ORIGINAL RESEARCH

Immunity, Inflammation and Disease

WILEY Open Access

Immunoglobulin profile and B-cell frequencies are altered with changes in the cellular microenvironment independent of the stimulation conditions

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Abstract

Introduction: B-cells are essential in the defense against *Mycobacterium tuberculosis*. Studies on isolated cells may not accurately reflect the responses that occur in vivo due to the presence of other cells. This study elucidated the influence of microenvironment complexity on B-cell polarization and function in the context of tuberculosis disease.

Methods: B-cell function was tested in whole blood, peripheral blood mononuclear cells (PBMCs), and as isolated cells. The different fractions were stimulated and the B-cell phenotype and immunoglobulin profiles analyzed.

Results: The immunoglobulin profile and developmental B-cell frequencies varied for each of the investigated sample types, while in an isolated cellular environment, secretion of immunoglobulin isotypes immunoglobulin A (IgA), IgG2, and IgG3 was hampered. The differences in the immunoglobulin profile highlight the importance of cell-cell communication for B-cell activation. Furthermore, a decrease in marginal zone B-cell frequencies and an increase in T1 B-cells was observed following cell isolation, indicating impaired B-cell development in response to in vitro antigenic stimulation in isolation.

Conclusion: Our results suggest that humoral B-cell function and development was impaired likely due to a lack of costimulatory signals from other cell types. Thus, B-cell function should ideally be studied in a PBMC or whole blood fraction.

KEYWORDS
B cells, IgG, PBMC, *M. tuberculosis*, microenvironment

1 | INTRODUCTION

Over the past three decades, researchers have increased the use of cell culture and isolated tissue samples for biological research as an alternative to in vivo animal studies, due to ethical considerations, the large cost and strict regulatory conditions associated with in vivo studies.[1-3] For this reason, cell culture studies have formed the fundamental basis of a variety of research topics. While the information gained from these isolated cell studies provides valuable insight into

biological mechanisms under investigation, they do not account for the many factors that control these physiological responses in vivo. Numerous studies have illustrated the ability of various cell types to modulate the host immune response during tuberculosis (TB) disease,[4-13] and the presence and activation of these cell types may contribute significantly to the function of a cell type of interest, through directing the mounting immune response. As such, the absence of these cells during isolated cell studies may result in artificial observations and inaccurate assumptions regarding the role of a cell population during health and disease. The effects of isolation on investigated cellular responses is evident in many studies,[3,14,15] in which a particular condition produced a measured immune response in whole organism, while having minimal or no effect on the isolated target cells or tissue (vice versa).

Recent studies investigating the role of B-cells during TB revealed impaired B-cell function and decreased B-cell frequencies during active disease.[16,17] A regulatory B-cell (B_{reg}) subtype, killer B-cells, was recently discovered and has been implicated in a variety of immune conditions, including *Mycobacterium tuberculosis* infection and disease.[18,19] Emerging evidence has acknowledged B-cells as essential in the defense against *M. tuberculosis*. Before the participation of B-cells in host immune responses, development and migration of precursor cells known as transitional B-cells from the bone marrow to the spleen is required; where they give rise to mature B-cells in response to antigenic stimulation.[20,21] Immature transitional 1 (T1) B-cells ($CD19^+CD21^-CD23^-$) form the foundation from which transitional 2 (T2) B-cells ($CD19^+CD21^+CD23^+$) and mature B-cells sequentially derive.[22] However, T2 B-cells have been found to be more receptive to cellular activation and proliferation, in comparison to T1 B-cells.[23,24] As such, the presence and regulation of these T2 B-cells dramatically affect the course of the mounted immune responses.

Functional analysis of these cells in numerous health settings identified the role of these cells as potential immune modulators responsible for controlling the immune response during disease and infection.[18,25-28] A study by van Rensburg et al[29] investigated the frequency of these killer B_{regs} during active TB disease and found a decrease in the frequency of these cells during TB diseased individuals when compared to controls. Upon successful TB treatment, these killer B-cell frequencies returned to levels comparable to that of healthy controls, suggesting a pivotal role of these B_{regs} in protective anti-TB immunity.[16,17]

The objective of this study was to elucidate the influence of microenvironment complexity on B-cell polarization and function in the context of TB disease. Current research findings inferring the role of B-cells during *M. tuberculosis* infection utilized isolated B-cell cultures to investigate their functional capacity. Thus, we sought to first assess and characterize B-cell function in whole blood, peripheral blood mononuclear cells (PBMCs), and as an isolated culture in response to various antigenic stimuli, and second to determine the significance of the use of isolated cell culture techniques in studies inferring the role of B-cells during TB disease.

2 | METHODS

2.1 | Ethics statement

Ethical approval was obtained from the health research ethics committee of Stellenbosch University (N16/05/070) and the City of Cape Town City Health. The study was conducted according to the Helsinki Declaration and the International Conference of Harmonization guidelines. Written informed consent was obtained from all study participants.

2.2 | Study participants

For this pilot study, we recruited 23 healthy individuals (15 individuals with a negative Quantiferon [QFN] status). The positive QFN status was suggestive of exposure to *M. tuberculosis*. Recruited participants did not present with any clinical symptoms of TB and had no previous record of active disease. All participants for this study were HIV negative. QFN positive and negative participants were matched according to socioeconomic background.

2.3 | B-cell isolation

Heparinized peripheral blood (18 mL) was collected, of which 3 mL was set aside for stimulation. From the remaining whole blood, PBMCs were isolated using the ficoll-histopaque (GE Healthcare Life Sciences, Marlborough, MA) density gradient method. A fraction of the PBMCs (3×10^6 cells) was set aside for stimulation. Subsequently, B-cells were negatively isolated from the remaining PBMCs using the magnetic-activated cell sorting bead technology, according to the manufacturer's instructions, with the B-Cell Isolation Kit II (Miltenyi Biotec, Centurion, South Africa). Once all the sample fractions had been collected, the cells were stimulated as described below. The purity of the enriched B-cell samples was confirmed by flow cytometry using anti-human CD19 mAb. All samples with resulting gated purity of above 90% were included in analysis.

