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TLR5 Signaling Enhances the Proliferation of Human Allogeneic CD40-Activated B Cell Induced CD4^{hi}CD25⁺ Regulatory T Cells

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Abstract

Although diverse functions of different toll-like receptors (TLR) on human natural regulatory T cells have been demonstrated recently, the role of TLR-related signals on human induced regulatory T cells remain elusive. Previously our group developed an *ex vivo* high-efficient system in generating human alloantigen-specific CD4^{hi}CD25⁺ regulatory T cells from naïve CD4⁺CD25⁻ T cells using allogeneic CD40-activated B cells as stimulators. In this study, we investigated the role of TLR5-related signals on the generation and function of these novel CD4^{hi}CD25⁺ regulatory T cells. It was found that induced CD4^{hi}CD25⁺ regulatory T cells expressed an up-regulated level of TLR5 compared to their precursors. The blockade of TLR5 using anti-TLR5 antibodies during the co-culture decreased CD4^{hi}CD25⁺ regulatory T cells proliferation by induction of S phase arrest. The S phase arrest was associated with reduced ERK1/2 phosphorylation. However, TLR5 blockade did not decrease the CTLA-4, GITR and FOXP3 expressions, and the suppressive function of CD4^{hi}CD25⁺ regulatory T cells. In conclusion, we discovered a novel function of TLR5-related signaling in enhancing the proliferation of CD4^{hi}CD25⁺ regulatory T cells by promoting S phase progress but not involved in the suppressive function of human CD40-activated B cell-induced CD4^{hi}CD25⁺ regulatory T cells, suggesting a novel role of TLR5-related signals in the generation of induced regulatory T cells.

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Introduction

Natural regulatory T cells (nTregs) and induced regulatory T cells (iTregs) are important to the self-tolerance of the human body and the tolerance to transplanted organs or tissues [1,2]. Impairments in the development or functions of these cells can cause autoimmune diseases such as immunodysregulation poly-endocrinopathy enteropathy X-linked syndrome [3], and systemic lupus erythematosus [4], which is either fatal or severely reduces the quality of life of patients, and graft rejection in transplantation. Although many efficient strategies have been developed to treat autoimmune diseases and graft rejection, their severe side effects lead to an urgent need for novel therapeutic strategies, such as adoptive transfer of antigen-specific regulatory T cells [5]. As a result, investigation in the biology of regulatory T cells is crucial for understanding these diseases and the development of novel therapeutic strategies for treating and managing autoimmune diseases and graft rejections.

It is known that activation and function of regulatory T cells require signals from both T cell receptor (TCR) [6] and CD28 [7,8]. However, as increasing number of co-stimulatory molecules, such as OX-40 and PD-1, were discovered to be implicated in the activation and function of regulatory T cells [9,10], it is speculated

that co-stimulatory molecules may also play diverse and crucial roles in the activation and function of these cells [11]. Reports about the non-absolute requirement of TCR signal in T cell function further support this speculation [12,13]. As a result, investigation in the role of co-stimulatory molecules in regulatory T cells is warranted. Although toll-like receptors (TLR) are thought to mainly participate in the antigen recognition and activation of innate immune cells [14], they are also crucial co-stimulatory molecules involved in the function of T cells. *In vitro* data suggested that TLR2, 4, 5, 7, and 8 could promote the proliferation of CD4⁺ T cells [15,16], and compelling evidence from the experiment of Marsland *et al.* demonstrated that CpG DNA stimulation could activate CD4⁺ T cells from PKC- $\theta^{-/-}$ mice and causing EAE, indicating that TLR stimulation could support the activation and differentiation of CD4⁺ T cells in the absence of TCR signaling [17]. TLRs are also involved in the activation and function of nTregs. Direct stimulation of mice CD4⁺ nTregs with TLR2 ligand Pam3Cys increased the proliferation and concomitantly abrogated the function of the cells [18,19], while stimulation of human nTregs with TLR4 ligand LPS and IL-2 up-regulated FOXP3 expression and the suppressive function [20]. *In vivo* result from TLR9^{-/-} mice also

suggested that TLR9 signaling enhanced nTregs function through induction of IDO [21].

TLR5 is expressed in both CD4⁺ T cells and nTregs [22,23]. Since the TLR5 ligand, flagellin, is commonly expressed in different bacteria species [24,25], TLR5 may be particularly important to the induction of tolerance to intestinal commensal bacteria and of oral tolerance [26]. Currently, there is only a single report investigated on the direct effect of TLR5-related signals on human nTregs. Crellin *et al.* reported that stimulation of human nTregs with anti-CD3/CD28 and flagellin up-regulated FOXP3 expression and the suppressive function [27]. Since the direct effect of TLR5-related signals on iTregs remains unexamined, the function of TLR5 in human iTregs is investigated in this study.

Previously our laboratory has developed a simple and cost effective novel protocol of large-scale *in vitro* induction and expansion of human alloantigen specific CD4^{hi}CD25⁺ regulatory T cells with therapeutic potential from naive CD4⁺CD25⁻CD45RO⁻ precursors using human allogeneic CD40-activated B cells as stimulators without the use of exogenous cytokine. Co-culture of human naive CD4⁺CD25⁻ T cells with allogeneic CD40-activated B cells at T cell to B cell ratio of 10:1 induced a population of CD4^{hi}CD25⁺ regulatory T cells [28]. The CD4^{hi}CD25⁺ T cells were alloantigen specific CD45RO⁺CCR7⁻CD62L⁺ memory T cells and expressed FOXP3, IFN- γ , CTLA-4, and GITR [28,29]. Suppressive MLR experiment demonstrated that these cells could suppress T cell proliferation in a cell-cell contact dependent manner which was partially dependent on the surface CTLA-4, indicating that these cells are iTregs [28]. In this experiment, we investigated the role of TLR5-related signals in the generation and function of human CD4^{hi}CD25⁺ regulatory T cells induced by allogeneic CD40-activated B cells and have unveiled a novel function of TLR5-related signaling in iTregs. Our results indicated that TLR5-related signaling enhances the proliferation but not the suppressive function of human CD4^{hi}CD25⁺ regulatory T cells induced by allogeneic CD40-activated B cells.

