODN was considerably more effective than systemic administration for the treatment of tumors[45] and resulted in enhanced tumor rejection of established tumor when combined with heterologous p53 immunization [46]. Also, mice who received mesothelioma tumors and were then treated with CpG combined with poly (I:C) strongly suppressed tumor growth and resulted in a long-term tumor-free survival coincided with increases in CD8+ T cell responses [47]. The triple combination of plasmid DNA encoding CD40L plus CpG and polyinhibited tumor growth and increased survival in B16F10 melanoma murine models with a significant increase in CD8+ T cells [47].

This study shows that CpG-conditioning of bulk immune cells in vitro can enhance proliferation of antigen-specific $CD8^+$ T cells upon their adoptive transfer and concomitant administration of poly(I:C) and vaccine. The implications of including TLRLs, namely TLR3 and TLR9, to improve $CD8^+$ T cell expansion post-transfer warrants further investigation in other preclinical models and human subjects.

# Declarations

**Acknowledgment:** Not applicable

**Author contributions:** Mohamed Salem and Sohaila Khalil wrote the manuscript. Ashraf and rehab prepared the figures. All authors revised the manuscript**.**

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**Competing interests:** The authors declared that there was no competing interest.

**Ethic approval and consent to participate**

The research study was approved by the Ethical Committee, Faculty of Science, Tanta University, Egypt, before the commencement of the study. All methods were carried out in accordance with relevant guidelines and regulations. The use of animal in the study is in accordance with ARRIVE guidelines.

**Consent for publication:** Not Applicable.

# References


1. Moreno, V., et al., *Adoptive cell therapy for solid tumors: Chimeric antigen receptor T cells and beyond*. Curr Opin Pharmacol, 2021. **59**: p. 70–84.
2. Wang, Z. and Y.J. Cao, *Adoptive Cell Therapy Targeting Neoantigens: A Frontier for Cancer Research*. Front Immunol, 2020. **11**: p. 176.
3. Chan, J.D., et al., *Cellular networks controlling T cell persistence in adoptive cell therapy*. Nat Rev Immunol, 2021.
4. Rohaan, M.W., et al., *Adoptive transfer of tumor-infiltrating lymphocytes in melanoma: a viable treatment option*. J Immunother Cancer, 2018. **6**(1): p. 102.
5. Morotti, M., et al., *Promises and challenges of adoptive T-cell therapies for solid tumours*. Br J Cancer, 2021. **124**(11): p. 1759–1776.
6. Ndejembi, M.P., A.L. Tang, and D.L. Farber, *Reshaping the past: Strategies for modulating T-cell memory immune responses*. Clin Immunol, 2007. **122**(1): p. 1–12.
7. Bonavida, B. and S. Chouaib, *Resistance to anticancer immunity in cancer patients: potential strategies to reverse resistance*. Ann Oncol, 2017. **28**(3): p. 457–467.
8. Diaz-Montero, C.M., et al., *Synergy of brief activation of CD8 T-cells in the presence of IL-12 and adoptive transfer into lymphopenic hosts promotes tumor clearance and anti-tumor memory*. Am J Cancer Res, 2011. **1**(7): p. 882–96.
9. Rubinstein, M.P., et al., *Ex vivo interleukin-12-priming during CD8(+) T cell activation dramatically improves adoptive T cell transfer antitumor efficacy in a lymphodepleted host.* J Am Coll Surg, 2012. **214**(4): p. 700-7; discussion 707-8.
10. Rubinstein, M.P., et al., *Interleukin-12 enhances the function and anti-tumor activity in murine and human CD8(+) T cells*. Cancer Immunol Immunother, 2015. **64**(5): p. 539–49.
11. Andrijauskaite, K., et al., *IL-12 conditioning improves retrovirally mediated transduction efficiency of CD8+ T cells*. Cancer Gene Ther, 2015. **22**(7): p. 360–7.

