



## MAINTENANCE OF MEMORY CD4 CELLS; THE ROLE OF TLR2 ENGAGEMENT

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**ABSTRACT... Objective:** Ligation of TLR by distinct pathogen components provides essential signals for T cell priming, although how individual TLR engagement affects memory T cells induction and maintenance in vivo is not well defined. The aim of the present study was to investigate the role of TLR2 engagement in the maintenance of memory T cells. **Method:** Ova specific KJ-1 cells from DO-11 mice were adoptively transferred to Balb/c mice. T cells were activated with Ova in the host of adoptive cells to induce memory. To examine the function and maintenance of memory cells in vivo, CD4<sup>+</sup> T cells were transferred to mice, which were then challenged with Ova-BLP and looked for memory cell proliferation. Furthermore, the memory T cells harvested from lymph node and spleen of Balb/c mice were treated with Ova and BLP in vitro to establish the effects of TLR2 ligation on proliferation of memory T cells. Two different protocols were used to confirm the same phenomenon. **Results:** Two different protocols show that memory T cells proliferation in vivo and in vitro can be maintained by TLR2 agonist (BLP). We demonstrate that antigen specific CD4 T cells undergo extensive proliferation in the presence of Ova and TLR2 agonist, in fact with TLR2 priming results in greater expansion. Moreover, TLR2 agonist priming of ova-specific CD4 T cells resulted in a higher frequency of persisting ova/BLP specific memory CD4 T cells which facilitated strong secondary responses upon challenge with ova antigen. **Conclusions:** Ligation of TLR2 agonist BLP (Pam3Cys) alone is sufficient to maintain the proliferation of Ova specific CD4<sup>+</sup> T cells without the need of antigen. Which might suggest that long-term functional capacities of T cells are set by innate signals during early phases of an infection

**Key words:** Cd4 cells, T Cell, Memory cells, Immunity, KJ cells.

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### INTRODUCTION

Humans are protected against pathogenic infections by immunity, which can be divided into innate and adaptive arms. The innate immune response is the first line defense characterized by immediate, non-specific and rapid response to limit the spread of infection. Whereas, the adaptive immune response is second line defense mediated through T (cellular) and B (humoral) components. These components are known to target only pathogens specifically with no response to self. These responses are long lasting and rapid on subsequent exposure because of memory cells. After antigenic stimulation naïve T cells differentiate into T helper 1 (Th1), Th2 or Th17 T cells subject to antigen affinity, costimulation at

the immune synapse and cytokine environment<sup>1,2</sup>.

Pattern-recognition receptors of the innate immune system, such as Toll like receptors TLRs, differentiate between infectious non-self and self. This leads to initiation of adaptive immune response<sup>3</sup>. Toll was first recognized in *Drosophila* as part of the host resistance in fruit flies against fungal infections<sup>4</sup>. So far 12 TLRs have been reported in humans<sup>5</sup>. TLRs act as the main sensors of microbial products and the subsequent downstream signaling leads to induction of immune and inflammatory genes<sup>6</sup>. Engagement of TLR during the initial stages of infection by pathogen-associated patterns trigger costimulatory signals for inducing adaptive

immune responses. Individual TLR have distinct pathogen-associated pattern specificities, TLR2 specifically binds bacterial lipoproteins<sup>7</sup> with TLR4 recognizing LPS<sup>8</sup>, while TLR3 and TLR9 bind viral and bacterial nucleic acids, respectively<sup>6,9,10</sup>. It was initially thought that TLRs are primarily expressed on antigen presenting cells (APCs), and their activation would in turn elicit adaptive immune system, especially T cells. However, the growing evidence suggestive of TLRs role in recognition and activation of components of pathogens in adaptive immune response proved it incorrect. In fact different T cell subsets such as natural killer cells, conventional  $\alpha\beta$  T cells,  $\delta$ -T cells and regulatory T cells have been confirmed to express TLRs<sup>11,12</sup>. In addition, TLR-3, TLR-6, 7 and 9 have been reported to be expressed on CD4 T cells<sup>13</sup>. We demonstrated for the first time that TLR2, and TLR2 only, is functionally expressed on the surface of activated T cells and memory cells<sup>11,14,15</sup>.