2.4 | In vitro stimulation assays

According to the literature, 1 mL of blood contains ~1 million PBMCs,[30] of which B-cells make-up roughly 10%.[31,32] As such, the cell concentrations used within this study to investigate B-cell function across investigated microenvironment complexities were based on these cell frequencies. Whole blood (1 mL blood/well), PBMCs (1×10^6 cells/well) and isolated B-cells (100 000 cells/ well) were stimulated under three conditions: unstimulated, H37Rv (1×10^6 CFU) or TLR9a (Sigma-Aldrich, St. Louis, MO) at 50 ng/mL. PBMCs and isolated B-cells were cultured in 96-well round-bottom plates in a total of 200 μL complete media (Roswell Park Memorial Institute plus L/Glutamine [Sigma-Aldrich]) supplemented with 10% Fetal Calf Serum (FCS, Lonza, South Africa). Whole blood samples were incubated in a 24-well flat-bottom plate in a total of 1.1 mL (stimulants were diluted in complete media and added to 1 mL blood). All sample fractions were incubated at 37°C and 5% CO_2 for 24 hours. Following incubation, the plasma (in case of whole blood) and culture supernatants (in the case of PBMCs and isolated B-cells) were harvested, passed through a filter of 0.22 μm (to remove any bacilli that may be contained within the sample) and stored at −80°C for measurement of immunoglobulin secretion. The cells were then fixed with 4% paraformaldehyde (eBioscience, San Diego, CA) for 30 minutes at 37°C, washed with phosphate-buffered saline (Lonza) and cryopreserved (90% FCS and 10% dimethyl sulfoxide; Sigma-Aldrich) in liquid nitrogen for future analysis by flow cytometry.

2.5 | Immunoglobulin isotype analysis by Luminex technology

Quantification of immunoglobulins within the plasma and culture supernatant, following the 24-hour stimulation, was determined using the MAGPix and Bioplex platforms (Bio-Rad Laboratories, Irvine, CA). The immunoglobulins included immunoglobulin A (IgA), IgE, IgG1, IgG2, IgG3, IgG4, and IgM. The experiments were performed according to the kit manufacturer's recommendations.

2.6 | Phenotype analysis by flow cytometry

The antibody panel for cell surface receptor analysis consisted of: CD19-BV605, CD21-PE/Dazzle, CD23-BV421, CD5-PerCP/Vio770, CD125 (IL5RA)-PE (All from Biolegend, San Diego, CA), CD3-FITC (BD Biosciences,

Germany) and CD178 (FASL)-APC (Miltenyi Biotec). Cells were stained for 1 hour at room temperature in the dark, washed, and acquired on a BD LSR II (BD Biosciences). The resulting data were analyzed using the FlowJo v10 software (Treestar, Woodburn, OR). The gating strategy is depicted in Figure S1.

2.7 | Statistical analysis

Quantification of the immunoglobulin isotypes by Luminex was expressed as a percentage of the total immunoglobulin and statistical analysis performed on the relative percentage contribution of each of the isotypes within a sample. Data analysis for all Luminex results was performed using Statistica 12 software (Statsoft) and Prism 7 Software (San Diego, CA). Raw data were checked for normality using normality plots. Statistical differences between sample fractions (whole blood, PBMC, and B-cells), QFN status, and culture conditions (unstimulated, TLR9a, and H37v) were calculated using a four-way mixed model analysis of variance. Comparisons within groups were calculated using the Fisher's least significant difference post hoc test. A two-way step-up Benjamini, Krieger, and Yekutieli false discovery rate (FDR) approach, with an FDR of 1%, was used to correct for multiple testing.

Data analysis of the flow cytometric plots was done using FlowJo V10 (Treestar) and the resulting cell frequencies analyzed using Statistica and Prism 7 Software. Raw data were checked for normality using normality plots and winsorized using the Huber mean and median absolute deviation as necessary to reduce the residual distribution into close agreement with a normal distribution. Statistical significance is indicated by an asterisk, in which the $P < .05$ (*), $P < .01$ (**), and $P < .001$(***) or by letters, in which groups denoted with different letters indicate statistical differences.

3 | RESULTS

3.1 | The B-cell immunoglobulin profile varies considerably according to cellular microenvironment/sample fraction

The mechanisms employed by B-cells toward facilitating enhanced anti-TB immunity remain unresolved. Subsequently, the effects of microenvironment complexity, that is, the degree of cellular isolation on B-cell function were investigated. This was achieved by examining the immunoglobulin expression patterns within culture supernatants following stimulation of B-cells in various sample fractions (whole blood, PBMCs, and isolated B-cells).

Altered immunoglobulin profiles were observed across the various sample fractions, regardless of stimulation conditions; however, most notable was the observation of increased IgM release from isolated B-cells (Figure 1). A significant difference in the immunoglobulin profile was observed for all isotypes, except IgG4, following isolation of B-cells from whole blood (Figure 2A). In some instances, the relative abundance of an isotype within a given sample set, such as IgE ($P = .0001$), IgG3 ($P < .00001$), and IgA (ns, $P > .05$), was observed to increase following PBMC isolation. However, in the majority of cases, a decrease in the relative abundance of an immunoglobulin isotype, specifically IgG1 ($P < .00001$), IgG2 (ns, $P > .05$) IgG4 (ns, $P > .05$), and IgM (ns, $P > .05$), within a given sample set was observed following PBMC isolation. A significant decrease in the relative abundance of most isotypes, including IgA ($P < .00001$), IgG1 ($P < .00001$), and IgG2 ($P < .00001$), was observed following isolation of B-cells compared to whole blood, whilst an increase in the relative abundance of IgE ($P = .0004$) and IgM ($P < .00001$) was found. Likewise, a substantial decrease in the relative abundance of most isotypes, IgA ($P < .00001$), IgG1 ($P = .0001$), IgG2 ($P < .00001$), and IgG3 ($P < .00001$), was observed following isolation of B-cells compared to the PBMC sample fraction, whereas an increase in the relative

