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

Ping-Lung Chan, Jian Zheng, Yinping Liu, Kwok-Tai Lam, Zheng Xiang, Huawei Mao, Yuan Liu, Gang Qin, Yu-Lung Lau, Wenwei Tu*

Department of Paediatrics and Adolescent Medicine, Li Ka Shing Faculty of Medicine, University of Hong Kong, Hong Kong SAR, China

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 naive 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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* E-mail: wwtu@hku.hk

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 polyendocrinopathy 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 vivo 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-9^{−/−} 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 iTregs function through induction of IDO [21].

TLR5 is expressed in both CD4+ T cells and iTregs [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 iTregs. Crellin et al. reported that stimulation of human iTregs 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+CD25+ regulatory T cells with therapeutic potential from naïve CD4+CD25-CD45RO- precursors using human allologenic CD40-activated B cells as stimulators without the use of exogenous cytokine. Co-culture of human naïve CD4+CD25- T cells with allologenic CD40-activated B cells at T cell to B cell ratio of 10:1 induced a population of CD4+CD25+ regulatory T cells [28]. The CD4+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+CD25+ regulatory T cells induced by allologenic 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+CD25+ regulatory T cells induced by allologenic 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) supplemented with the 2μg/ml of IL-4 (R&D systems, MN), 5.5 x 10^-7 M of cyclosporine A (Sigma-Aldrich, MO), 50 μg/ml of transferrin (Sigma-Aldrich, MO), 5 μg/ml of insulin (Sigma-Aldrich, MO), 15 μg/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 Naïve CD4+CD25-CD45RO- T Cells and Induction of CD4+CD25+ Regulatory T Cells

The CD4+CD25+ regulatory T cells were induced by the co-culture of the CD4+CD25-CD45RO- T cells with the allelogenic 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 naïve 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 naïve 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 μg/ml of anti-TLR5 mAb, and its relevant isotype control (Invivogen, CA) were used for the blockade of TLR5. 20 μM 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 allelogenic 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, naïve CD4+CD25-CD45RO- T cells were stained with CFSE before co-culturing with allelogenic 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+CD25+ regulatory T cells generated with or without the blockade of TLR5 were sorted using FACS Aria after 9 days of co-culture. The sorted CD4+CD25+ regulatory T cells were titrated and co-cultured with 5 x 10⁴ responder CD4+CD25- T cells from the same donor of the CD4+CD25+ regulatory T cells and 5 x 10⁴ 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+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 naive 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^{hi}CD25^{+} T cells, surface TLR5 expression level was lower than that of naive 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 cells 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/propidium 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. Approximate 5% of CD4^{hi}CD25^{+} T cells and 2% of CD4^{+}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. Lr5 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^{+}CD25^{+} regulatory T cells generated on Day 6 (right panel) from naive CD4^{+}CD25^{+}CD45RO^{+} T cells (left panel). (B) Flow cytometric analysis of the expression of surface TLR5 in freshly isolated naive 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 naive 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 naive 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 naive 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^{hi}CD25^{+} T cells (lower panel) after 6 days of co-culture of naive 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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generation was the result of decreased CD4+ T cells proliferation. CFSE staining demonstrated that CD4hiCD25+ 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 CD4hiCD25+ 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 CD4hiCD25+ regulatory T cells (p, 0.05) (Figure 2A, right panel). This result supported our hypothesis that TLR5-related signals enhanced the proliferation of CD4hiCD25+ 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 CD4hiCD25+ Regulatory T Cells

To elucidate the molecular mechanism of the TLR5-blockade induced-S phase arrest, the ERK1/2 phosphorylation was investigated [33]. Flow cytometric analysis indicated that the blockade of TLR5 reduced phosphorylated ERK1/2 (p-ERK1/2) in CD4hiCD25+ regulatory T cells (Figure 3A, left panel). The MFI of p-ERK1/2 in CD4hiCD25+ 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 blockade might reduce CD4hiCD25+ regulatory T cells proliferation by inducing S phase arrest. (A) Flow cytometric analysis of the CFSE signal in CD4hiCD25+ 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 CD4hiCD25+ regulatory T cells. Data show Mean+SEM, n = 6. (right panel). (B) Cell cycle analysis of CD4hiCD25+ 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 CD4hiCD25+ regulatory T cells in S phase (left panel). Statistical analysis of percentage of CD4hiCD25+ 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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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 cell cycle progression of CD4$^{hi}$CD25$^{+}$ regulatory T cells was 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 nTregs [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
knowledge, is the first report concerning TLR5-related signals in iTregs.

Here we found an increase of TLR5 expression in CD4hiCD25+ regulatory T cells. This was probably the consequence of CD4+ T cell activation during the co-culture. NF-κB and AP-1 binding sites are situated around the promoter region of TLR5 locus [37]. NF-κB 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-κB 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 upregulated in

Figure 4. TLR5-related signals did not affect the function of CD4hiCD25+ 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 CD4hiCD25+ 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 naive 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 CD4hiCD25+ 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.
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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 CD4hiCD25+ regulatory T cells through S phase arrest but did not increase the apoptosis of CD4hiCD25+ 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 CD4hiCD25+ 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], phosphorylation after TLR5 blockade. It is known that S phase be explained by the associated reduction of the ERK1/2 expression by activating STAT5, which binds to the promoter of TCR [47], CD28 [48], and IL-2 [49]. IL-2 promotes Foxp3 peripheral thymic derived regulatory T cells requires signals from stimulation of natural regulatory T cells enhanced the FOXP3 et al.

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