12. Diaz-Montero, C.M., et al., *Priming of naive CD8+ T cells in the presence of IL-12 selectively enhances the survival of CD8+CD62Lhi cells and results in superior anti-tumor activity in a tolerogenic murine model*. Cancer Immunol Immunother, 2008. **57**(4): p. 563–72.
13. Kawasaki, T. and T. Kawai, *Toll-like receptor signaling pathways*. Front Immunol, 2014. **5**: p. 461.
14. Khanmohammadi, S. and N. Rezaei, *Role of Toll-like receptors in the pathogenesis of COVID-19*. J Med Virol, 2021. **93**(5): p. 2735–2739.
15. Lester, S.N. and K. Li, *Toll-like receptors in antiviral innate immunity*. J Mol Biol, 2014. **426**(6): p. 1246–64.
16. Khan, A.A., Z. Khan, and S. Warnakulasuriya, *Cancer-associated toll-like receptor modulation and insinuation in infection susceptibility: association or coincidence?* Ann Oncol, 2016. **27**(6): p. 984–997.
17. Salem, M.L., Z.I. Attia, and S.M. Galal, *Acute inflammation induces immunomodulatory effects on myeloid cells associated with anti-tumor responses in a tumor mouse model*. J Adv Res, 2016. **7**(2): p. 243–53.
18. Madan-Lala, R., P. Pradhan, and K. Roy, *Combinatorial Delivery of Dual and Triple TLR Agonists via Polymeric Pathogen-like Particles Synergistically Enhances Innate and Adaptive Immune Responses*. Sci Rep, 2017. **7**(1): p. 2530.
19. Luchner, M., S. Reinke, and A. Milicic, *TLR Agonists as Vaccine Adjuvants Targeting Cancer and Infectious Diseases*. Pharmaceutics, 2021. **13**(2).
20. Salem, M.L., et al., *The TLR3 agonist poly(I:C) targets CD8+ T cells and augments their antigen-specific responses upon their adoptive transfer into naive recipient mice*. Vaccine, 2009. **27**(4): p. 549–57.
21. Salem, M.L., et al., *Kinetics of rebounding of lymphoid and myeloid cells in mouse peripheral blood, spleen and bone marrow after treatment with cyclophosphamide*. Cell Immunol, 2012. **276**(1-2): p. 67–74.
22. Apostolico, J.S., et al., *Poly(I:C) Potentiates T Cell Immunity to a Dendritic Cell Targeted HIV-Multiepitope Vaccine*. Front Immunol, 2019. **10**: p. 843.
23. Salem, M.L., et al., *The Toll-Like Receptor 3 Agonist Polyriboinosinic Polyribocytidylic Acid Increases the Numbers of NK Cells with Distinct Phenotype in the Liver of B6 Mice.* J Immunol Res, 2020. **2020**: p. 2489407.
24. Renavikar, P.S., et al., *IL-12-Induced Immune Suppressive Deficit During CD8+ T-Cell Differentiation*. Front Immunol, 2020. **11**: p. 568630.
25. Cui, W. and S.M. Kaech, *Generation of effector CD8+ T cells and their conversion to memory T cells*. Immunol Rev, 2010. **236**: p. 151–66.
26. Ando, M., et al., *Memory T cell, exhaustion, and tumor immunity*. Immunol Med, 2020. **43**(1): p. 1–9.
27. Dzhandzhugazyan, K.N., P. Guldberg, and A.F. Kirkin, *Adoptive T cell cancer therapy*. Nat Mater, 2018. **17**(6): p. 475–477.