Several studies have confirmed that binding of specific ligand with their TLR can induce CD4 T cell priming and effector differentiation, which might result in T helper (Th1-Th2) skewing and subsequently induction of inflammation and/or promoting regulatory activity. TLR4 is primarily known to produce Th1 responses<sup>16,17</sup> and is upstream of Th17 differentiation<sup>18-20</sup>. Unlike TLR2 engagement which predominantly favor Th2 responses in some cases<sup>20</sup>, While at other time it support IFN- $\gamma$  production<sup>21</sup>. TLR2 is also known to promote IL-10 production and enhance Treg activity in vivo<sup>22</sup>. It has been confirmed to also regulate IL-17 production; however, different infection models have shown aggravation or reduction in these effects<sup>23</sup>, and its direct effect on IL-17 production by Ag-specific T cells is not clear. Previously, we showed that CD4<sup>+</sup>CD45RO<sup>+</sup> memory T cells from adult peripheral blood constitutively expressed TLR2 and rapidly produced more IFN $\gamma$  in response to BLP than CD4<sup>+</sup>CD45RA<sup>+</sup> T cells cultured with immobilized anti CD3 antibody. Interestingly, BLP also significantly enhanced the proliferation and IFN $\gamma$  production of memory CD4<sup>+</sup>CD45RO<sup>+</sup> (but not naïve CD4<sup>+</sup>CD45RA<sup>+</sup>) T cells cultured with IL-2 or IL-15 alone. These results therefore show that

TLR2 serves as a co stimulator receptor for antigen driven T cell development, and may have a role in maintaining T cell memory. Priming of the T cells and differentiation to their effector cells also results in the generation of long-lived memory T cells, which then facilitate efficient secondary immune responses at the site of infection<sup>24</sup>. TLR signaling induced generation of memory CD4 T cells was validated in vivo. Whereas, mice lacking the common signal transducer MyD88 were unable to produce antigen-specific memory T cell responses<sup>25</sup>. Antigenic peptide priming in the presence of LPS as a TLR4 ligand stimulate memory CD4 T cell production<sup>26</sup>, although at a low rate. TLR ligation with agonists in vivo not only targets DC, but can also bind to TLR expressed by T cells themselves<sup>27,28</sup>. Notably, surface TLR2 expression has been confirmed in memory and regulatory CD4 T cells<sup>29</sup>, and TLR2 ligation can support Treg function<sup>30</sup>. Moreover, engagement of TLR2 on CD8 T cells has been recently demonstrated to facilitate memory T cell development from effector cells<sup>31</sup>. Whether TLR2 engagement in vivo can mediate CD4 T cell priming and memory due to direct effect on the T cells has not been examined yet. The ability of other TLR agonists, including those that trigger TLR2 engagement, to generate memory T cell responses and subsequent secondary responses upon rechallenge is not well understood.

Majority of the studies on TLRs have concentrated on cells of the innate immune system, as TLRs are closely associated with innate immune response. Therefore, we have established TLR2 expression on activated T cells of adaptive immunity as a co-stimulatory receptor<sup>12</sup>. Moreover, the aim of this study is to investigate if TLR2 is directly involved in proliferation and maintenance of memory cells.

## METHODS AND MATERIAL

### Mice

The transgenic D011.10 mice on Balb/cJ background express the KJ-1 cells that can be tracked if adoptively transferred to other mice. The mice were bred and maintained at the central animal facility. All experiments were undertaken

within the terms of Animal (Scientific Procedures) Project License issued by the Home Office of the U.K.

### In vivo priming / memory T cell generation

#### A. Induction of memory cells

As the proportion of T cells is higher in lymph nodes, therefore KJ-1 T cells were harvested from lymph nodes of DO11 transgenic mice. These cells were then purified by negative selection following depletion of CD8 T cells and MHC class II-expressing cells yielding 98% purity. The KJ-1<sup>+</sup> T cells were adoptively transferred ( $5 \times 10^6$  cells/mouse) into 5-6 weeks old BALB/c (Thy1.1) mice. To induce memory, these mice were primed with Ova plus complete Freund's adjuvant (CFA) subcutaneously on day 1 as a primary exposure. Later, on day 16 the mice were given subcutaneous injection of ova plus incomplete Freund's adjuvant. On day 19 the lymph node cells were harvested and looked for CD4<sup>+</sup> and KJ1<sup>+</sup> memory T cells.

#### B. Function of memory cells

To confirm in vivo memory cells function two groups of mice were used, both the control mice (n=5) and the test group (n=5) were injected intravenously with CD4<sup>+</sup> / KJ1 cells. Later, on day 1 after the adoptive transfer the control mice were subcutaneously injected with PBS alone. whereas, the test group was challenged with Ova+BLP. After this on day 3 cells were harvested from the lymph nodes. Later, the harvested cells were challenged in vitro with two different concentration of ova (1/100 and 1/1000) and  $\pm$ BLP. This protocol was repeated and the results were reproducible.

### Second protocol for priming / memory T cell generation

#### A. Induction of memory cells

First the KJ-1 cells ( $5 \times 10^6$  cells) from the D011 mice were adoptively transferred to Balb/c mice. These mice were then primed with Ova + complete Freund's adjuvant subcutaneously on day 1 and without further boosting the immune response, on day 15 the CD4<sup>+</sup> KJ1 positive cells were taken from the lymph nodes and the ova specific

memory KJ-1 cells were separated for the adoptive transfer. Around two weeks period between the priming and harvesting the cells to ensure memory cells generation.

