Toll-Like Receptor (TLR)9-signaling and its effects on B cell receptor (BCR) signaling in B-1 cells

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Introduction

What are Toll-like receptors (TLRs) and what is the role of TLR9 in infection?

- Part of the innate immune system
- A type of pattern recognition receptor (PRR) that recognize structurally conserved motifs among pathogens, also known as pathogen associated molecular patterns or PAMPs
- TLR9 is located in the endosomes and recognizes unmethylated DNA that is present in pathogens, but not in mammals
- TLR activation leads to an inflammatory response including the release of cytokines and the transcription of genes involved in the

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Methods

Cell isolation

- B-1 cells were isolated from the peritoneal and pleural cavities via lavage and B-2 cells were isolated from the spleen; both were from BALB/c mice
- CD5+ B-1 cells and B-2 cells were sorted using Fluorescence Activated Cell Sorting on the FACSAria

Flow cytometry and ELISA

- B-1 and B-2 cells were cultured for 72h at 37°C with the proliferation dye eFluor670 (1.25 mM) and either CpG (5 mg/mL), anti-IgM (Fab)2 (10 mg/mL), CpG+anti-IgM, or no stimulation
- Cells were used for Flow cytometry and the supernatant used for ELISA

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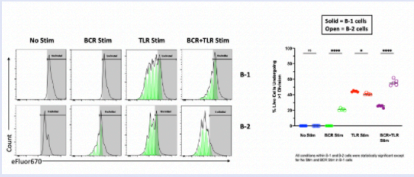
Figure 1. TLR but not BCR stimulation results in activation, proliferation, and differentiation of B-1 cells



(A) Representative histograms of the proliferation dye eFluor670 for B-1 and B-2 cells cultured for 72 hours with no stimulation, anti-IgM(Fab)2 (BCR stimulation), CpG (TLR stimulation), or anti-IgM+CpG (BCR+TLR stimulation) (left). Graph of proliferation measured as % of live cells dividing more than once for B-1 and B-2 cells (right).

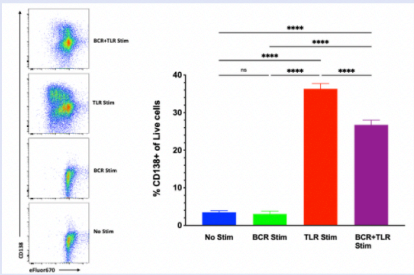


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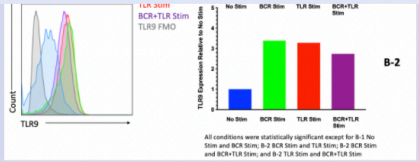
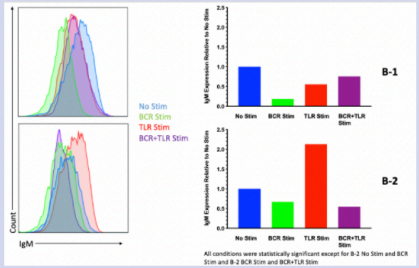
Figure 2. Reciprocal induction of IgM-BCR and TLR9 following their stimulation in B-2 but not B-1 cells

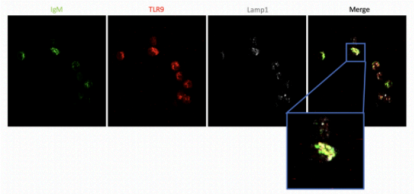


(A) Representative histograms of TLR9 (left) and a graphical representation of normalized TLR9 expression in B-1 and B-2 cells cultured for 72 hours with no stimulation, anti-IgM(Fab)2 (BCR stimulation), CpG (TLR stimulation), or anti-IgM+CpG (BCR+TLR stimulation) measured by flow cytometry (n=3) (right).



(B) Representative histograms of IgM (left) and a graphical representation of normalized IgM expression in B-1 and B-2 cells cultured as indicated in (A) (right).

Figure 3. The BCR colocalizes with TLR9 and Lamp-1 (late endosome) following BCR internalization in B-1 and B-2 cells



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Conclusions

- The outcome of BCR and/or TLR stimulation differs significantly between B-1 and B-2 cells as observed in proliferation, activation and differentiation
- B-1 cells are unable to proliferate in response to BCR stimulation, but are able to differentiate into CD138+ plasma cells with combined BCR+TLR stimulation
- The effect of BCR and/or TLR stimulation on their own expression and the expression of each other also differs in B-1 and B-2 cells
- The BCR colocalizes with TLR9 and Lamp-1 (late endosome) in both B-1 and B-2 cells