28. Restifo, N.P., M.E. Dudley, and S.A. Rosenberg, *Adoptive immunotherapy for cancer: harnessing the T cell response*. Nat Rev Immunol, 2012. **12**(4): p. 269–81.
29. Berraondo, P., et al., *Cytokines in clinical cancer immunotherapy*. Br J Cancer, 2019. **120**(1): p. 6–15.
30. Matsumoto, M., et al., *Defined TLR3-specific adjuvant that induces NK and CTL activation without significant cytokine production in vivo*. Nat Commun, 2015. **6**: p. 6280.
31. Li, Q., et al., *Toll-Like Receptor 7 Activation Enhances CD8+ T Cell Effector Functions by Promoting Cellular Glycolysis*. Front Immunol, 2019. **10**: p. 2191.
32. Pahlavanneshan, S., et al., *Toll-Like Receptor-Based Strategies for Cancer Immunotherapy*. J Immunol Res, 2021. **2021**: p. 9912188.
33. Zhang, Y., et al., *TLR1/TLR2 agonist induces tumor regression by reciprocal modulation of effector and regulatory T cells*. J Immunol, 2011. **186**(4): p. 1963–9.
34. Guo, S., et al., *Induction of protective cytotoxic T-cell responses by a B-cell-based cellular vaccine requires stable expression of antigen*. Gene Ther, 2009. **16**(11): p. 1300–13.
35. Haller, S., et al., *Interleukin-35-Producing CD8alpha(+) Dendritic Cells Acquire a Tolerogenic State and Regulate T Cell Function*. Front Immunol, 2017. **8**: p. 98.
36. Akkaya, M., et al., *Toll-like receptor 9 antagonizes antibody affinity maturation*. Nat Immunol, 2018. **19**(3): p. 255–266.
37. Krieg, A.M., et al., *CpG motifs in bacterial DNA trigger direct B-cell activation*. Nature, 1995. **374**(6522): p. 546–9.
38. Kennedy, R. and E. Celis, *T helper lymphocytes rescue CTL from activation-induced cell death*. J Immunol, 2006. **177**(5): p. 2862–72.
39. Xu, Z., et al., *Immunoglobulin class-switch DNA recombination: induction, targeting and beyond*. Nat Rev Immunol, 2012. **12**(7): p. 517–31.
40. Kaiko, G.E., et al., *Immunological decision-making: how does the immune system decide to mount a helper T-cell response?* Immunology, 2008. **123**(3): p. 326–38.
41. Jahrsdorfer, B., et al., *CpG DNA increases primary malignant B cell expression of costimulatory molecules and target antigens*. J Leukoc Biol, 2001. **69**(1): p. 81–8.
42. Krieg, A.M., *Toll-like receptor 9 (TLR9) agonists in the treatment of cancer*. Oncogene, 2008. **27**(2): p. 161–7.
43. Mathan, T.S., C.G. Figdor, and S.I. Buschow, *Human plasmacytoid dendritic cells: from molecules to intercellular communication network*. Front Immunol, 2013. **4**: p. 372.
44. Barrow, A.D., et al., *Natural Killer Cells Control Tumor Growth by Sensing a Growth Factor*. Cell, 2018. **172**(3): p. 534-548 e19.
45. Zhao, B.G., et al., *Combination therapy targeting toll like receptors 7, 8 and 9 eliminates large established tumors*. J Immunother Cancer, 2014. **2**: p. 12.
46. Ishizaki, H., et al., *Heterologous prime/boost immunization with p53-based vaccines combined with toll-like receptor stimulation enhances tumor regression*. J Immunother, 2010. **33**(6): p. 609–17.

47. Stone, G.W., et al., *Regression of established AB1 murine mesothelioma induced by peritumoral injections of CpG oligodeoxynucleotide either alone or in combination with poly(I:C) and CD40 ligand plasmid DNA*. J Thorac Oncol, 2009. **4**(7): p. 802–8.