Materials and Methods

Ethics Statement

Written consent for the use of buffy coat for research purposes was obtained from the donors by the Hong Kong Red Cross Blood Transfusion Services at the time of blood donation. The use of buffy coat for this experiment was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster (IRB Reference Number: UW 07-390).

Generation of CD40-activated B Cells

PBMC were isolated from buffy coat of healthy adult donors from the Hong Kong Red Cross Blood Transfusion Services. CD40-activated B cells were generated from PBMC via CD40 stimulation using lethally irradiated (96Gy) NIH3T3 cells transfected with the human CD40 ligand (t-CD40-L cells) as stimulator in B cell medium as we described previously [30,31]. Briefly, isolated PBMC were co-cultured with the lethally irradiated (96Gy) t-CD40-L cells in IMDM (GIBCO-BRL, Life Technologies, CA) supplement with the 2ng/ml of IL-4 (R&D systems, MN), 5.5×10^{-7} M of cyclosporine A (Sigma-Aldrich, MO), 50 μ g/ml of transferrin (Sigma-Aldrich, MO), 5 μ g/ml of insulin (Sigma-Aldrich, MO), 15 μ g/ml of gentamycin (GIBCO-BRL, Life Technologies, CA), and 10% of heat-inactivated human AB serum (Innovative Research, MI) at 37°C in 5% CO₂. Cells were sub-cultured to new 6-well plates of t-CD40-L cells every 3 to 4 days. After 14 days of co-culture, more than 95% of the viable

suspended cells are routinely CD19 positive. These B cells were cryopreserved in 10% DMSO medium for future use.

Isolation of Naive CD4⁺CD25⁻CD45RO⁻ T Cells and Induction of CD4^{hi}CD25⁺ Regulatory T Cells

The CD4^{hi}CD25⁺ regulatory T cells were induced by the co-culture of the CD4⁺CD25⁻CD45RO⁻ T cells with the allogeneic CD40-activated B cells at a T-cell: B-cell ratio of 10:1 for 6 days as described previously [28] unless otherwise specified. Human naive CD4⁺CD25⁻CD45RO⁻ T cells were isolated from healthy donors PBMC by CD4⁺ T cell enrichment using the human CD4 T Cells Enrichment Cocktails (StemCell Technologies, Canada), followed by negative selection using a human naive CD4⁺ T Cell Isolation Kit and LD Column (Miltenyi Biotec, Germany) according to manufacturer's instructions.

TLR5 Blockade and Chemical Inhibition of ERK1/2 Phosphorylation

10 μ g/ml of anti-TLR5 mAb, and its relevant isotype control (Invivogen, CA) were used for the blockade of TLR5. 20 μ M of PD98059 and its solvent control DMSO (Merck, Germany) were used for chemical inhibition of ERK1/2 phosphorylation. Antibodies and PD98059 were added to CD4⁺CD25⁻CD45RO⁻ T cells one hour before co-culturing with allogeneic CD40-activated B cells and were replenished when cell culture medium was changed.

Flow Cytometric Assays

All fluorescence-conjugated antibodies were from BD-Biosciences unless otherwise specified: CD4-pacific blue (Biolegend, CA), CD25-APC-Cy7, CTLA-4-PE, GITR-PE, TLR5-PE (Imgenex, CA), human Foxp3 staining kit (clone: PCH101) (eBiosciences, CA), p-p44/42 (Thr202/Tyr204)-AlexaFluor-488 (Cell Signaling, MA). Annexin V/propidium iodide (Gibco-BRL, Life Technologies, CA) was used for measuring apoptosis. Propidium iodide (Gibco-BRL, Life Technologies, CA) was used for cell cycle analysis. For measuring cell proliferation, naive CD4⁺CD25⁻CD45RO⁻ T cells were stained with CFSE before co-culturing with allogeneic CD40-activated B cells. Cells were analyzed using FACS LSRII (BD Biosciences, CA) and results were analyzed using FlowJo v8.8.2 (Tree Star, OR). Cell cycle analysis results were analyzed using ModFit (Verity Software House, ME).

Mixed Lymphocyte Reaction (MLR) Assays

CD4^{hi}CD25⁺ regulatory T cells generated with or without the blockade of TLR5 were sorted using FACSAria after 9 days of co-culture. The sorted CD4^{hi}CD25⁺ regulatory T cells were titrated and co-cultured with 5×10^4 responder CD4⁺CD25⁻ T cells from the same donor of the CD4^{hi}CD25⁺ regulatory T cells and 5×10^4 γ -irradiated target PBMC from the donor of the CD40-activated B cells as stimulator for 3 days. ³H-thymidine was added to the co-culture in the last 18 hours and the proliferation was analyzed by ³H-thymidine incorporation assay as we described previously [28].