abundance of IgM ($P < .00001$) was found. Incidentally, the effect of sample type on the abundance of the various immunoglobulin isotypes was investigated to discern the effect of cellular isolation on the magnitude of subsequent B-cell responses. A significant difference in quantified immunoglobulin levels was observed for all isotypes following each successive isolation procedure (data not shown). To account for the limitations involved in comparing plasma supernatants to culture supernatants, only differences between PBMCs and isolated B-cells were considered. Here, significant decreases in the observed concentration of all immunoglobulin isotypes were observed for isolated B-cell culture supernatants compared to those of PBMC samples.

In addition, the effects of QFN status and stimulation condition on immunoglobulin profiles were investigated. We investigated whether or not *M. tuberculosis*-exposed (QFN-positive) individuals with immune memory would respond differently to *M. tuberculosis* challenge compared to QFN-negative individuals. In all instances, QFN status was found to have no effect on the relative abundance of the various immunoglobulin isotypes (Figure 2B). To conclude, the impact of stimulation condition on immunoglobulin profiles was investigated to determine the effect of *M. tuberculosis* infection on B-cell performance.
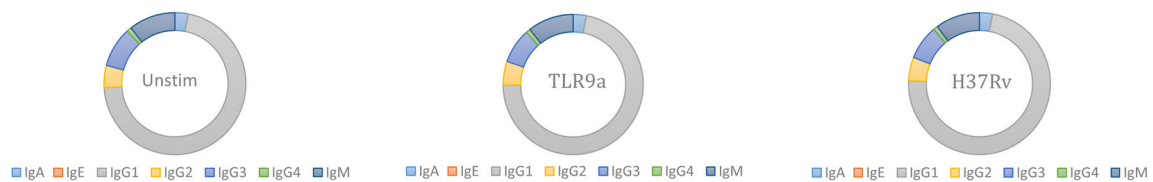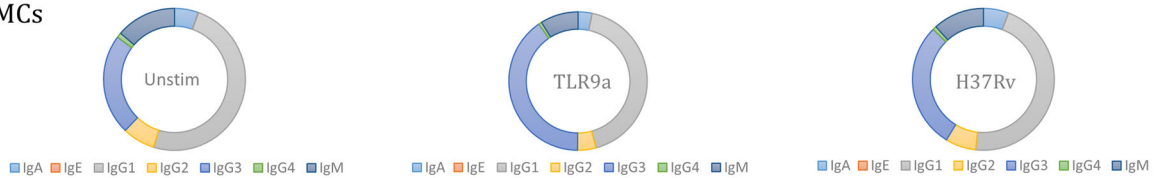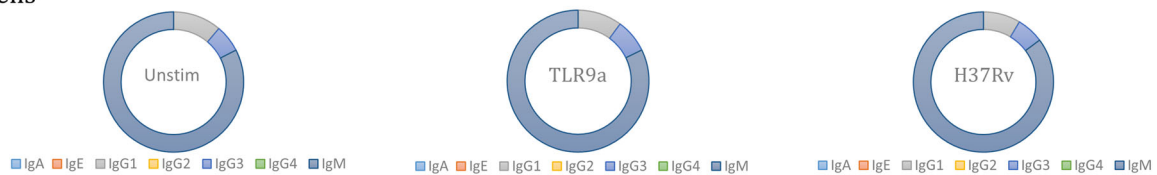