# Figures

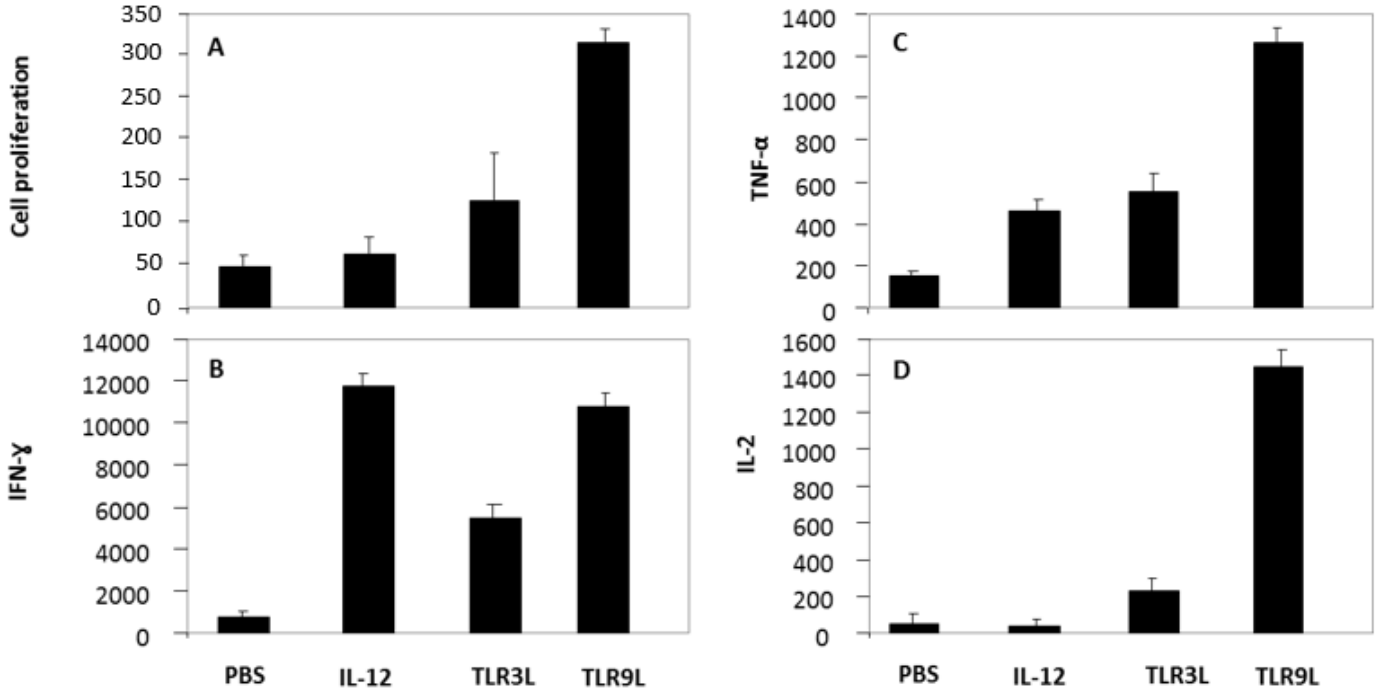
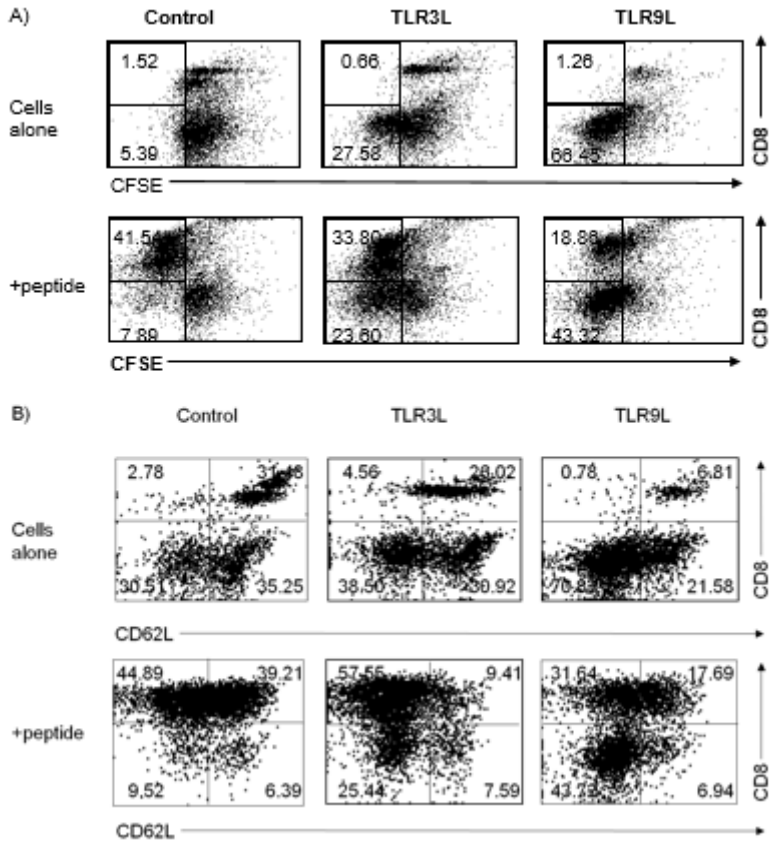