Statistical Analysis

Graphs and statistical analysis were performed using Prism 5.0 for Windows software (GraphPad Software, CA). One-way ANOVA with Tukey's pairwise comparisons was used for comparing the percentage of regulatory T cells, apoptotic T cells, and percentage of CD4^{hi}CD25⁺ regulatory T cells in S phase. *p* value of <0.05 was considered to be significant.

Results

TLR5-related Signals Enhance the Generation of CD4^{hi}CD25⁺ Regulatory T Cells Independent of Cell Apoptosis

We first investigated the TLR5 expression in the CD4^{hi}CD25⁺ regulatory T cells. A population of CD4^{hi}CD25⁺ regulatory T cells and a population of CD4⁺CD25⁻ T cells without any regulatory function could be identified in the co-culture of naïve CD4⁺CD25⁻CD45RO⁻ T cells with allogeneic CD40-activated B cells for 6 days (Figure 1A). As shown in Figure 1 B-E, CD4^{hi}CD25⁺ regulatory T cells exhibited an up-regulated surface (Figure 1B and C) and total TLR5 protein expression (Figure 1D and E). Interestingly, in CD4⁺CD25⁻ T cells, surface TLR5 expression level was lower than that of naïve CD4⁺CD25⁻CD45RO⁻ T cells while total TLR5 expression was the same (Figure 1 B-E).

The up-regulated TLR5 expression in CD4^{hi}CD25⁺ regulatory T cells prompted us to investigate the effect of TLR5-related signals on the generation and function of CD4^{hi}CD25⁺ regulatory T cells. It was found that the blockade of TLR5 using anti-TLR5 blocking antibodies decreased CD4^{hi}CD25⁺ regulatory T cell generation (Figure 1F and G). Frequency of CD4^{hi}CD25⁺

regulatory T cells decreased from 61% of total CD4⁺ T cells to about 36% after 6 days of co-culture ($p < 0.001$) (Figure 1G), indicating that TLR5 signaling was involved in CD4^{hi}CD25⁺ regulatory T cells generation. Since TLR5 was reported to be anti-apoptotic [32], and could promote the survival of cells and mice subjected to lethal irradiation [33,34], we further studied whether the reduced CD4^{hi}CD25⁺ regulatory T cells generation was due to increased apoptosis of CD4⁺ T cells. Surprisingly, cell death analysis using annexin V/podium iodide staining indicated that the blockade of TLR5 did not increase the apoptosis of either CD4^{hi}CD25⁺ regulatory T cells or CD4⁺CD25⁻ T cells. Approximately 5% of CD4⁺CD25⁻ T cells and 2% of CD4^{hi}CD25⁺ regulatory T cells were in either early or late apoptotic phase and TLR5 blockade did not alter the percentage (Figure 1H). These results indicated that the reduction of CD4^{hi}CD25⁺ regulatory T cell generation by blocking TLR5-related signals is not dependent on cell apoptosis.

TLR5-related Signals Endorse the Proliferation of CD4^{hi}CD25⁺ Regulatory T Cells by Promoting the Process of S Phase

Unaltered apoptosis of CD4⁺ T cells after the blockade of TLR5 suggested that the reduced CD4^{hi}CD25⁺ regulatory T cells