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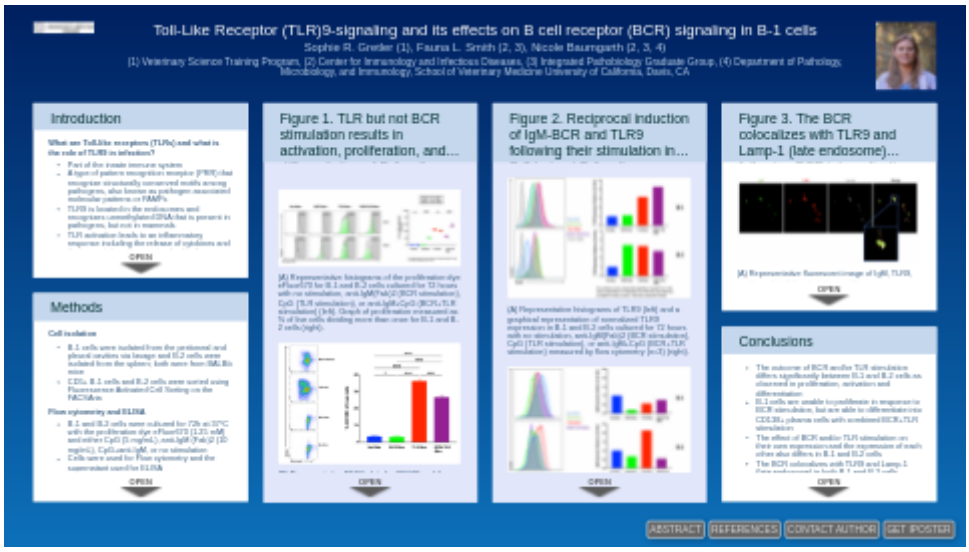

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PRESENTED AT:

AAVMC American Association of Veterinary Medical Colleges

NVSS National Veterinary Scholars Symposium

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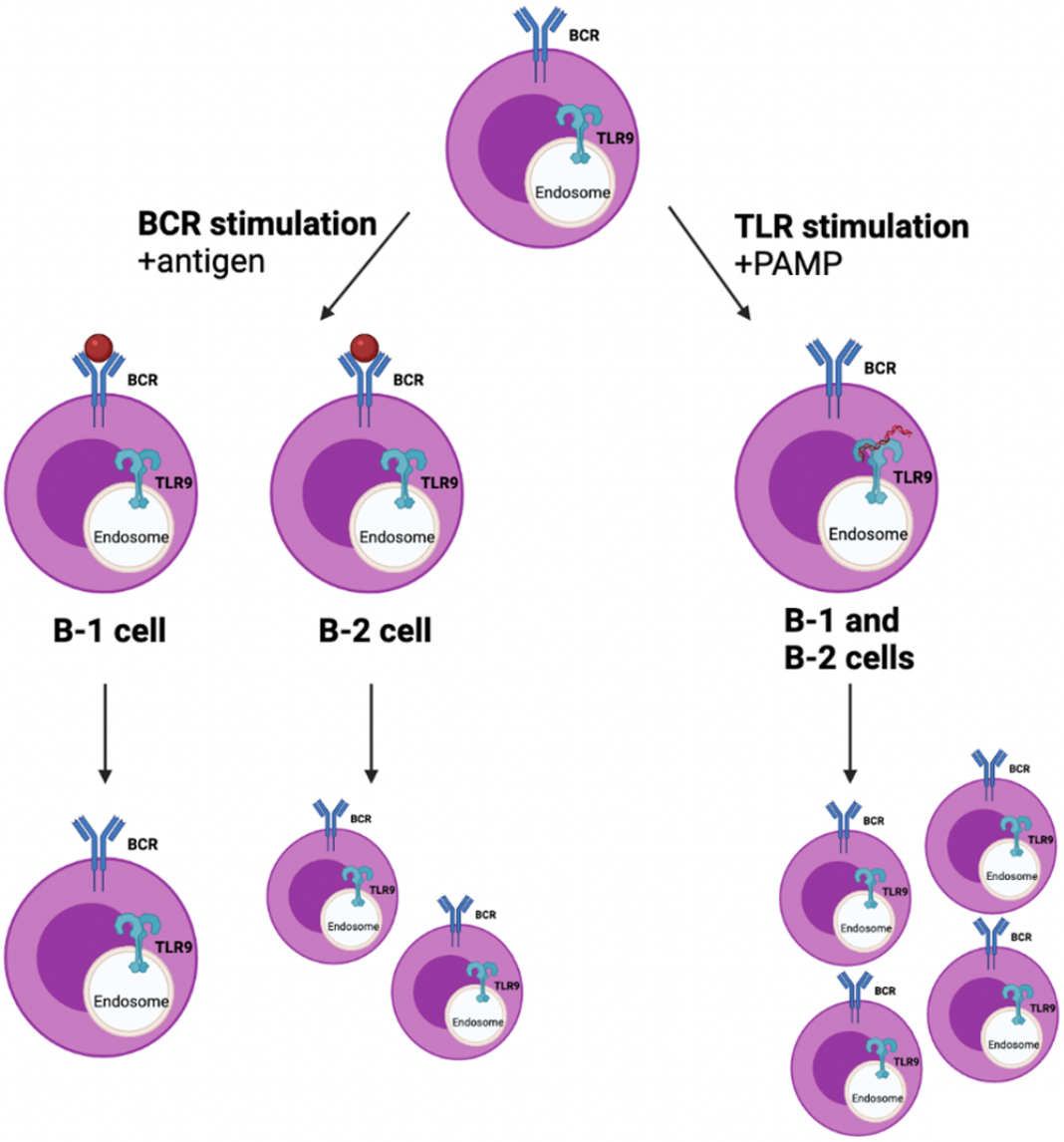
- TLR activation leads to an inflammatory response including the release of cytokines and the transcription of genes involved in the immune response