#### A. Function of memory cells

To confirm the in vivo memory cell generation two groups of Balb/c mice were adoptively transferred with ova specific memory KJ1 cells. Thereafter the test group was injected twice with BLP on day 1 and 4, subsequently on day 7 these mice were challenged with Ova + IFA. Finally on day 10 lymph node and spleen cells were harvested.

#### Cells and culture

Cells are harvested from lymph nodes and spleen of Balb/c mice. The cells were cultured in RPMI medium 1640 + 10% FCS at 37°C in 5% CO<sub>2</sub> ( $2 \times 10^6$  cells per ml) in 24-well plates for different time points (24, 48 and 72 h) in the presence or absence of ova (0.03  $\mu$ g/ml) and bacterial lipoprotein (BLP, 20 ng/ml, EMC, Tuebingen, Germany). Cell proliferation assays (<sup>3</sup>H]thymidine incorporation) were determined at different time points.

#### Flow Cytometry

Purified ex vivo T cells from lymph nodes and spleen were stained directly with conjugated antibodies: CD4 (FITC) from Sigma, KJ-1 (PE) from BD Bioscience, and normal IgG control (PE or ApC) from BD Bioscience. Aliquots of freshly harvested cells ( $3 \times 10^5$  cells/tube) were suspended in buffer containing 2% FCS and 0.02% sodium azide in PBS, preincubated with CD16/32 (BD Biosciences) for 30 min, and stained with specific FITC-, PE-conjugated mAb for 20–30 min. The isotype-matched antibodies were included as controls. Cells were analyzed by FACSCalibur using CellQuest software (BD Biosciences) or FlowJo (Celeza, Olten, Switzerland).

#### Statistics

Results are expressed as mean  $\pm$  SEM (n =5; \*, P < 0.05; \*\*, P < 0.01 by Student's t test). It should be noted that the experiments were performed using two different protocols and nevertheless the

results are similar for T cells proliferation. All experiments were performed at least two times with five mice per group. Results presented are representative from a single group.

**RESULTS**

**TLR2-primed memory CD4 T cells mediate robust secondary responses to BLP challenge in vivo.**

To induce memory Blab/c mice were first adoptively transferred with ova specific KJ-1 cells. Three days after the transfer the mice were primed with Ova+CFA to activate the ova specific KJ-1 cells. Activation of CD4<sup>+</sup> T cells are known to mediate expression of TLR2 receptors. Two weeks later to initiate the memory cell dependent secondary immune response mice were boosted with Ova-IFA. To confirm the effects of TLR2 engagement three days after the boost on day 19 CD4<sup>+</sup> and KJ-1 cells were harvested from lymph node and spleen to examine them for total CD4<sup>+</sup> T cells and absolute KJ-1 cells.

Thereafter, equal number of purified memory KJ-1 cells from these mice were adoptively transferred to the Blab/c mice (n=5) and then for the first time the host mice were challenged with Ova-BLP on day 1 and finally 2 days later the lymph node and spleen cells were harvested. The antigen challenge caused a significant increase not only in the total CD4<sup>+</sup> T cells of the lymph nodes but also the ova specific memory KJ-1 cells. Despite the

overall increase in the CD4<sup>+</sup> T cells the percentage of Ova specific KJ-1 cells was found significantly increased (Figure 1 A). In fact a comparable configuration was observed in the spleen cells for both absolute KJ-1 cells and their percentage (Figure 1, B).

CD4<sup>+</sup> T cells from both lymph nodes and spleen were labelled with FITC and KJ-1 with PE. The double positive cells were sorted out by FACS aria. A) Lymph node cells from mice treated with Ova+BLP show a significant rise in total KJ-1, CD4<sup>+</sup> T cells. These mice also have a increased percentage of KJ-1 cells as compared with the control mice. B) A similar pattern is appreciated in the spleen cells, with significant rise in KJ-1 cell number and percentage, as well as total CD4<sup>+</sup> T cells. Vertical bars represents SEM (n=5), results are representatives of two experiments. \*, P<0.05; \*\* P<0.01 vs. cells from Ova+BLP treated mice.