FIGURE 1 Immunoglobulin profile of supernatants obtained from each of the stimulatory conditions of the various cellular fractions. Following a 24-hour stimulatory period, plasma/culture supernatants of each of the stimulatory conditions for all sample types were collected and the immunoglobulin secretion profile determined using Luminex. A, Representation of the average secretion of each isotype within whole blood for each stimulatory condition. B, Representation of the average secretion of each isotype within the PBMC fraction for each stimulatory condition. C, Representation of the average secretion of each isotype within the isolated B-cell fraction for each stimulatory condition, n = 23. Ig, immunoglobulin; PBMC, peripheral blood mononuclear cells
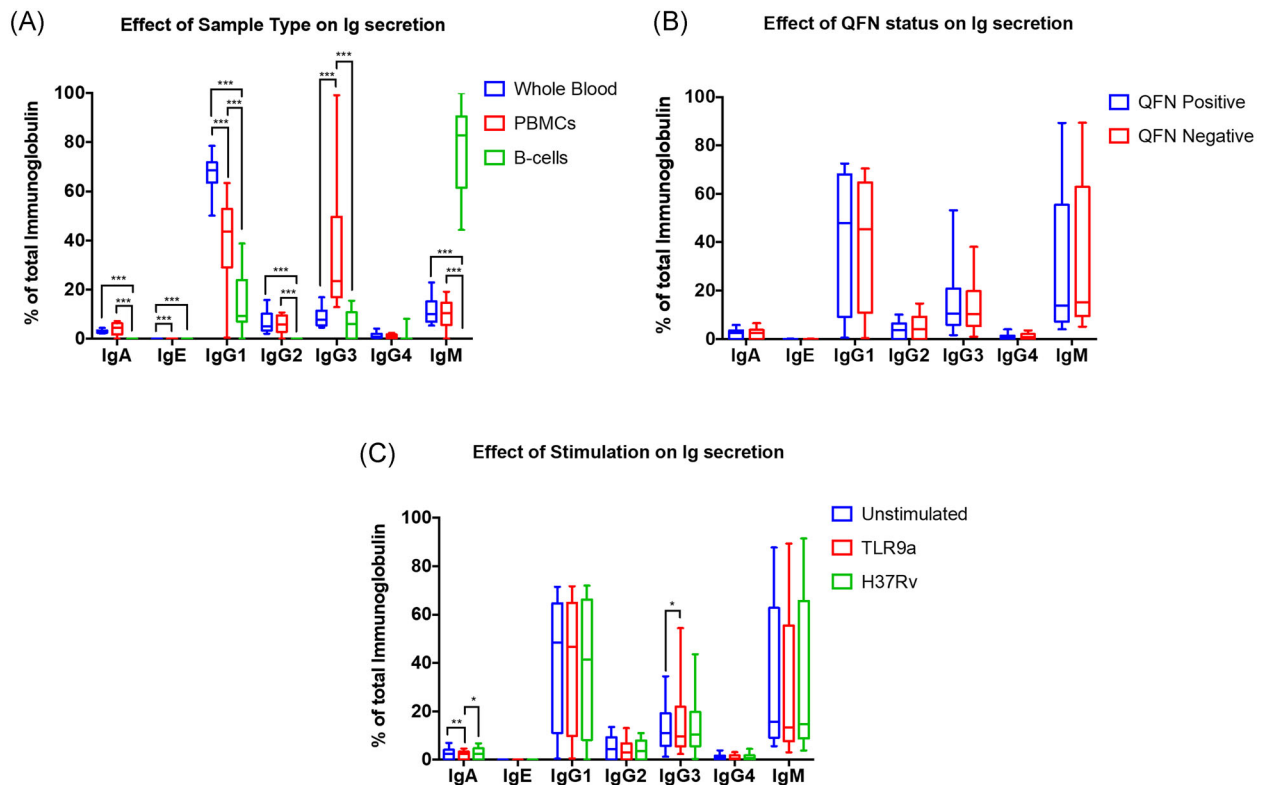
FIGURE 2 Evaluation of the effect of various experimental factors on B-cell immunoglobulin profile. Following a 24-hour stimulatory period, plasma/culture supernatants of each of the stimulatory conditions for all sample types were collected and the immunoglobulin profile determined using Luminex. The immunoglobulin isotype levels depicted are reported as a percentage of the total immunoglobulin within a given sample. Median with interquartile range plotted. Statistical differences between culture conditions was calculated using a four-way mixed-model ANOVA. Comparisons within groups were calculated using Fisher's LSD post hoc test. A two-way step-up Benjamini, Krieger, and Yekutieli false discovery rate (FDR) approach, with an FDR of 1% was used to correct for multiple testing. Statistical significance is indicated by an asterisk, in which the $P < .05$ (*), $P < .01$ (**), and $P < .001$ (***). A, Effects of sample type, that is, microenvironment complexity on immunoglobulin isotype abundance for all stimulation conditions. B, Effects of quantiferon (QFN) status on immunoglobulin isotype abundance across all sample types and stimulation conditions. C, Effects of stimulatory conditions on immunoglobulin isotype abundance for all sample types. ANOVA, analysis of variance; LSD, least significant difference; PBMC, peripheral blood mononuclear cells

For the majority of immunoglobulin isotypes, stimulation with different antigens had no effect on the measured immunoglobulin abundance (Figure 2C). However, a significant decrease in IgA levels was observed following TLR9 stimulation for all sample types compared to unstimulated controls ($P = .0038$) and H37Rv stimulated ($P = .0292$) samples. Conversely, an increase in IgG3 levels was observed following TLR9 stimulation for all sample types compared to unstimulated controls ($P = .0482$).

3.2 | B-cells isolation results in decreased frequencies of MZ B-cells

In addition to investigating alterations within the immunoglobulin profile of B-cells, variations in the phenotypic frequencies of various B-cell subsets were investigated to evaluate the effect of microenvironment complexity on cell function. The expression of the cell surface receptors CD21 and CD23 by B-cells was examined to determine the proportion of B-cells within various developmental stages following antigenic stimulation. Following a 24-hour stimulatory period, cells from all stimulatory conditions for each of the cellular fractions were collected and phenotypic frequencies of the various B-cell population determined using flow cytometry. It is important to note that due to limited cell numbers, isolated B-cell samples (B-cells only) were pooled according to QFN status and stimulatory conditions before flow cytometry analysis, prohibiting the assessment of individual sample distribution and statistically significant differences between B-cells only and other microenvironment conditions. Consequently, observations made from the resulting data focus principally on the difference between whole blood and PBMC, while inferring the

physiological implications of the trends observed for isolated B-cells. The effect of sample type on B-cell development was investigated, in which no significant difference in T1, T2, MZ, and FO B-cell frequencies was observed between whole blood and PBMCs. However, a shared pattern of decreased CD19$^+$CD21$^+$CD23$^-$ (MZ) B-cells, whilst an increase in CD19$^+$CD21$^+$CD23$^+$ (T2) B-cells, CD19$^+$CD21$^-$CD23$^+$ (FO) B-cells, and CD19$^+$CD21$^-$CD23$^-$ (T1) B-cells was observed for all sample types following each successive isolation procedure (Figure 3A). Regrettably, the significance of alterations in these B-cell frequencies for isolated B-cell samples cannot be analyzed (due to the extremely low number of B-cells remaining); however, a sizeable difference in the investigated frequencies is apparent. These results indicate the potential impact that B-cell isolation has on maturation in response to stimulation in vitro.

In addition, the effects of QFN status and stimulation conditions on B-cell development were investigated. For majority of the investigated populations, QFN status was found to have no significant effect on the observed B-cell frequencies (Figure 3B). A general trend of decreased frequencies for all populations was observed for QFN-negative individuals compared to QFN-positive individuals. In accordance, a significant decrease in the observed frequency of CD19$^+$CD21$^+$CD23$^+$ (T2) B-cells ($P = .0012$) was observed for QFN-negative individuals.