What are B-1 cells?

- Small subset of fetal and neonatally-derived B cells
- Play a role in the innate immune response through their constitutive and induced secretion of polyreactive IgM
- They are often self-reactive and are prevented from becoming activated upon BCR stimulation by the presence of inhibitory molecules such as CD5
- Respond to TLR stimulation by downregulating CD5 and becoming activated



Hypothesis: TLR stimulation in B-1 cells alters the location and expression levels of the BCR and results in activation of downstream BCR signaling pathways

METHODS

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- Cells were used for Flow cytometry and the supernatant used for ELISA

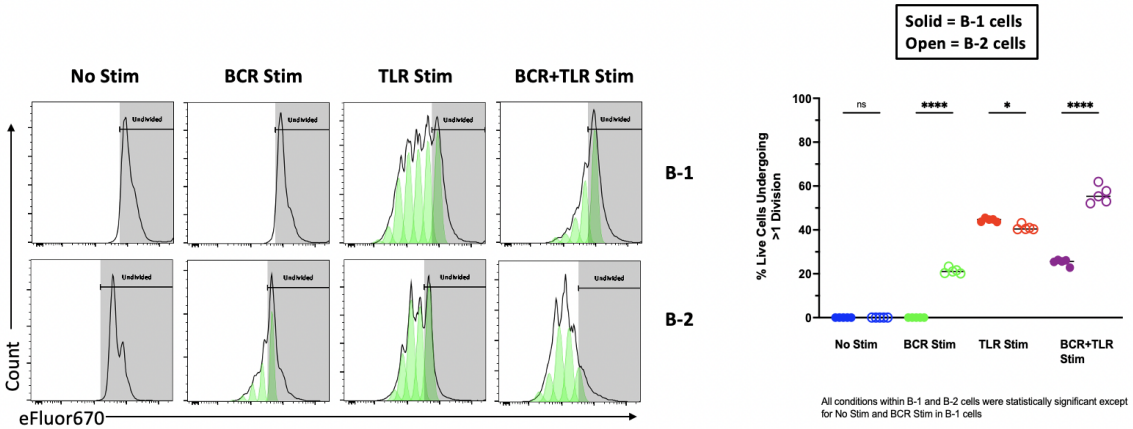
Immunofluorescence

- Immunofluorescence staining was performed on B-1 and B-2 cells pretreated for 30 min at 37°C with CpG, anti-IgM, CpG+anti-IgM, or no stimulation, followed by a 30 min internalization assay with anti-IgM-FITC
- Cells were imaged using a Leica confocal microscope