**TLR2 engagement increased the proliferation of antigen specific memory CD4<sup>+</sup> T cells in vitro**

We then asked whether the CD4<sup>+</sup> T cells proliferation in vitro is associated with the in vivo priming /memory and to establish antigen specific memory proliferation is ova independent. Memory CD4<sup>+</sup> T cells was challenged with TLR2 agonists (pam3Cys) and different concentration of ova in vitro. The CD4<sup>+</sup> T cells from PBS treated mice showed a significant increase in the proliferation with Ova 1/100 and BLP alone. Whereas, CD4<sup>+</sup> T

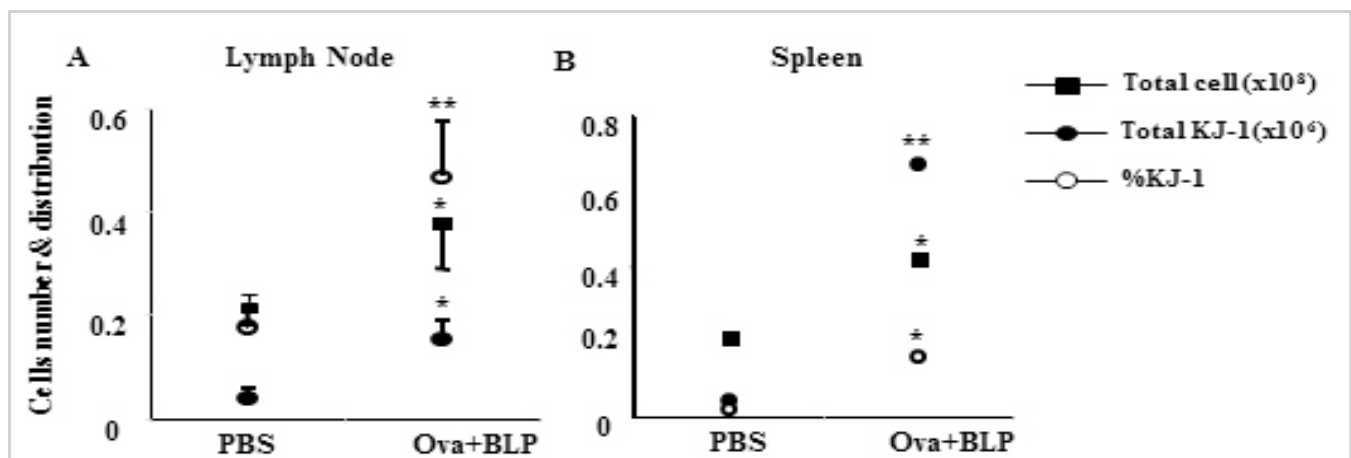
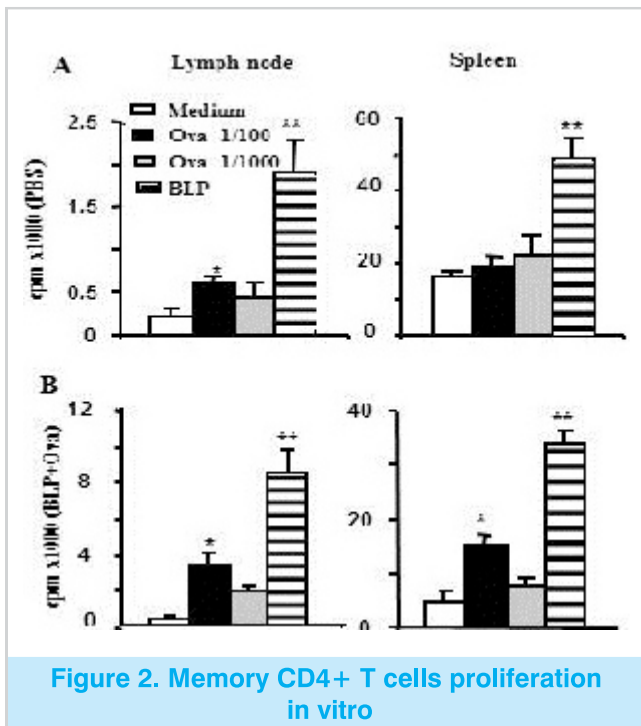


Figure 1. Memory T cells expansion in lymph node and spleen

cells from the spleen showed a substantial expansion only in the presence of BLP (Figure 2, A). This indicates that BLP independently caused the expansion of CD4<sup>+</sup> T cells.

To confirm the findings the lymph node and spleen cells from the ova-BLP treated mice were also challenged again in vitro with ±ova and ±BLP. The memory cells proliferation was significantly enhanced with the TLR2 agonist (BLP) for both lymph node and spleen cells. As expected CD4<sup>+</sup> memory T cells proliferation was substantially increased with the ova challenge as well, but the scale of increase seen with BLP was far greater (Figure 2, B)



Total lymph node and spleen cells were cultured in medium with different concentration of ± ova antigen and ± BLP. T cell proliferation was determined at 72 h. A) The lymph node cells from PBS treated mice show significant difference in proliferation with the ova 1/100 but not with ova 1/1000. However, the BLP treated spleen cells showed a significant rise in CD4<sup>+</sup> T cells.