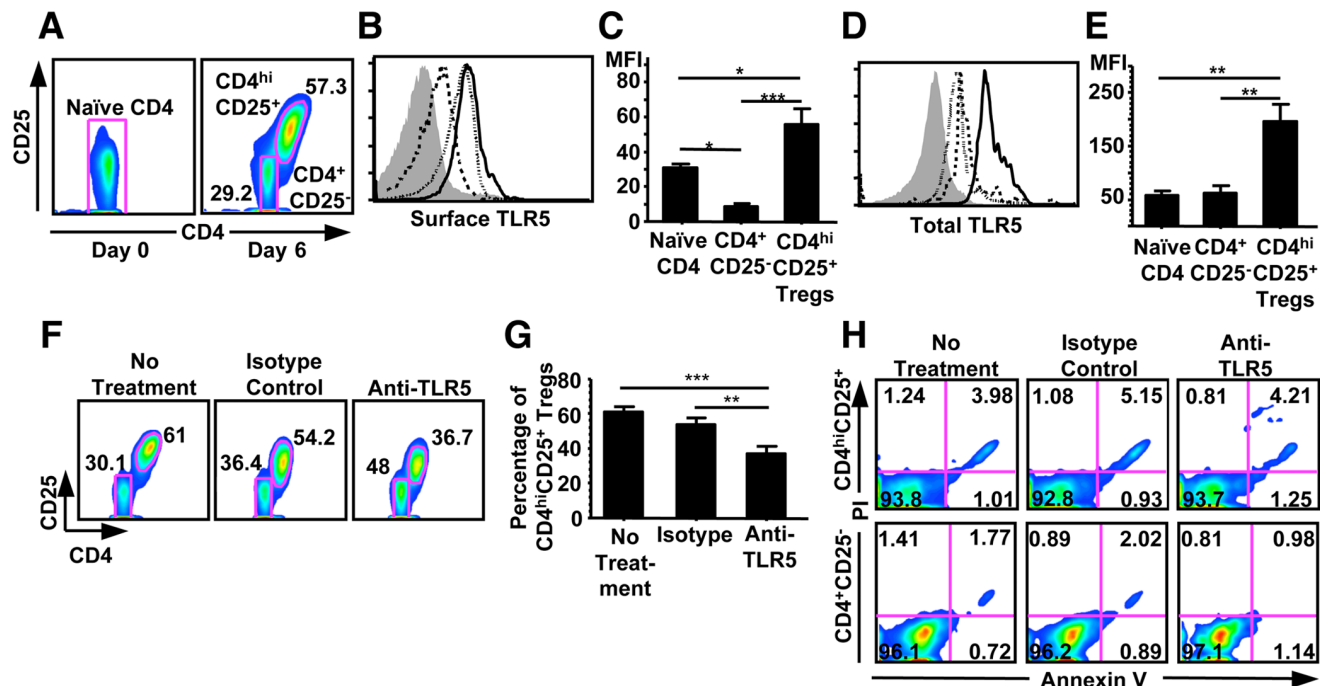


Figure 1. TLR5 blockade reduced the generation of CD4^{hi}CD25⁺ regulatory T cells and was independent of apoptosis. (A) Flow cytometric analysis of the percentage of CD4^{hi}CD25⁺ regulatory T cells generated on Day 6 (right panel) from naïve CD4⁺CD25⁻CD45RO⁻ T cells (left panel). (B) Flow cytometric analysis of the expression of surface TLR5 in freshly isolated naïve CD4⁺CD25⁻CD45RO⁻ T cells (dotted line), and CD4⁺CD25⁻ (dashed line) and CD4^{hi}CD25⁺ regulatory T cells (solid line) after 6 days of co-culture of naïve CD4⁺CD25⁻CD45RO⁻ T cells with allogeneic CD40-activated B cells. Filled histogram indicates the staining obtained from isotype-matched mAb controls. (C) Mean fluorescence intensity (MFI) of the expression of surface TLR5. Data show Mean+SEM, n = 6. (D) Flow cytometric analysis of total TLR5 in freshly isolated naïve CD4⁺CD25⁻CD45RO⁻ T cells (dotted line), CD4⁺CD25⁻ (dashed line), and CD4^{hi}CD25⁺ regulatory T cells (solid line) after 6 days of co-culture of naïve CD4⁺CD25⁻CD45RO⁻ T cells with allogeneic CD40-activated B cells. Filled histogram indicates the staining obtained from isotype-matched mAb control. (E) Mean fluorescence intensity (MFI) of the expression of total TLR5. Data show Mean+SEM, n = 6. (F) Flow cytometric analysis of the generation of CD4^{hi}CD25⁺ regulatory T cells with no treatment (left panel), with isotype-matched mAb (middle panel), and with anti-TLR5 blocking mAb (right panel) during the co-culture. (G) Mean percentage of CD4^{hi}CD25⁺ regulatory T cells generated with no treatment, with isotype-matched mAb, and with anti-TLR5 blocking mAb. Data shown Mean+SEM, n = 6. (H) Flow cytometric analysis of the percentage of apoptotic CD4^{hi}CD25⁺ regulatory T cells (upper panel) or CD4⁺CD25⁻ T cells (lower panel) after 6 days of co-culture of naïve CD4⁺CD25⁻CD45RO⁻ T cells with allogeneic CD40-activated B cells. All results shown are representative of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one way ANOVA with Tukey's pairwise comparisons.

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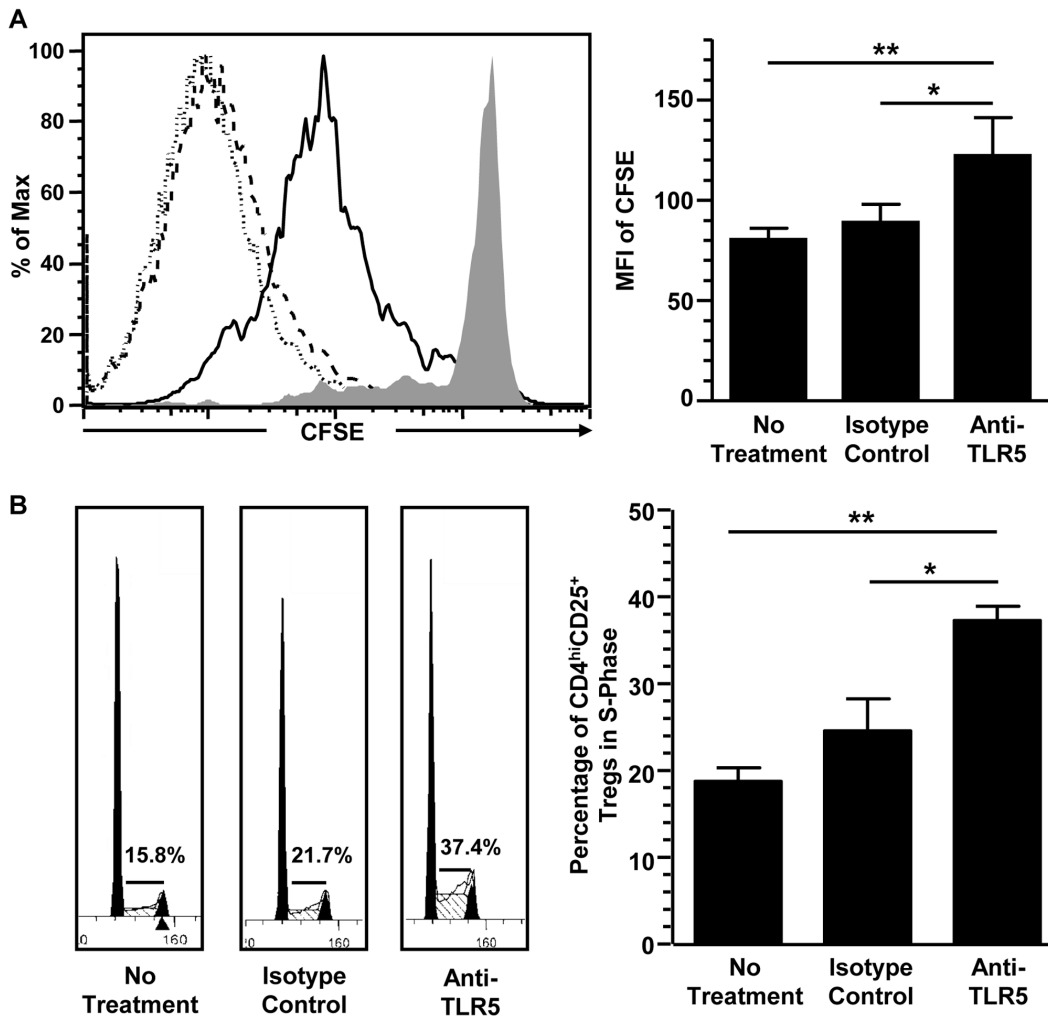