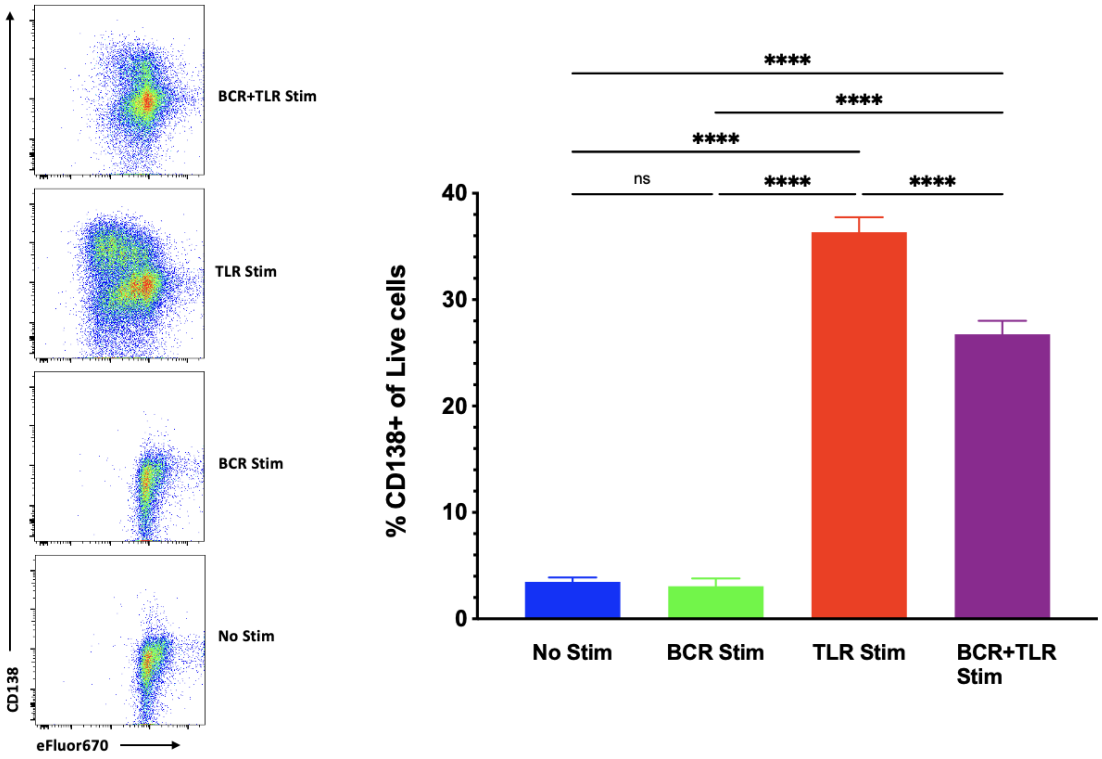
Statistical Analysis

- Analysis was done using a One-way ANOVA with multiple comparisons
- *=$p<0.05$, **=$p<0.005$, ***=$p<0.0005$, ****=$p<0.00005$

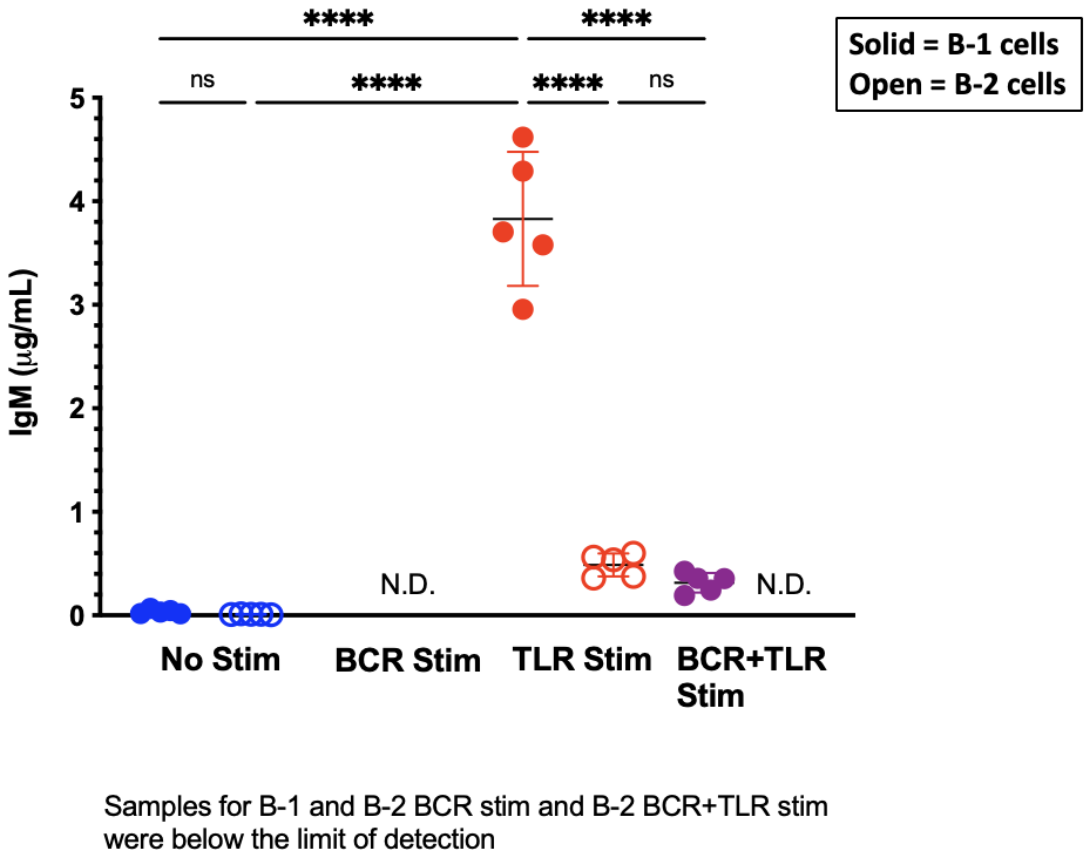
FIGURE 1. TLR BUT NOT BCR STIMULATION RESULTS IN ACTIVATION, PROLIFERATION, AND DIFFERENTIATION OF B-1 CELLS