B) As for the cells from BLP+Ova treated group, there is a significant difference between medium

and ova 1/100 both in spleen and lymph node. In addition, when BLP was added to both the lymph node and spleen cells the proliferation increased massively. Vertical bars represent SEM of two experiments. \*P<0.05; \*\*, P<0.01 vs. column ova/100 and BLP respectively.

**BLP induced, antigen independent proliferation of memory T cells**

We then examined the effect of different time course on the BLP induced memory CD<sup>+</sup> T cells proliferation. The earlier in vitro observation, established that TLR2 engagement enhances memory CD4<sup>+</sup> T cells proliferation both in lymph nodes and spleen (Figure 2). Therefore, here we examine the effect of five days exposure on lymph node memory CD4<sup>+</sup> T cells proliferation.

We observed a significantly enhanced expansion of CD4<sup>+</sup> T cells from PBS treated mice exposed to ± ova / ±BLP in vitro (Figure 3, upper panel). This reinforces the finding that BLP is independently enhancing the CD4<sup>+</sup> T cell expansion. Despite a longer exposure (5 days) ova challenge could not surpass the effect produced by TLR2 agonist. Whereas the cells from Ova-BLP mice were already exposed to the antigen and therefore a repeat exposure with ova 1/100 significantly increased the proliferation (Figure 3, lower panel). However, it could not reach the expansion level achieved by BLP exposure. It is now clear that increase in ova concentration or duration together fails to reach the level of BLP induced proliferation of CD4<sup>+</sup> T cells.

Total lymph node and spleen CD4<sup>+</sup> cells were cultured in medium with different concentration of ± ova antigen and ± BLP. T cell proliferation was determined after 5 days of exposure. Upper panel) lymph node cells from the PBS treated mice were exposed to different concentrations of Ova and BLP. The proliferation induced by Ova 1/100 is significantly less than the BLP induced proliferation lower panel) Interestingly, the cells from the Ova-BLP mice show a significant rise in the proliferation with ova 1/100.

Vertical bars represent SEM (n=5) of two

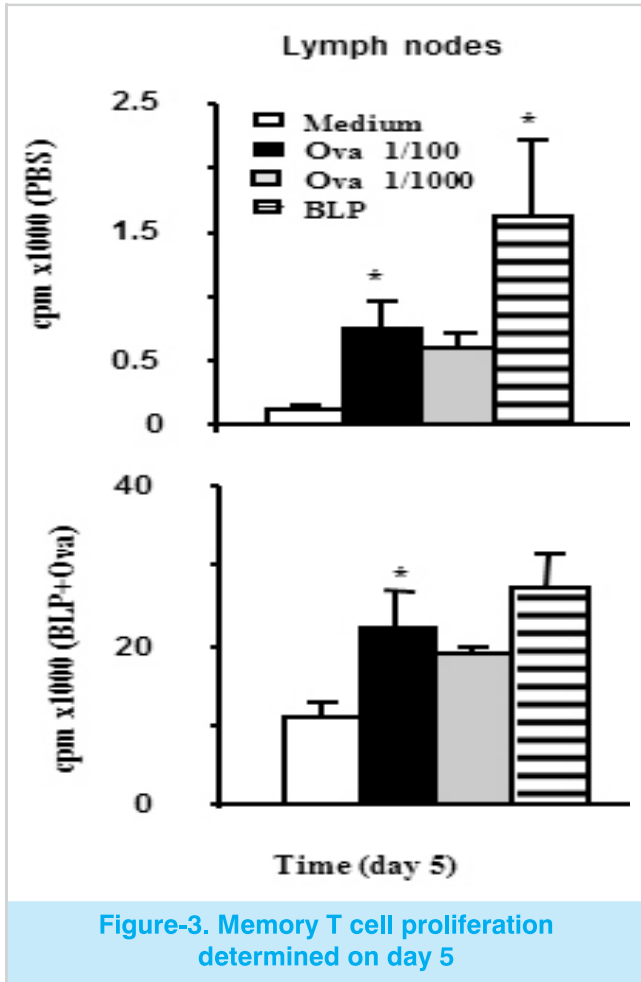


Figure-3. Memory T cell proliferation determined on day 5

experiments. \*P<0.05; \*\*, P<0.01 vs. column ova/100 and BLP respectively.

**Another protocol to establish the role of TLR2 engagement in the proliferation of activated CD4<sup>+</sup> T cells**

Since the complexity involved in the induction and maintenance of memory cell is not well understood, therefore we looked at the same phenomenon via a different protocol using the Balb/c mice. T cells were first activated in vivo to express the TLR2 receptors. Thereafter, we evaluated the ability of TLR2 agonists to trigger proliferation of activated CD4 T cells using an in vivo transfer model in which small numbers of activated KJ-1 T cells are transferred into intact adoptive hosts and subsequently primed and challenged with BLP (Pam3Cys). There was around two weeks period between

priming the naïve cells with ova antigen and harvesting the cell for adoptive transfer. This was to ensure activation of T cells.