Furthermore, the effects of stimulation condition were examined (Figure 3C). A common shift of increased B-cell frequencies was observed for most of the population subsets investigated following TLR9a stimulation compared to unstimulated controls. A significant increase in the frequency of CD19$^+$CD21$^+$CD23$^-$ (MZ) B-cells was observed following TLR9a stimulation ($P = .0000$) and *M. tuberculosis* infection ($P = .0000$), compared to unstimulated cells. Similarly, a significant increase in CD19$^+$CD21$^+$CD23$^+$ (T2) B-cells and CD19$^+$CD21$^-$CD23$^+$ (FO) B-cells was observed in response to TLR9a stimulated compare to unstimulated ($P = .0000$; $P = .0061$) and H37Rv stimulated ($P = .0000$; $P = .0198$) samples, respectively. Contrariwise, a significant decrease in CD19$^+$CD21$^-$CD23$^-$ (T1) B-cells was observed for samples stimulated with either TLR9a ($P = .0000$) or H37Rv ($P = .0000$) compared to unstimulated controls.

4 | DISCUSSION

The standard application of isolated cell studies for the investigation of cell function has proven to be invaluable; however, these studies do not account for the multifaceted effects that surrounding cells types have on each other in vivo. This cellular communication results in
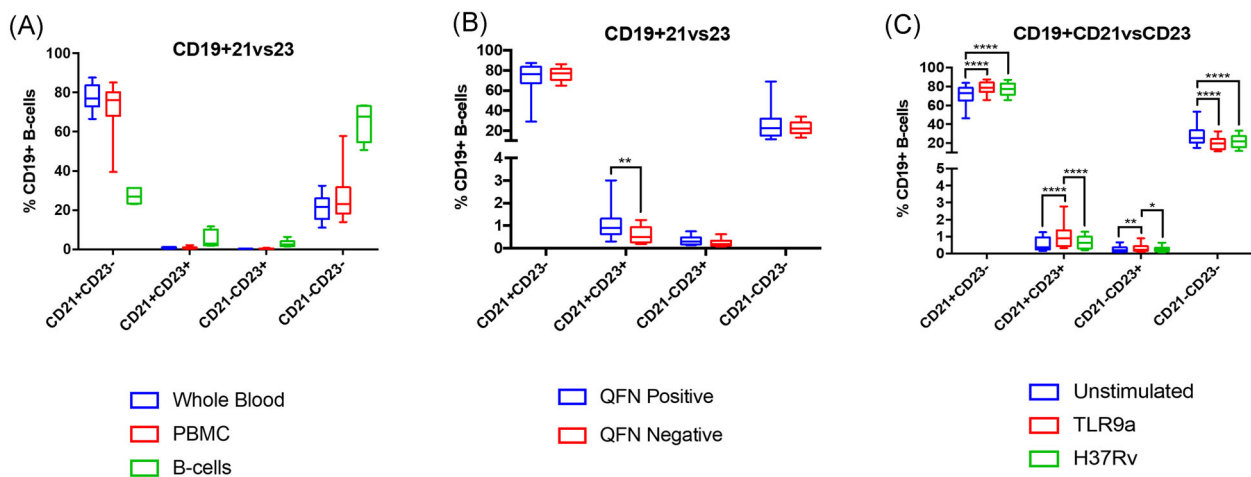

FIGURE 3 Analysis of developmental B-cell phenotypic frequencies. Following a 24-hour stimulatory period, cells from each of the stimulatory conditions, for all sample types, were collected and the B-cell phenotypic frequencies determined using flow cytometry. Notable, statistical significance was only investigated for whole blood and PBMC sample type as isolated B-cell samples for the respective QFN groups were pooled (according to stimulation condition) before flow analysis, due to inadequate cell numbers. Whiskers denote 10 to 90 percentile. Statistical differences between culture conditions were calculated using a four-way mixed-model ANOVA. Comparisons within groups were calculated using the Fisher's LSD post hoc test. A two-way step-up Benjamini, Krieger, and Yekutieli false discovery rate (FDR) approach, with an FDR of 1% was used to correct for multiple testing. Statistical significance is indicated by an asterisk, in which the $P < .05$ (*), $P < .01$ (**), and $P < .001$ (***). A, Effects of sample type on the developmental state of B-cells for all stimulation conditions. B, Effects of quantiferon (QFN) status on the developmental state of B-cells across all sample types and stimulation conditions. C, Effects of stimulation condition on the developmental state of B-cells for all sample types. ANOVA, analysis of variance; LSD, least significant difference; PBMC, peripheral blood mononuclear cells

physiological events that shape the immune response to various antigenic stimuli. In accordance, the purpose of this pilot study was to determine the effect of micro-environment complexity on B-cell function and to determine the significance of the use of isolated cell culture techniques in studies inferring the role of B-cells during TB disease.

Recent research findings have signified the importance of B-cells during *M. tuberculosis* infection, in which the absence or impaired function of this immune cell type has been associated with poor disease prognosis.[33-37] For decades, the primary function of B-cells, apart from antigen presentation, was considered to be antibody secretion, forming part of the adaptive humoral response.[38,39] These humoral immune responses were considered to be effective in controlling the growth and survival of extracellular invading pathogens exclusively. However, investigations analyzing the efficiency of antibody-mediated immunity against several intracellular pathogens, including *M. tuberculosis*, have since disproven this notion.[40,41] In addition, studies have indicated non-humoral B-cell function, such as immune modulation through receptor engagement and cytokine expression, as key mechanisms by which these cells contribute to the successful control of *M. tuberculosis* infection.[19,33,37] As such, the influence of in vitro isolated cell culture studies on B-cell development and function is of great importance, as the majority of current observational findings inferring the physiological role of these cells during TB disease utilize these techniques[19,36] and form the foundation upon which complex in vivo studies investigating potential TB drugs, host-directed therapies, and TB vaccines are based.