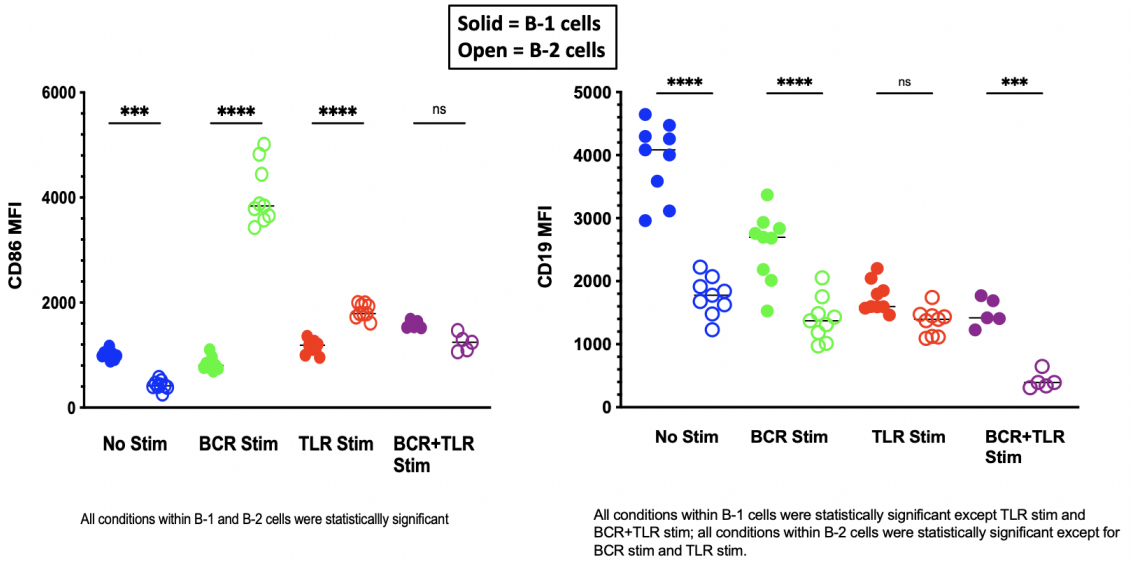
(A) Representative histograms of the proliferation dye eFluor670 for B-1 and B-2 cells cultured for 72 hours with no stimulation, anti-IgM(Fab)2 (BCR stimulation), CpG (TLR stimulation), or anti-IgM+CpG (BCR+TLR stimulation) (left). Graph of proliferation measured as % of live cells dividing more than once for B-1 and B-2 cells (right).



(B) Representative FACS plots for CD138 and the proliferation dye eFluor670 (left) and graph of Mean % CD138+ B-1 cells ± SD cultured for 72 hours with no stimulation, anti-IgM(Fab)2 (BCR stimulation), CpG (TLR stimulation), or anti-IgM+CpG (BCR+TLR stimulation) (n=5) (right).