To establish the effects of repeated exposure of TLR2 agonist on ova specific activated T cell proliferation two groups of Balb/c mice were adoptively transferred with ova specific memory KJ1 cells. Thereafter the test group was injected twice with BLP on day 1 and 4, subsequently on day 7 these mice were challenged with Ova + IFA for a secondary response. Finally on day 10 lymph node and spleen cells were harvested.

Lymph node cells showed an overall rise in the T cells indicating a rapid proliferation in response to the antigen and TLR2 agonist. Notably, the absolute KJ-1 cells rise and their percentage increase reflects that the proliferation was induced by specific antigen (Figure 4). In addition, similar pattern is observed with the spleen cells. Despite the fact that spleen has B cell dominance, these cells show increased total T cells as well as absolute KJ-1 cell and their percentage (Figure 4)

CD4<sup>+</sup> T cells from both lymph nodes and spleen were labelled with FITC and KJ-1 with PE. The double positive cells were sorted out by FACS aria. A) Lymph node cells from mice treated with Ova+BLP show a significant rise in total T cells and KJ-1 cells. These mice also have an increased percentage of KJ-1 cells as compared with the control mice. B) A similar pattern was appreciated in the spleen cells, with significant rise in KJ-1 cell number and percentage, as well as total CD4<sup>+</sup> T cells. Vertical bars represents SEM (n=5), results are representatives of two experiments. \*, P<0.05; \*\* P<0.01 vs. cells from Ova+BLP treated mice.

**TLR2 engagement increased the proliferation of memory CD4<sup>+</sup> T cells in antigen independent manner**

Later, the harvested cells were looked for the memory cell proliferation by challenging the cells in vitro with two different concentration of ova (1/100 and 1/1000) and BLP. Unlike the first protocol here the effects of proliferation were

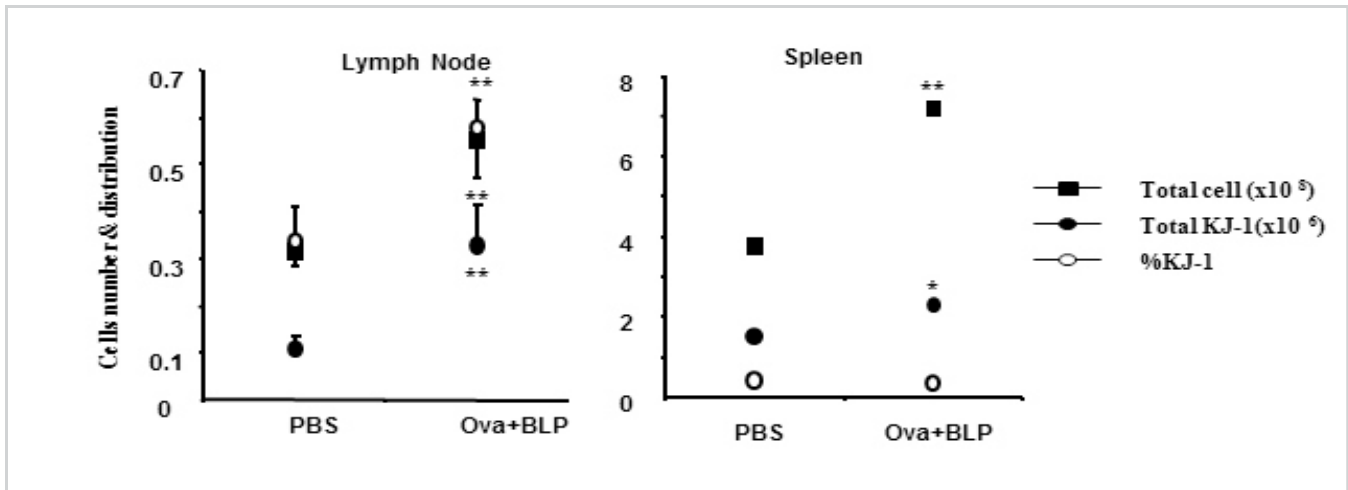


Figure-4. Ova specific activated T cells proliferation

examined at earlier time points (24 and 48h). We observed a rise in the proliferation of CD4<sup>+</sup> T cells with TLR2 agonist after 24h, which became massive after 48h. Since, the CD4<sup>+</sup> T cells from the control mice were not primed/challenged with a specific antigen or TLR2 agonist in vivo. Therefore, this in vitro response suggest that CD4<sup>+</sup> T cells proliferation is antigen independent and maintained by BLP engagement with its receptor (Figure 5, A). Moreover, the CD4<sup>+</sup> T cells from the spleen also showed a rise in proliferation with ova 1/1000 and BLP at 48h. Perhaps a longer incubation and high concentration of antigen together induced the proliferation of spleen cells. Whereas, the effect of BLP on CD4<sup>+</sup> T cells of spleen are strongly noticed (Figure 5, upper panel)