Figure 2. The blockade of TLR5 reduced CD4^{hi}CD25⁺ regulatory T cells proliferation by inducing S phase arrest. (A) Flow cytometric analysis of the CFSE signal in CD4^{hi}CD25⁺ regulatory T cells generated with no treatment (dotted line), with isotype-matched mAb (dashed line), and with anti-TLR5 blocking mAb (solid line). Filled histogram is the CFSE signal on Day 0 (left panel). Statistical analysis of the MFI of the CFSE in CD4^{hi}CD25⁺ regulatory T cells. Data show Mean±SEM, n = 6. (right panel). (B) Cell cycle analysis of CD4^{hi}CD25⁺ regulatory T cells generated with no treatment (left), with isotype-matched mAb (middle), and with anti-TLR5 blocking mAb (right). Numbers indicate the percentage of CD4^{hi}CD25⁺ regulatory T cells in S phase (left panel). Statistical analysis of percentage of CD4^{hi}CD25⁺ regulatory T cells in S phase. Data show Mean±SEM, n = 6 (right panel). All data shown are representative from three independent experiments. **p*<0.05, ***p*<0.01, one way ANOVA with Tukey's pairwise comparisons.

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generation was the result of decreased CD4⁺ T cells proliferation. CFSE staining demonstrated that CD4^{hi}CD25⁺ regulatory T cells underwent extensive proliferation and blockade of TLR5 reduced their proliferation (Figure 2A, left panel). The mean fluorescence intensity (MFI) of the CFSE in CD^{hi}CD25⁺ regulatory T cells generated without any treatment or with isotype matched mAb were about 80.5 and 89.1 respectively on Day 5. TLR5 blockade increased the MFI to about 122.3, indicating a reduction in proliferation of the CD4^{hi}CD25⁺ regulatory T cells (*p*<0.05) (Figure 2A, right panel). This result supported our hypothesis that TLR5 blockade decreased the generation of CD4^{hi}CD25⁺ regulatory T cells by reducing its proliferation. Since cell proliferation is a direct result of cell cycle, effect of TLR5 blockade on cell cycle progress of CD4^{hi}CD25⁺ regulatory T cells was investigated. After co-culture with allogeneic CD40-activated B cells, about 15% of CD4^{hi}CD25⁺ regulatory T cells were in S phase whereas their percentage was increased to about 40% with

the blockade of TLR5 (*p*<0.05) (Figure 2B), indicating an arrest in S phase. Therefore, it is concluded that TLR5-related signals enhanced the proliferation of CD4^{hi}CD25⁺ regulatory T cells by promoting the process of S phase.

Reduced ERK1/2 Signaling by the Blockade of TLR5 might Contribute to S Phase Arrest in CD4^{hi}CD25⁺ Regulatory T Cells

To elucidate the molecular mechanism of the TLR5-blockade induced-S phase arrest, the ERK1/2 phosphorylation was investigated [35]. Flow cytometric analysis indicated that the blockade of TLR5 reduced phosphorylated ERK1/2 (p-ERK1/2) in CD4^{hi}CD25⁺ regulatory T cells (Figure 3A, left panel). The MFI of p-ERK1/2 in CD4^{hi}CD25⁺ regulatory T cells generated without any treatment or with isotype matched mAb were about 33.6 and 29.7 respectively. TLR5 blockade decreased the MFI to about 26.3 (*p*<0.05) (Figure 3A, right panel), indicating that TLR5