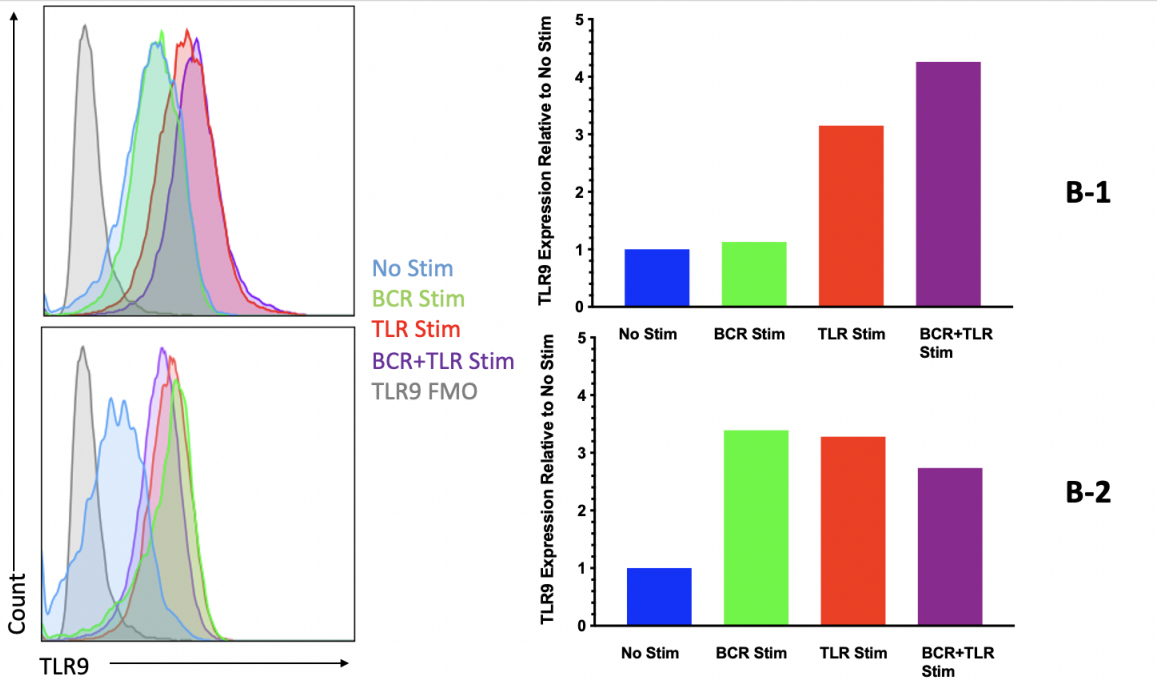


(C) Concentration of IgM (ug/mL) in the supernatant of B-1 and B-2 cells cultured for 72 hours with no stimulation, anti-IgM(Fab)2 (BCR stimulation), CpG (TLR stimulation), or anti-IgM+CpG (BCR+TLR stimulation) measured by ELISA (n=5).

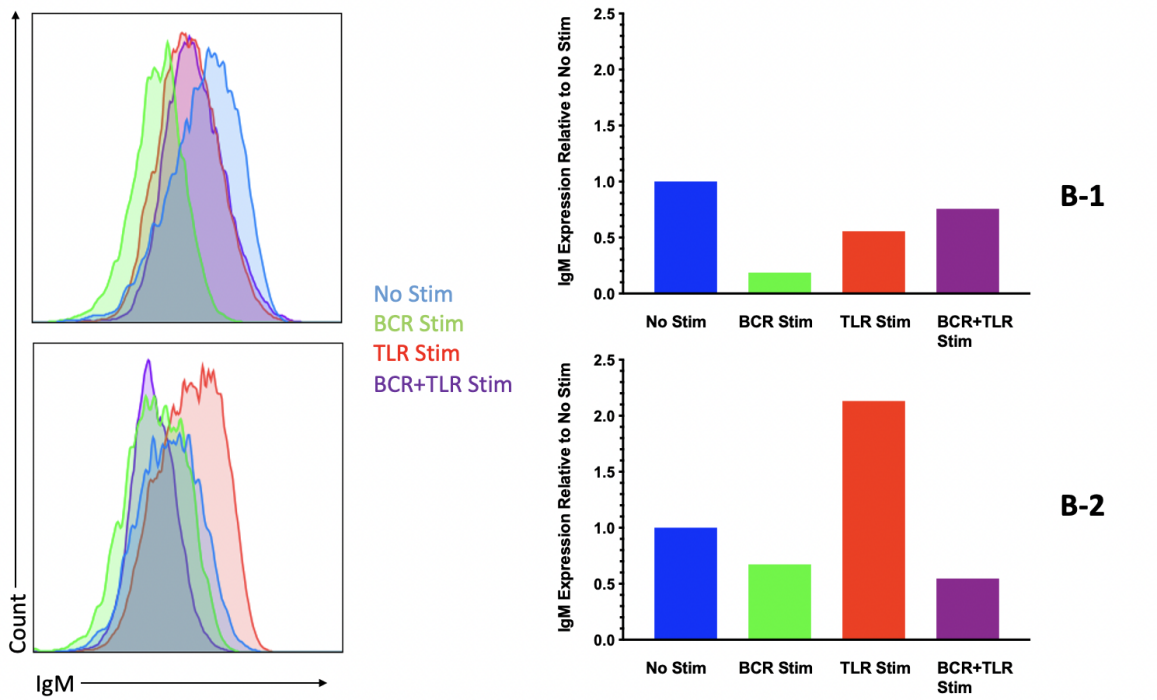


(D) MFI ± SD of CD86 (left) and CD19 (right) in B-1 and B-2 cells cultured for 72 hours with no stimulation, anti-IgM(Fab)2 (BCR stimulation), CpG (TLR stimulation), or anti-IgM+CpG (BCR+TLR stimulation) measured by flow cytometry (n=5-9).