The Blab/c mice treated with Ova-BLP does show a gradual increase in their lymph node CD4<sup>+</sup> T cells proliferation in response to challenge with Ova 1/100, 1/1000 and BLP at 48h. Whereas, the spleen CD4<sup>+</sup> T cells proliferation is enhanced at both 24h and 48h. This further establish the finding that BLP alone can promote or maintain the proliferation of memory T cells. In fact the magnitude of proliferation in response to TLR2 engagement with BLP is much stronger than the ova induced proliferation (Figure 5, lower panel). Total lymph node and spleen cells were cultured in medium with different concentration of ± ova antigen and ± BLP. T cell proliferation was determined at 24h and 48 h. A) The lymph node

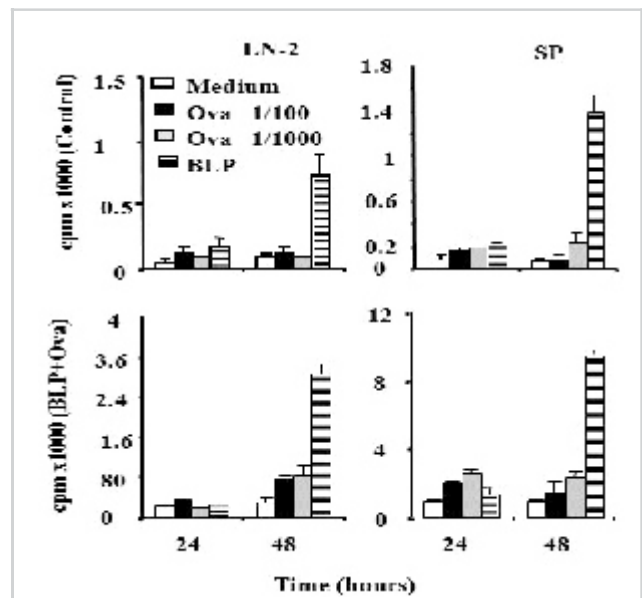


Figure-5. CD4<sup>+</sup> T cells expansion at earlier time points

cells from PBS treated mice show no significant difference in proliferation with Ova treatment. There is a significant increase with BLP at 48h. However, the spleen cells from PBS treated mice show rise in both Ova 1/1000 and BLP after 48h.

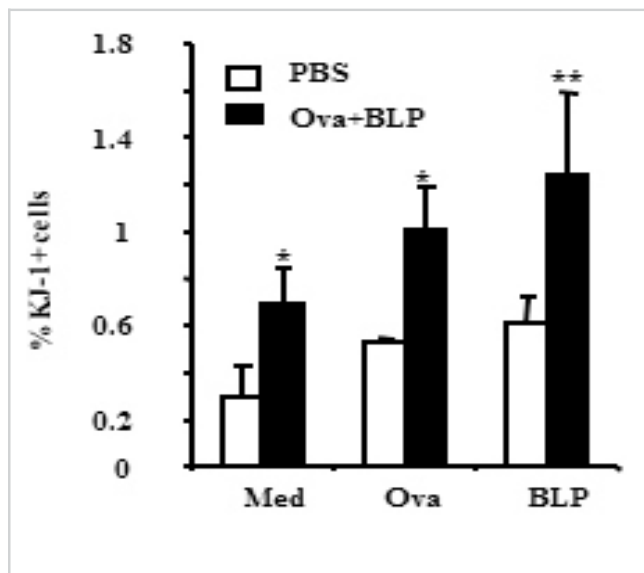
B) As for the cells from BLP+Ova treated group, there is a significant difference with both ova 1/100 and Ova 1/1000 both in spleen and lymph node. However, when BLP was added to both the lymph node and spleen cells the proliferation increased massively after 48h. Vertical bars represent SEM of

two experiments. \*P<0.05; \*\*, P<0.01.

**BLP induced, antigen independent proliferation of KJ-1 cells**

Finally, we looked at the proportion of KJ-1 cells harvested from both PBS and Ova-BLP treated mice. These memory cells from both the groups were treated with Ova, BLP and compared with unchallenged group. The unchallenged group revealed the the base line level of memory T cell in the PBS and Ova-BLP group. The base line level were compared with ova and BLP treated memory cells. All the three conditions show relatively increased percentage of KJ-1 cells. However, the maximum proliferation of memory T cells was observed with BLP treatment, which shows that TLR2 engagement is sufficient and can induce proliferation of memory T cells in an antigen independent manner (Figure 6).