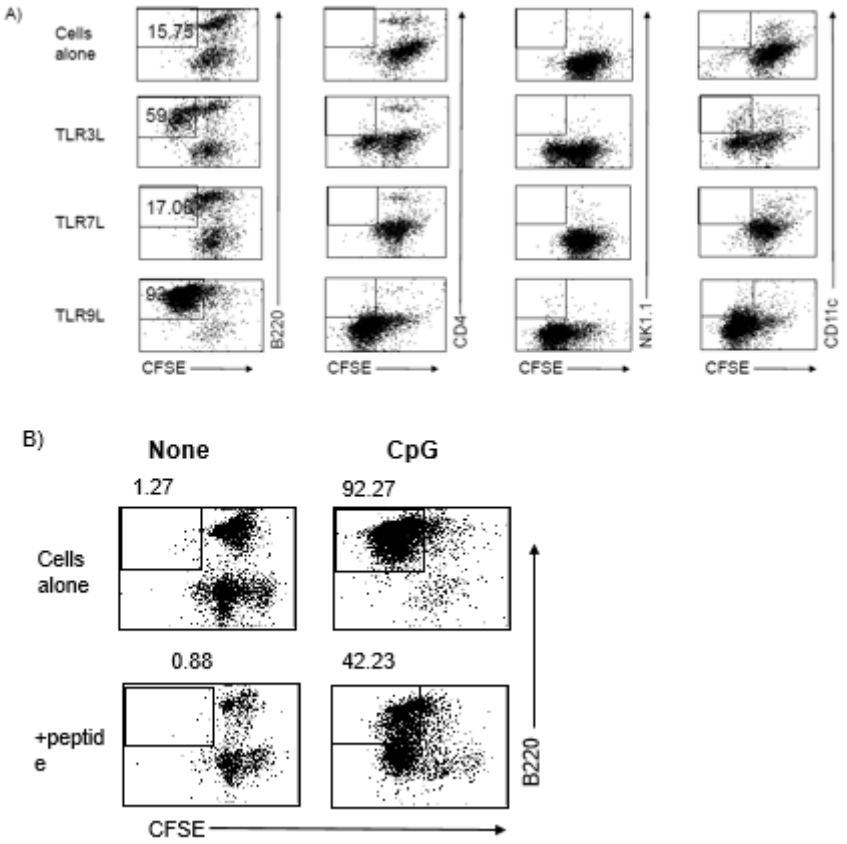

**Figure 1**

**Conditioning of pmel-1 cells with TLR ligands in the presence of antigen stimulation enhances their proliferation and function.** Unfractionated pmel-1 splenocytes were cultured with hgp100 (1ug/mL) peptide alone (1ug/mL) or with TLR3L (50ng/mL), TLR9L (10ng/mL), or IL-12 (10ng/mL). Cell proliferation was measured by MTT assay. Cytokine production was assessed measuring TNF-α, IFN-γ and IL-2 levels in the supernatant of the cultured cells.