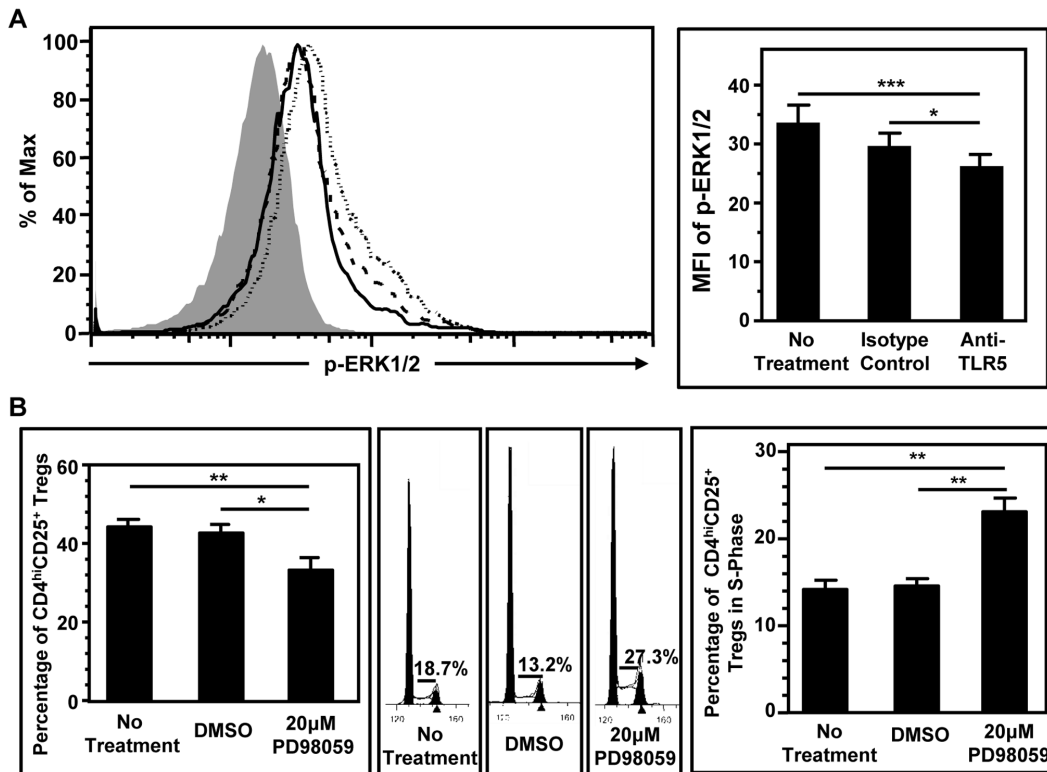


Figure 3. Reduced phosphorylated ERK1/2 might contribute to S phase arrest in CD4^{hi}CD25⁺ regulatory T cells. (A) Flow cytometric analysis of the expression of phosphorylated ERK1/2 in CD4^{hi}CD25⁺ regulatory T cells generated with no treatment (dotted line), isotype-matched mAb (dashed line), and with anti-TLR5 blocking mAb (solid line). Filled histogram is the staining obtained from isotype-matched mAb control for staining antibody (left panel). Statistical analysis of the MFI of p-ERK1/2 in CD4^{hi}CD25⁺ regulatory T cells. Data show Mean+SEM, n = 10. All data shown are representative from five independent experiments (right panel). (B) Statistical analysis of the percentage of CD4^{hi}CD25⁺ regulatory T cells generated on Day 6 with or without the inhibition of ERK1/2 phosphorylation by PD98059. DMSO treated group is the control for PD98059. Data show Mean+SEM, n = 6. All results shown are from 3 independent experiments (left panel). Cell cycle analysis of CD4^{hi}CD25⁺ regulatory T cells generated on Day 6 with or without the inhibition of ERK1/2 phosphorylation by PD98059. DMSO treated group is the control for PD98059. Numbers indicate the percentage of CD4^{hi}CD25⁺ regulatory T cells in S phase. All results shown are from 3 independent experiments (middle panel). Mean percentage of CD4^{hi}CD25⁺ regulatory T cells in S phase with the inhibition of ERK1/2 phosphorylation by PD98059. Data show Mean+SEM, n = 6. All results shown are from 3 independent experiments (right panel). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, one way ANOVA with Tukey's pairwise comparisons.

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blockade reduced the phosphorylation of ERK1/2. This also suggested that phosphorylation of ERK1/2 might contribute to the S phase arrest. To confirm this, the effect of ERK1/2 phosphorylation inhibition on the generation and the cell cycle progress of CD4^{hi}CD25⁺ regulatory T cells were investigated using MEK1/2 inhibitor PD98059 [36]. Naïve CD4⁺CD25⁻CD45RO⁻ T cells were co-cultured with allogeneic CD40-activated B cells in the presence of PD98059. Inhibition of ERK1/2 phosphorylation decreased the generation of CD4^{hi}CD25⁺ regulatory T cells from about 45% to about 35% ($p < 0.05$) (Figure 3B, left and middle panel), and the percentage of CD4^{hi}CD25⁺ regulatory T cells in S phase increased from about 18% to about 28% (Figure 3B, right panel). Taken together, these results indicated that reduced ERK1/2 phosphorylation might contribute to TLR5-blockade induced-S phase arrest.

TLR5-related Signals do not Affect CD4^{hi}CD25⁺ Regulatory T Cells Function

Previous study by Crellin *et al.* demonstrated that flagellin stimulation up regulated Foxp3 expression and the suppressive function of iTregs [27]. In this study, the effect of TLR5-related signals on the suppressive function of the human allogeneic CD40-

activated B cell induced CD4^{hi}CD25⁺ regulatory T cells was examined. Previous study from our group showed that the function of CD4^{hi}CD25⁺ regulatory T cells is partially dependent on the surface expression of CTLA-4 [28]. Therefore, the expression levels of CTLA-4, GITR, and FOXP3 were measured using FACS after TLR5 blockade. It was found that the blockade of TLR5 did not alter the surface and total expression of these molecules and no statistically significant reduction in the MFI of these concerned molecules was detected (Figure 4A). In addition, MLR results indicated that CD4^{hi}CD25⁺ regulatory T cells generated in either condition exhibited similar suppressive capacity even at the regulatory T cells: responders ratio of 1:1 (Figure 4B). Taken together, these data suggested that blockade of TLR5 did not alter the function of CD4^{hi}CD25⁺ regulatory T cells.