In this study, antibody profiles were assumed to be a direct indication of the relative functional capacity of B-cells within the investigated samples. Notably, the presence of circulating antibody within the plasma samples compromises the inference of B-cell activity within whole blood samples and is a limitation of the study. Considerable changes in the immunoglobulin profile were observed across the different sample types, in which the relative percentage contribution of each of the measured isotypes, with the exception of IgG4, differed significantly (Figure 1). The sample type, rather than stimulation condition, had a significant effect on the observed immunoglobulin profile. More specifically, a significant decrease in the relative abundant of IgG1 was observed following PBMC isolation compared to whole blood samples, while a significant increase in the relative abundance of IgG3 was found. The same pattern in the immunoglobulin expression was observed when comparing isolated B-cell samples with PBMCs. Importantly, the observed "increase/decrease" in antibody levels is not equivalent to the concentration of these isotypes within a given sample but rather indicates the relative

immunoglobulin diversity within the cellular micro-environment. The physiological implications of altered immunoglobulin production have been extensively reviewed in several disease states, where deficiency has been associated with increased susceptibility to bacterial infection.[42,43]

Immunoglobulins are known to have a half-life of between 5 to 21 days.[44,45] Thus, circulating levels of antibodies were present within the plasma of whole blood samples before stimulation, whereas cells within the PBMC and isolated B-cell fraction were incubated in fresh media, containing no baseline antibody levels (Figure 2A). This may have resulted in possible artefactual observations in the relative reduction in immunoglobulin levels for whole blood samples compared to PBMC and isolated B-cell samples. As such, the significance of the observed (Ig) decrease was only considered between PBMCs and isolated B-cells. Collectively, these results illustrated that isolation procedures profoundly hindered the ability of B-cells to secrete several immunoglobulin isotypes; underscoring the fact that the presence of additional cell types is required for augmented B-cell activation and function.

Research has implicated IgA and IgG as leaders in protective anti-TB humoral immunity.[34,46,47] The exact mechanisms by which these immunoglobulins achieve the protective effect is still unknown, and further investigation into their cellular targets is needed to better understand the role they play during TB disease.[5]

Investigation of isolated cell studies on B-cells at the site of infection may provide valuable insight into anti-TB immunity. Mechanisms by which T-cells and dendritic cell induce this process are directly through cell-cell interactions with adjacent B-cells and indirectly through the secretion of various soluble molecules.[48,49] Studies utilizing animal models to study anti-TB humoral immunity in vivo and ex vivo analysis of human samples from healthy and active TB participants have proved that humoral responses do in fact aid in the defense against *M. tuberculosis* infection.[34,40,41] This further emphasizes the need to validate experimental observations in several independent experiments, utilizing different techniques in health and disease.

The effect of sample type of B-cell development was investigated via evaluation of the relative frequencies on T1, T2, MZ, and FO B-cells within each sample type following antigenic stimulation (Figure 3). Interestingly, no significant difference in the frequency of all B-cell subsets was observed between whole blood and PBMCs. The involvement of MZ B-cells in T-independent early adaptive immune responses is well established, in which increased plasma cell differentiation and superior induction of Th1 expansion has been shown by MZ B-cells in comparison to FO B-cells.[50-52]

As such, MZ B-cells are regarded as primarily responsible for protective humoral and effector T-cell immune response. In contrast, the activation of FO B-cells occurs via T-cell-dependant mechanisms and is thus involved in late immune responses.[53,54] These results indicate the potential impact of cell isolation on B-cell derived immune responses, in which decreased frequencies of MZ B-cells were found. Thus, impaired B-cell development, as a result of diminished micro-environment complex due to cellular isolation, may result in the manifestation of inappropriate cellular responses to antigenic stimulation in vitro.

The effect of stimulation condition (TLR9 vs H37Rv) on immunoglobulin isotype abundance and B-cell frequencies was investigated. A general pattern of enhanced B-cell development indicated by the increase of MZ B-cells was observed following TLR9a stimulation. This increase in MZ B-cells can be linked to the increase in IgG3 secretion observed following TLR9 stimulation. According to the literature, MZ B-cells have been shown to exhibit an activated phenotype allowing their rapid proliferation and differentiation into Ab-producing cells, preferentially secreting IgM and IgG via class-switch recombination.[55,56] The observed results in response to H37Rv stimulation are in agreement with previous findings[57] and indicate that *M. tuberculosis* challenge evokes early nonhumoral B-cell responses that may play an important role in the host defense against *M. tuberculosis* infection. These results underscore the ability of B-cells to actively respond to the *M. tuberculosis* challenge and suggest that B-cells are involved in directing and shaping the immune response during TB disease.

Our study demonstrates the influence that the micro-environment complexity (ie, sample type) had a profound impact on the activation and function of B-cells. These complex interactions underscore the basis for the use isolated cell studies to investigate cellular function, in an attempt to limit the degree of external factors influencing the observed results. This allows for the assumption, with complete certainty that the measured output for a cell population is in response to a particular drug or stimulus. However, it is important to remember that these isolated interactions are not indicative of whole blood scenarios.[58] Composite cellular interactions exist in vivo that may drastically influence the function of the (B-) cell type of interest resulting in a different reaction of these cells to the same drug or stimulus in whole organism.

SU-IRG CONSORTIUM

Bongani Motaung, Andriette Hiemstra, Stephanus Malherbe, Gerhard Walzl, Belinda Kriel, Marika Flinn, and Ayanda Shabangu.

CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

All the data for this study are included in the manuscript. Flow cytometry (FCS files) and Luminex (CSV files) data are available on request.