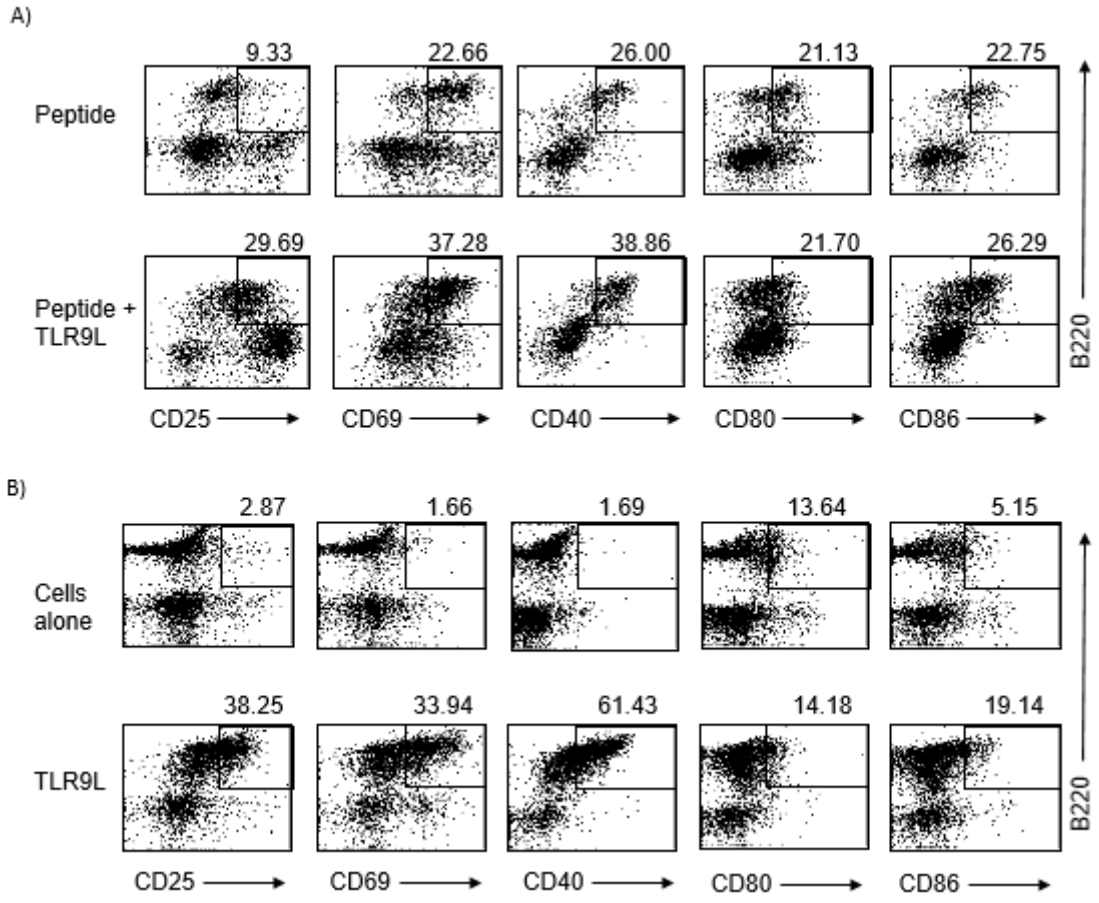

## Figure 2

**TLR3L and TLR9L induce proliferation of non-CD8 cells with a CD62L- phenotype.** Unfractionated Pmel-1 cells were labeled with 0.5uM CFSE and cultured with either TLR3L (50ng/mL) or TLR9L (10ng/mL) in the absence or presence of hgp100 (1ug/mL) stimulation. After 3 days, CD8 (A) proliferation and (B) CD62L expression were measured.