The KJ-1 cells were purified from lymph node and spleen of PBS and ova-BLP treated mice shown in white and black columns respectively. These cells were treated in vitro under 3 different conditions medium, ova and BLP. Vertical bars represent



**Figure-6. Proliferation of KJ-1 cell in response to ova and BLP**

SEM; results are representative of two different experiments. \*P<0.05; \*\*, P<0.01.

**DISCUSSION**

The functions of TLRs were intensively investigated by us and others<sup>3,6,8,12,14,16</sup>. Memory T cells differentiate from naive T cells after antigenic stimulation and protect the individual during a subsequent encounter with the same antigen. How the immune system remembers a previous encounter with a pathogen and responds more efficiently to pathogen has been one of the central interests of immunologists for over a century. Although immunological memory has been intensively studied in the past few decades, the mechanisms underlying the generation and maintenance of memory T cell during and after an immune response remain only partially understood. In previous studies, we have shown that CD4 memory cells express TLR2 and our finding suggested that engagement of TLR2 and bystander effect of cytokines such as IL-15 and IL-2 is associated with extensive proliferation of memory T cells<sup>12</sup>.

In this study, we took two different in vivo approaches to establish the effects of TLR2 engagement on the generation and maintenance of memory CD4<sup>+</sup> T cell responses. We generated memory cells in KJ-1 + cells from DO11 mice and then tracked them in vivo and in vitro. This was to highlight two main features; firstly, the memory cells proliferates in response to antigen and it is even more exaggerated in the presence of BLP. Secondly, when the memory cells are formed, the antigenic stimulation is no more required. Therefore the cells respond to BLP in antigen independent manner, which is appreciated as increase in their numbers in proliferation assays.

Our results are consistent with others, which highlight the importance of engagement of TLRs in generation of memory cells. Medzhitov and colleagues reported ablation of MyD88 mice impairs not only differentiation of TH1, TH17 but also generation and function of memory cells<sup>32</sup> or Long-lasting IgG T cell independents response require MyD88 signal<sup>33</sup>. TLR agonists not only are important to CD4 helper cells, but are also vital in generation of CD8 CTL and B cells<sup>34,35</sup>.



We found that TLR2 engagement after antigen priming resulted in increased frequency of memory CD4<sup>+</sup> T cells proliferation, which was upheld without antigen presence. Therefore these results suggest that TLR engagement at the early phases of T cell activation in vivo can influence the maintenance and proliferation of antigen specific memory cells even in the absence of the antigen.

Differential TLR engagement has been shown to have variable effects on the priming and differentiation of CD4 T cells that can be influenced by the type of Ag, DC, location, and dose of TLR agonist<sup>11,36</sup>. TLR agonists can act directly on the APC and/or the T cells themselves as certain TLR such as TLR2 are expressed by activated T cells and T cell subsets<sup>21,30</sup>. Together, these results point to adjuvant effects of both LPS and Pam3Cys (BLP), but Pam3Cys exhibited less toxic effects in vivo compared with LPS. Furthermore, although use of high-dose LPS for in vivo priming is associated with morbidity, use of higher doses of Pam3Cys resulted in an enhanced frequency of activated T cells without any associated toxicity. It is possible that the enhanced frequency of CD4 T cell priming resulting from using Pam3Cys compared with LPS as an adjuvant may be due to increased availability of activated DC and their associated pro-inflammatory cytokines during initiation of T cell activation.

We found enhanced memory CD4 T cell survival with TLR2 agonist priming, supporting a model that innate immune signals may determine memory vs effector T cell development at very early stages in vivo<sup>37</sup>, and that memory T cell fate is determined at the very earliest stages of T cell activation<sup>38</sup>. We show here that TLR2-primed memory CD4 T cells mediated more robust secondary responses to BLP challenge compared with Ova mediated memory CD4 T cells in Vitro. We further observed a more extensive ova antigen CD4 T cells expansion of TLR2 primed memory CD4<sup>+</sup> T cells, indicating that functional capacities of TLR2 agonist on memory CD4 T cells and their potential to coordinate secondary immune responses are determined at the earlier priming stage.

We show here that TLR2 agonist expand memory CD4 T cells in greater numbers in response to BLP engagement alone. Which continued to persist even at greater frequency following a secondary challenge, suggesting that early IL-2 production programmed the development of memory CD4 T cells with enhanced capacities for secondary expansion<sup>14</sup>. Keeping together that BLP apply less toxicity and results presented here may have important implications for vaccine development, where use of specific TLR agonists can determine the long-term outcome of persisting immunity.

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