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REFERENCES

1. Brown GE Jr. *30 Years of the Animal Welfare Act. Animal Welfare Information Center Newsletter*. 1997;8:1-2.
2. Adams B, Larson J. *Legislative History of the Animal Welfare Act. Animal Welfare Information Center Resource Series* 41, Beltsville, MD: USDA NAL Animal Welfare Information Center. 2007.
3. Murphy HC. The use of whole animals versus isolated organs or cell culture in research. *Trans Nebraska Acad Sci*. 1991; XVIII:105-108.
4. Kaufmann SH. Protection against tuberculosis: cytokines, T cells, and macrophages. *Ann Rheum Dis*. 2002;61:ii54-ii58.
5. Li H, Wang X, Wang B, et al. Latently and uninfected health-care workers exposed to TB make protective antibodies against *Mycobacterium tuberculosis. Proc Nat Acad Sci USA*. 2017; 114(19):5023-5028.
6. Maglione PJ, Xu J, Chan J. B cells moderate inflammatory progression and enhance bacterial containment upon pulmonary challenge with *Mycobacterium tuberculosis. J Immunol*. 2007;178:7222-7234.
7. Manivannan S, Rao VN, Ramanathan VD. Role of complement activation and antibody in the interaction between *Mycobacterium tuberculosis* and human macrophages. *Indian J Exp Biol*. 2012;50:542-550.
8. Millington KA, Innes JA, Hackforth S, et al. Dynamic relationship between IFN-γ and IL-2 profile of *Mycobacterium tuberculosis*-specific T cells and antigen load. *J Immunol*. 2007;178:5217-5226.
9. Orme IM, Roberts AD, Griffin JP, Abrams JS. Cytokine secretion by CD4 T lymphocytes acquired in response to *Mycobacterium tuberculosis* infection. *J Immunol*. 1993;151:518-525.
10. Phuah J, Wong EA, Gideon HP, et al. Effects of B cell depletion on early *Mycobacterium tuberculosis* infection in cynomolgus macaques. *Infect Immun*. 2016;84:1301-1311.

11. Redford PS, Murray PJ, O'garra A. The role of IL-10 in immune regulation during M. tuberculosis infection. *Mucosal Immunol.* 2011;4:261-270.

12. Ribeiro-Rodrigues R, Resende Co T, Rojas R, et al. A role for CD4+ CD25+ T cells in regulation of the immune response during human tuberculosis. *Clin Exp Immunol.* 2006;144:25-34.

13. Warsinske HC, Pienaar E, Linderman J, Joshua M, Kirschner D. Deletion of TGF-β1 increases bacterial clearance by cytotoxic T cells in a tuberculosis granuloma model. *Front Immunol.* 2017; 8:1843.

14. Kondo T, Magee DF. Evidence for antral inhibition of pentagastrin from experiments using mucosal cooling. *J Physiol.* 1977;270(1):37-50.

15. Sanders MJ, Amirian DA, Ayalon A, Soll AH. Regulation of pepsinogen release from canine chief cells in primary monolayer culture. *Am J Physiol.* 1983;245(5):G641-G646.

16. Joosten SA, van Meijgaarden KE, Del Nonno F, et al. Patients with tuberculosis have a dysfunctional circulating B-cell compartment, which normalizes following successful treatment. *PLoS Pathog.* 2016;12(6):e1005687.

17. Van Rensburg IC, Wagman C, Stanley K, et al. Successful TB treatment induces B-cells expressing FASL and IL5RA mRNA. *Oncotarget.* 2017;8(2):2037-2043.

18. Lundy SK, Klinker MW, Fox DA. Killer B lymphocytes and their fas ligand positive exosomes as inducers of immune tolerance. *Front Immunol.* 2015;6:122.

19. van Rensburg IC, Loxton AG. Killer (FASL regulatory) B cells are present during latent TB and are induced by BCG stimulation in participants with and without latent tuberculosis. *Tuberculosis.* 2018;108:114-117.

20. Abbas AK, Lichtman AH, Pillai S. *Basic Immunology: Functions and Disorders of the Immune System.* St. Louis, MO: Elsevier Health Sciences; 2014.

21. Paul WE. *Fundamental immunology.* 7th ed. Philadelphia, PA: Lippincott Williams & Wilkins; 2013.

22. Petro JB, Gerstein RM, Lowe J, Carter RS, Shinners N, Khan WN. Transitional type 1 and 2 B lymphocyte subsets are differentially responsive to antigen receptor signaling. *J Biol Chem.* 2002;277:48009-48019.

23. Chung JB, Silverman M, Monroe JG. Transitional B cells: step by step towards immune competence. *Trends Immunol.* 2003; 24(6):342-348.

24. Mackay F, Browning JL. BAFF: a fundamental survival factor for B cells. *Nat Rev Immunol.* 2002;2(7):465-475.

25. Chesneau M, Michel L, Degauque N, Brouard S. Regulatory B cells and tolerance in transplantation: from animal models to human. *Front Immunol.* 2013;4:497.

26. Lundy SK. Killer B lymphocytes: the evidence and the potential. *Inflamm Res.* 2009;58(7):345-357.

27. Lundy SK, Boros DL. Fas ligand-expressing B-1a lymphocytes mediate CD4+-T-cell apoptosis during schistosomal infection: induction by interleukin 4 (IL-4) and IL-10. *Infect Immun.* 2002;70(2):812-819.

28. Matsushita T, Yanaba K, Bouaziz JD, Fujimoto M, Tedder TF. Regulatory B cells inhibit EAE initiation in mice while other B cells promote disease progression. *J Clin Invest.* 2008;118(10): 3420-3430.

29. van Rensburg IC, Kleynhans L, Keyser A, Walzl G, Loxton AG. B-cells with a FasL expressing regulatory phenotype are induced following successful anti-tuberculosis treatment. *Immun Inflamm Dis.* 2017;5(1):57-67.

30. Corkum CP, Ings DP, Burgess C, Karwowska S, Kroll W, Michalak TI. Immune cell subsets and their gene expression profiles from human PBMC isolated by vacutainer cell preparation tube (CPTTM) and standard density gradient. *BMC Immunol.* 2015;16:48.