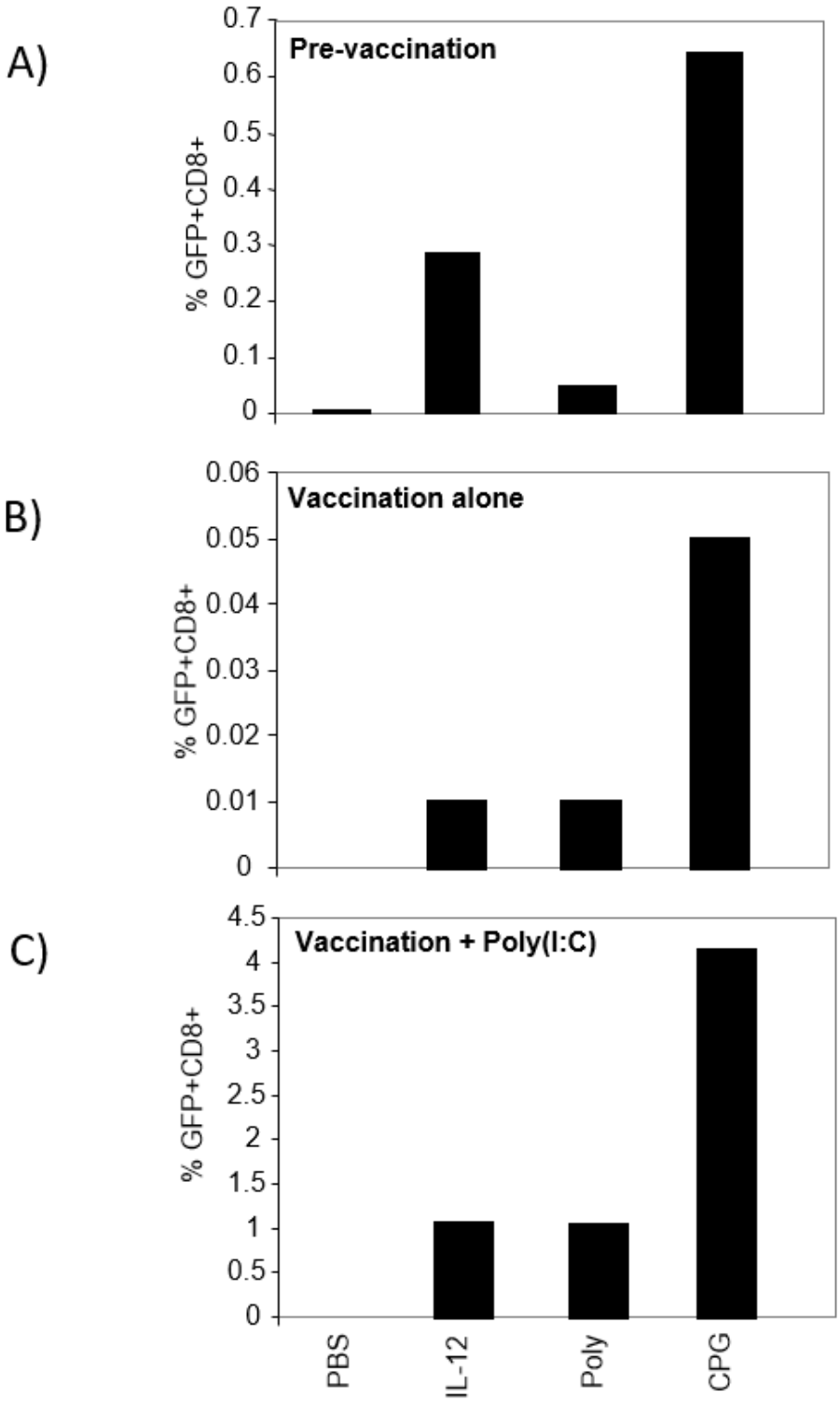

## Figure 3

**TLR3L and TLR9L induce proliferation of B220+ cells.** Unfractionated Pmel-1 cells were labeled with CFSE (0.5uM) and cultured with TLR3L (50ng/mL) or TLR9L (10ng/mL) in the presence or absence of hgp100 (1ug/mL) stimulation. (A) After 3 days, cells were harvested and stained for activation markers (B220, CD4, NK1.1, CD11c). (B) B220 proliferation was measured in the presence and absence of peptide during CpG stimulation.