FIGURE 2. RECIPROCAL INDUCTION OF IGM-BCR AND TLR9 FOLLOWING THEIR STIMULATION IN B-2 BUT NOT B-1 CELLS

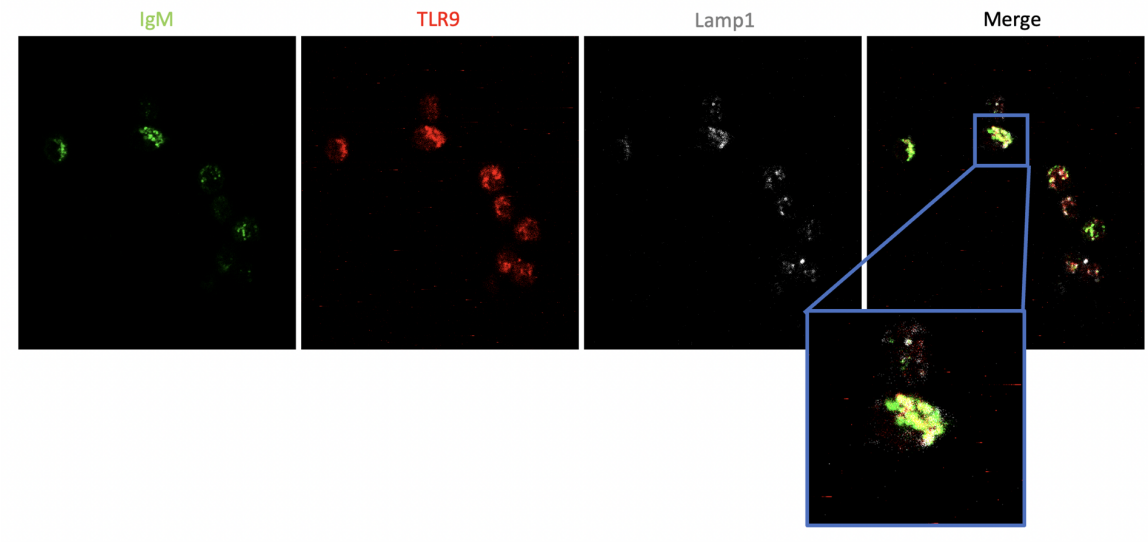


(A) Representative histograms of TLR9 (left) and a graphical representation of normalized TLR9 expression in B-1 and B-2 cells cultured for 72 hours with no stimulation, anti-IgM(Fab)2 (BCR stimulation), CpG (TLR stimulation), or anti-IgM+CpG (BCR+TLR stimulation) measured by flow cytometry (n=3) (right).

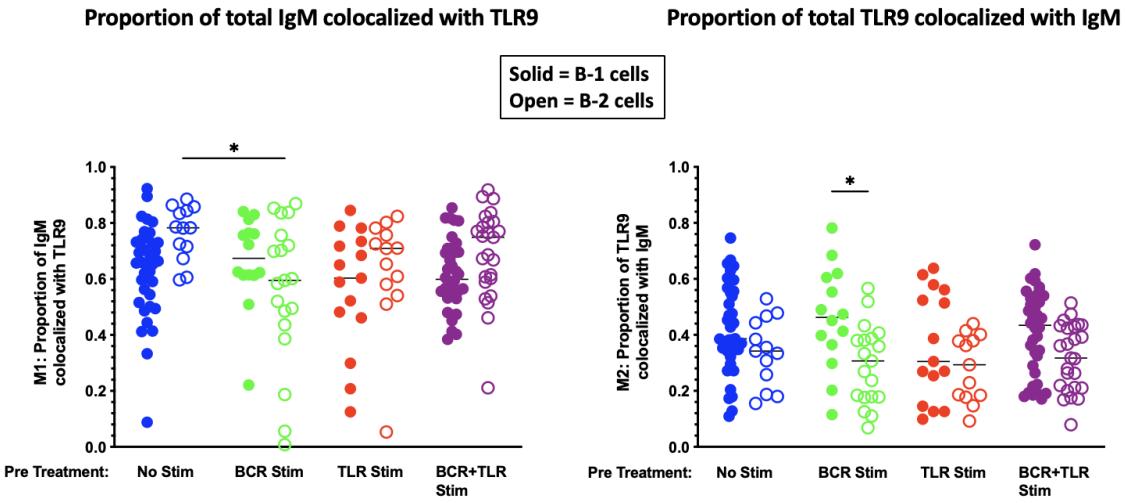


(B) Representative histograms of IgM (left) and a graphical representation of normalized IgM expression in B-1 and B-2 cells cultured as indicated in (A) (right).