31. Autissier P, Soulas C, Burdo TH, Williams KC. Evaluation of a 12-color flow cytometry panel to study lymphocyte, monocyte, and dendritic cell subsets in humans. *Cytom A.* 2010;77: 410-419. 2010.

32. Kleiveland CR. Peripheral blood mononuclear cells. In: Verhoeckx K, Cotter P, López-Expósito I, Lea T, Mackie A, Requena T, Swiatecka D, Wichers H, eds. *The Impact of Food Bioactives on Health.* Cham, Switzerland: Springer; 2015: 161-167.

33. Bénard A, Sakwa I, Schierloh P, et al. B cells producing type I IFN modulate macrophage polarization in tuberculosis. *Am J Respir Crit Care Med.* 2018;197(6):801-813.

34. Achkar JM, Chan J, Casadevall A. B cells and antibodies in the defense against *Mycobacterium tuberculosis* infection. *Immunol Rev.* 2015;264:167-181.

35. Rao M, Valentini D, Poiret T, et al. B in TB: B cells as mediators of clinically relevant immune responses in tuberculosis. *Clin Infect Dis.* 2015;61(suppl_3):S225-S234.

36. Du Plessis WJ, Kleynhans L, du Plessis N, et al. The functional response of B Cells to antigenic stimulation: a preliminary report of latent tuberculosis. *PLoS One.* 2016;11(4):e0152710.

37. Du Plessis WJ, Keyser A, Walzl G, Loxton AG. Phenotypic analysis of peripheral B cell populations during *Mycobacterium tuberculosis* infection and disease. *J Inflamm.* 2016;13(1):23.

38. Chan J, et al. The role of B cells and humoral immunity in *Mycobacterium tuberculosis* infection. *Semin Immunol.* 2014;26: 588-600.

39. Zabriskie JB. *Essential Clinical Immunology.* Cambridge, UK: Cambridge University Press; 2009.

40. Achkar JM, Casadevall A. Antibody-mediated immunity against tuberculosis: implications for vaccine development. *Cell Host Microbe.* 2013;13(3):250-262.

41. Franz A, Webster AD, Furr PM, Taylor-Robinson D. Mycoplasmal arthritis in patients with primary immunoglobulin deficiency: clinical features and outcome in 18 patients. *Br J Rheumatol.* 1997;36(6):661-668.

42. Hermans PE, Diaz-Buxo JA, Stobo JD. Idiopathic late-onset immunoglobulin deficiency: clinical observations in 50 patients. *Am J Med.* 1976;61(2):221-237.

43. Kim J, Hayton WL, Robinson JM, Anderson CL. Kinetics of FcRn-mediated recycling of IgG and albumin in human: pathophysiology and therapeutic implications using a simplified mechanism-based model. *Clin Immunol.* 2007;122(2): 146-155.

44. Anderson CL, Chaudhury C, Kim J, Bronson CL, Wani MA, Mohanty S. Perspective–FcRn transports albumin: relevance to immunology and medicine. *Trends Immunol.* 2006;27(7): 343-348.

45. Balu S, Reljic R, Lewis MJ, et al. A novel human IgA monoclonal antibody protects against tuberculosis. *J Immunol*. 2011; 186:1003189-3119.

46. Abebe F, Belay M, Legesse M, Kees L M C F, Ottenhoff T. IgA and IgG against *Mycobacterium tuberculosis* Rv2031 discriminate between pulmonary tuberculosis patients, *Mycobacterium tuberculosis*-infected and non-infected individuals. *PLoS One*. 2018;13(1):e0190989.

47. Fayette J, Dubois B, Vandenabeele S, et al. Human dendritic cells skew isotype switching of CD40-activated naive B cells towards IgA1 and IgA2. *J Exp Med*. 1997;185(11):1909-1918.

48. Le Bon A, Schiavoni G, D'Agostino G, Gresser I, Belardelli F, Tough DF. Type I interferons potently enhance humoral immunity and can promote isotype switching by stimulating dendritic cells in vivo. *Immunity*. 2001;14(4):461-470.

49. Crawford A, MacLeod M, Schumacher T, Corlett L, Gray D. Primary T cell expansion and differentiation in vivo requires antigen presentation by B cells. *J Immunol*. 2006;176(6): 3498-3506.

50. Lopes-Carvalho T, Foote J, Kearney JF. Marginal zone B cells in lymphocyte activation and regulation. *Curr Opin Immunol*. 2005;17(3):244-250.

51. Attanavanich K, Kearney JF. Marginal zone, but not follicular B cells, are potent activators of naive CD4 T cells. *J Immunol*. 2004;172(2):803-811.

52. Balázs M, Martin F, Zhou T, Kearney J. Blood dendritic cells interact with splenic marginal zone B cells to initiate T-independent immune responses. *Immunity*. 2002;17(3):341-352.

53. Martin F, Oliver AM, Kearney JF. Marginal zone and B1 B cells unite in the early response against T-independent blood-borne particulate antigens. *Immunity*. 2001;14(5):617-629.

54. Cerutti A, Cols M, Puga I. Marginal zone B cells: virtues of innate-like antibody-producing lymphocytes. *Nat Rev Immunol*. 2013;13(2):118-132.

55. Zouali M, Richard Y. Marginal zone B-cells, a gatekeeper of innate immunity. *Front Immunol*. 2011;2:63.

56. Lenert P, Brummel R, Field EH, Ashman RF. TLR-9 activation of marginal zone B cells in lupus mice regulates immunity through increased IL-10 production. *J Clin Immunol*. 2005;25(1):29-40.

57. Brodin P, Duffy D, Quintana-Murci L. A call for blood-in human immunology. *Immunity*. 2019;50(6):1335-1336. https://doi.org/10.1016/j.immuni.2019.05.012

58. Anderson NG, Wilbur KM. Studies on isolated cell components: the effect of various solutions on the isolated liver nucleus. *J Gen Physiol*. 1952;35(5):781-796.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.