Discussion

In this study, we demonstrated that TLR5 signaling was involved in the generation but not the function of human allogeneic CD40-activated B cells induced CD4^{hi}CD25⁺ regulatory T cells. Our data provided interesting information about the function of TLR5-related signals in iTregs. This, to the best of our

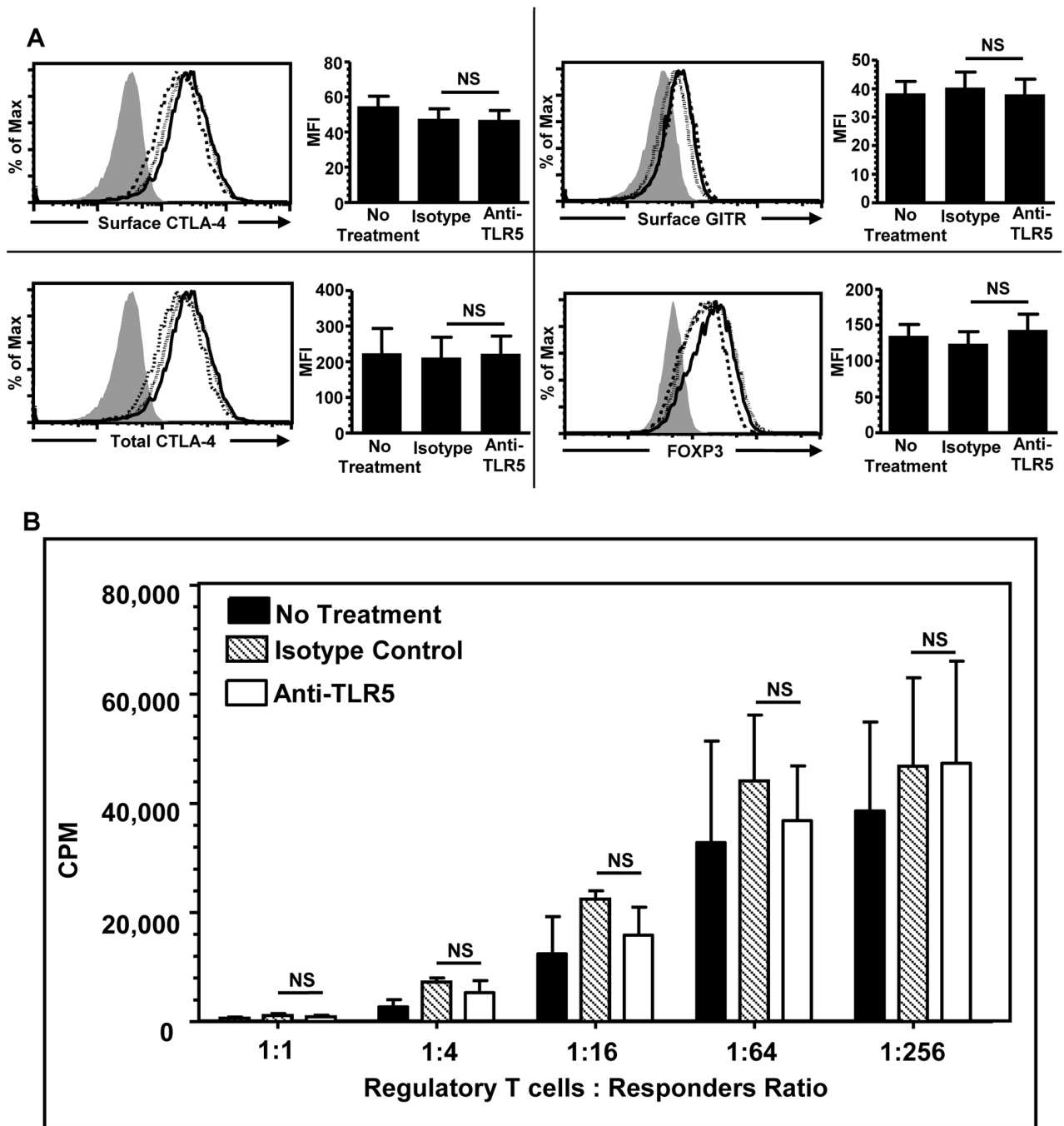


Figure 4. TLR5-related signals did not affect the function of CD4^{hi}CD25⁺ regulatory T cells. (A). Flow cytometric and statistical analysis of the expression of surface CTLA-4 (upper left panel), intracellular CTLA-4 (lower left panel), surface GITR (upper right panel), and FOXP3 (lower right panel) of CD4^{hi}CD25⁺ regulatory T cells generated with no treatment (dotted line), with isotype-matched mAb (dashed line), and with anti-TLR5 blocking mAb (solid line) after 6 days of co-culture of naïve CD4⁺CD25⁻CD45RO⁻ T cells and allogeneic CD40-activated B cells and filled histogram indicates staining obtained from the isotype-matched mAb for staining antibodies. Data were shown in Mean+SEM, n = 6. (B) ³H-thymidine incorporation of CD4^{hi}CD25⁺ regulatory T cells in suppressive MLR at different regulatory T cells: responders ratio. Data show Mean+SEM, n = 6. All data shown are from 3 independent experiments. NS, not significant, one way ANOVA with Tukey's pairwise comparisons. doi:10.1371/journal.pone.0067969.g004

knowledge, is the first report concerning TLR5-related signals in iTregs.