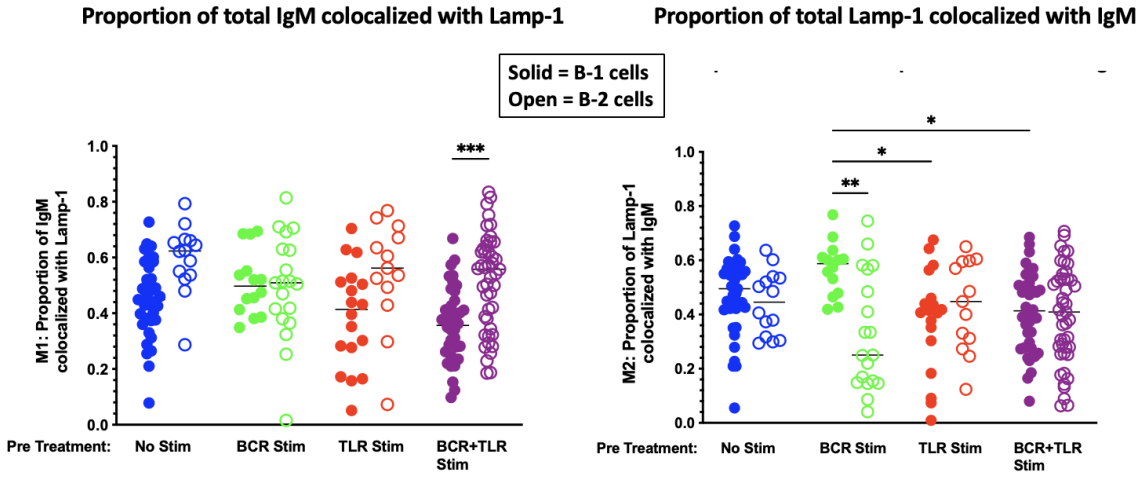
FIGURE 3. THE BCR COLOCALIZES WITH TLR9 AND LAMP-1 (LATE ENDOSOME) FOLLOWING BCR INTERNALIZATION IN B-1 AND B-2 CELLS



(A) Representative fluorescent image of IgM, TLR9, and Lamp1 in B-1 cells following 30 min internalization assay with anti-IgM-FITC.



(B) Thresholded Manders coefficients M1 (left) and M2 (right) for IgM and TLR9 in B-1 and B-2 cells pre-treated for 30 min with no stimulation, anti-IgM(Fab)2 (BCR stimulation), CpG (TLR stimulation), or anti-IgM+CpG (BCR+TLR stimulation) followed by 30 min internalization with anti-IgM-FITC (n=13-45).



(C) Thresholded Manders coefficients M1 (left) and M2 (right) for IgM and Lamp-1 in B-1 and B-2 cells stimulated as described in (B) (n=13-45).

CONCLUSIONS

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ABSTRACT

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B-1 cells are a subset of fetal and neonatal-derived B cells. They produce circulating natural IgM that help remove altered self and foreign antigens, including many pathogens. Antigen stimulation via the BCR fails to induce B-1 cell proliferation due to BCR-signaling inhibitors such as CD5, however, B-1 cells do proliferate in response to TLR-stimulation. We previously showed that TLR stimulation results in the downregulation of CD5 in B-1 cells, which is critical for their differentiation into plasma cells. However, the exact effects of TLR-mediated BCR-reorganization on the function of B-1 cells is unknown. The aim of this study was to evaluate the effects of these stimuli on BCR and TLR expression of B-1 and conventional B-2 cells using flow cytometry and confocal microscopy. Purified murine B-1 and B-2 cells were stimulated with CpG, a TLR9 agonist, and/or the BCR agonist anti-IgM $(Fab)_2$, for 72 hours. While CpG +/- anti-IgM increased TLR9 expression 2.8- and 3.5-fold, respectively, anti-IgM alone had no effect on TLR9. In contrast, B-2 cells increased TLR9 expression 3-to-4-fold after both TLR- and BCR- stimulation. TLR- but not BCR-stimulation induced IgM-secretion by B-1 cells, as measured by ELISA on cell culture supernatants. Although B-1 cells do not proliferate after anti-IgM stimulation, confocal image analysis showed rapid internalization of surface BCR. Ongoing analysis assesses the degree of TLR9 and BCR co-localization in the LAMP1+ endosome. The results expand our previous findings, indicating significant effects of TLR-stimulation on BCR internalization in B-1 and B-2 cells. Future studies will explore if TLR-mediated BCR internalization facilitates antigen-processing and presentation by B-1 cells.

Support from Students Training in Advanced Research (STAR) Fellowship (SG); NIH T-32 OD011147 (FLS); NIH/NIAID R01AI148652 (NB) and R21 AI159115 (NB).

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Savage HP, Kläsener K, Smith FL, Luo Z, Reth M, Baumgarth N. TLR induces reorganization of the IgM-BCR complex regulating murine B-1 cell responses to infections. Elife. 2019 Aug 21;8:e46997. doi: 10.7554/eLife.46997.

Lam JH, Smith FL, Baumgarth N. B Cell Activation and Response Regulation During Viral Infections. Viral Immunol. 2020 May;33(4):294-306. doi: 10.1089/vim.2019.0207.