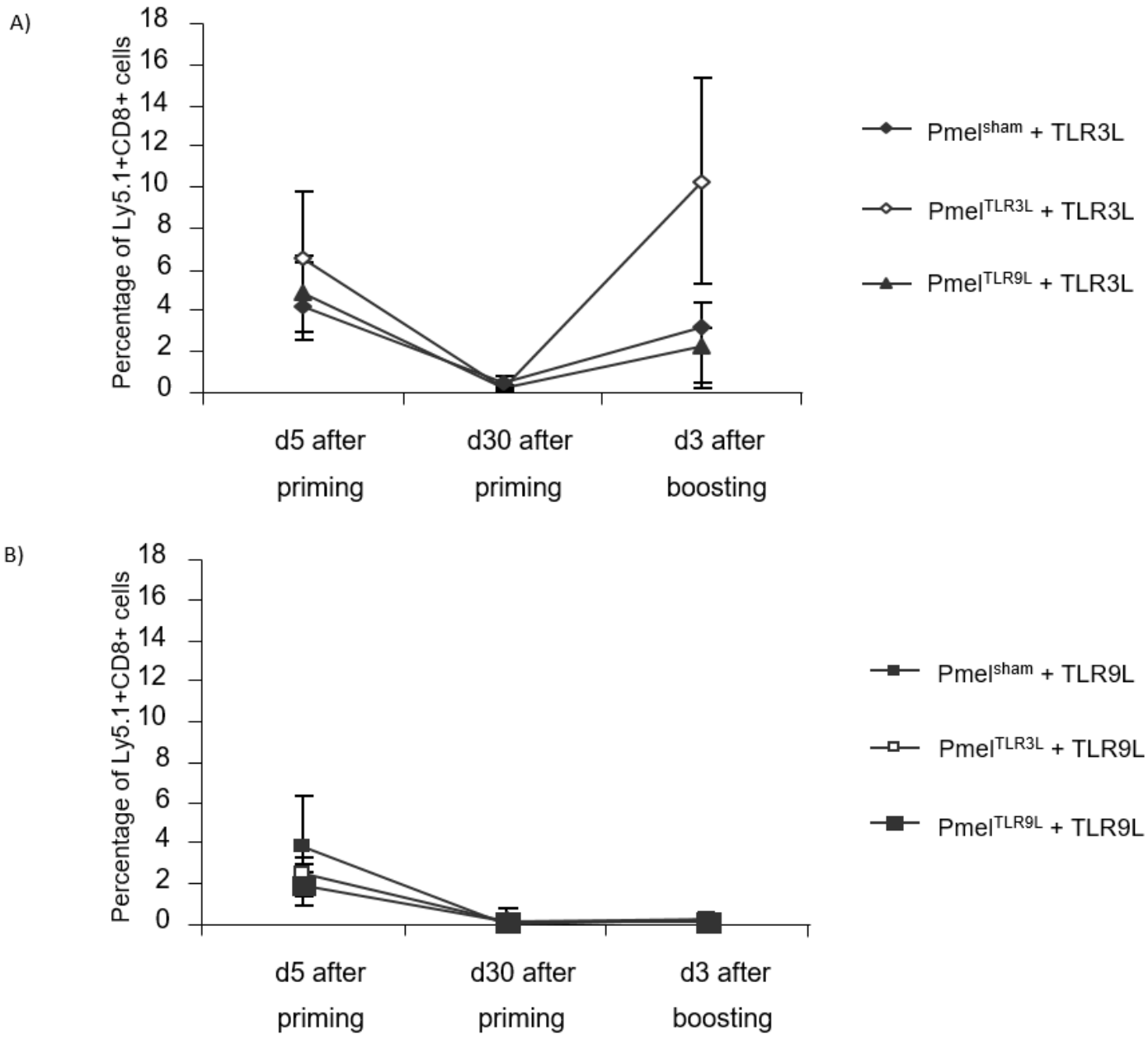

## Figure 4

**TLR9L-induced proliferation of B220+ cells is coincident with increased expression of activation markers.** Unfractionated Pmel-1 cells were cultured with TLR9L (10ng/mL) in the a) presence or b) absence of hgp100 (1ug/mL) stimulation. After 3 days, cells were harvested and stained for activation markers (CD25, CD69, CD40, CD80, CD86).




## Figure 5

**Conditioning cells with TLR3L and TLR9L during antigen presentation in vitro enhanced T cells in vivo.** Unfractionated Pmel splenocytes were cultured in vitro with hgp100 alone (1ug/mL) and in combination with either TLR3L (50ng/mL) or TLR9L (10ng/mL). After 72 hours, cells were adoptively transferred into naïve B6 mice on day 0. Mice were primed with hgp100 (100ug) alone or with TLR3L (200ug) on day 7. Mice were bled on days 5 and12, and the percentage of GFP-positive CD8+ cells was analyzed.




## Figure 6

**Conditioning cells with TLR3L and TLR9L in vitro in absence of antigen stimulation does not benefit Ag-specific expansion of T cells in vivo.** Unfractionated Pmel-1 splenocytes were cultured in vitro for 72 hours in the absence of antigen stimulation with either TLR3L or TLR9L. Mice were treated with CTX on day 1. Cells were then transferred into Ly5.2 mice day 0. Mice were primed on day 1 with hgp100 (100ug/mouse) and either TLR3L (200ug/mL) or TLR9L (10ng/mL). Boosting occurred 30 days after priming. Mice were bled on days 6 and 30 after priming and 3 days after boosting. The percentage of $CD8^+$ Pmel-1 $CD8^+$T cells was analyzed 7 days after priming (A) or after boosting (B).