Here we found an increase of TLR5 expression in CD4^{hi}CD25⁺ regulatory T cells. This was probably the consequence of CD4⁺ T cell activation during the co-culture. NF- κ B and AP-1 binding sites are situated around the promoter region of TLR5 locus [37]. NF-

κ B and AP-1 are synthesized during T cell activation [38,39] and may bind to the promoter of TLR5, resulting in the transcription of TLR5. Interestingly, TLR5 can also activate the synthesis of NF- κ B and AP-1 [14], thus it is possible that TLR5 was activated during the co-culture and positively feedback to the TLR5 expression. Since TLR5 expression was also up regulated in

resting nTregs [22], it is possible that Foxp3 also up regulate the TLR5 expression but the precise mechanism remains to be investigated.

In this study, we further found that blockade of TLR5 using anti-TLR5 blocking antibody reduced the proliferation of CD4^{hi}CD25⁺ regulatory T cells through S phase arrest but did not increase the apoptosis of CD4^{hi}CD25⁺ regulatory T cells or CD4⁺CD25⁻ T cells. Since TLR5 was reported to be anti-apoptotic [40], it was surprising that blockade of TLR5 did not increase the apoptosis of the cells. This may be explained by the observation from our previous investigation that large amount of IL-2 was produced by the CD40-activated B cells [28], thus it is possible that these IL-2 molecules rescued the CD4⁺ T cells from apoptosis.

The S phase arrest of the CD4^{hi}CD25⁺ regulatory T cells may be explained by the associated reduction of the ERK1/2 phosphorylation after TLR5 blockade. It is known that S phase exit or G2/M phase entry is controlled by cdk2 and cyclin A [41], the cdk2 is in turn activated by cdc25a [42], which could be activated and phosphorylated by p-ERK1/2 [43]. Therefore, it is speculated that the reduced ERK1/2 phosphorylation in the CD4^{hi}CD25⁺ regulatory T cells decreased the expression and activation of cdc25a, thus in turn, the cdk2 activation, causing S phase arrest. However, the precise molecular mechanism between the reduced ERK1/2 phosphorylation and the S phase arrest remains to be elucidated. In addition, the reduced proliferation of the CD4^{hi}CD25⁺ regulatory T cells may also be the result of reduced production of different cytokines. It was reported that stimulation of TLR5 using flagellin resulted in IL-8 production in epithelial cells and gastric cancer cells, increasing the proliferation of these cells [44,45], and the production of IFN- γ [46]. Therefore, it is possible that TLR5-related signals may enhance the production of IFN- γ , which in turn increases the proliferation of CD4^{hi}CD25⁺ regulatory T cells. However, the relative importance between cell cycle control and cytokine production in regulating the proliferation of the CD4^{hi}CD25⁺ regulatory T cells remains to be elucidated.

Our results demonstrated that TLR5 is not involved in the suppressive function of CD4^{hi}CD25⁺ regulatory T cells. This is in contrast with previous studies by Crellin *et al.* that flagellin stimulation of natural regulatory T cells enhanced the FOXP3 expression and function [27]. Maximal Foxp3 expression in peripheral thymic derived regulatory T cells requires signals from TCR [47], CD28 [48], and IL-2 [49]. IL-2 promotes Foxp3 expression by activating STAT5, which binds to the promoter of

Foxp3 locus. In Crellin *et al.* experiment, IL-2 was not used to activate nTregs [27] and the nTregs might express a low level of Foxp3, which could be up regulated by flagellin stimulation. Flagellin stimulation promoted AP-1 activation and the binding of AP-1 to the promoter of Foxp3 locus, thus in turn, the transcription of Foxp3 [50] while IL-2 secreted from the CD40-activated B cells [31] might compensate the effect of TLR5 blockade on the function of CD4^{hi}CD25⁺ regulatory T cells in our induction system. The lack of IL-2 in Crellin *et al.* experiment may also explain the contrasting results in CD4^{hi}CD25⁺ regulatory T cells proliferation. nTregs is hyporesponsive for proliferation and its proliferation requires the co-existence of CD3, CD28, and IL-2 signaling. It is possible that TLR5 signaling was not potent enough to break this hyporesponsiveness. In contrast, the CD4^{hi}CD25⁺ regulatory T cells are induced from naive CD4⁺CD25⁻CD45RO⁻ T cells, which are not hyporesponsive for proliferation, and the strength of TLR5 signaling may be sufficient in promoting the proliferation of CD4^{hi}CD25⁺ regulatory T cells.

In conclusion, our results demonstrated that TLR5-related signals were involved in the proliferation of CD4^{hi}CD25⁺ regulatory T cells by promoting the progress of S phase arrest and ERK1/2 signaling may be involved. However, TLR5-related signals did not affect the function of CD4^{hi}CD25⁺ regulatory T cells. The role of TLR5-related signaling in CD4^{hi}CD25⁺ regulatory T cells is more resemble to CD4⁺ effector T cells than CD4⁺ nTregs as reflected by the contrasting responses in proliferation and suppressive function after the blockade of TLR5. However, whether the same phenomenon can be observed in other types of iTregs such as Tr1 and Th3 remains to be elucidated. Our result also indicated that, unlike TLR2, TLR5-related signaling promoted the proliferation of CD4^{hi}CD25⁺ regulatory T cells without diminishing the suppressive function [19]. This suggests that flagellin may be a potential ligand for increasing the number of iTregs and suppressing inflammation in organs such as intestines where induced regulatory T cells are abundant.

Author Contributions

Conceived and designed the experiments: PLC WT. Performed the experiments: PLC JZ Yuan Liu KTL ZX HM GQ. Analyzed the data: PLC JZ Yingping Liu WT. Contributed reagents/materials/analysis tools: YLL. Wrote the paper: PLC WT.

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