Immune modulation by schistosomes: mechanisms of regulatory B cell induction and inhibition of allergic asthma

Obieglo, K.

Chapter 4

COMPARISON OF MURINE SPLENIC MARGINAL ZONE AND FOLLICULAR B CELL TRANSCRIPTOMES IN *S. MANSONI* INFECTION – SIMILARITIES AND DIFFERENCES

Katja Obieglo, Szymon M. Kielbasa,
Hailiang Mei, and Hermelijn H. Smits

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ABSTRACT

Chronic *Schistosoma mansoni* infections induce a network of regulatory immune cells, including interleukin (IL)-10-producing regulatory B (Breg) cells. The signals that drive Breg cell development and activation are however not well characterized, and multiple signals likely synergize to induce optimal Breg cell induction *in vivo*. We here compared the transcriptome of splenic marginal zone (MZ) B cells, which preferentially acquire a Breg cell phenotype, and follicular (FO) B cells of chronically *S. mansoni*-infected mice using RNA sequencing technology, in order to gain a global picture of signals and pathways involved in *S. mansoni*-induced Breg cell development *in vivo*. We report that MZ and FO B cells display distinct transcriptional profiles, and that both B cell subsets moreover undergo transcriptional changes in response to infection which are partially cell-type specific. Comparing the MZ and FO B cell transcriptome suggests the cytokines IL-1β, IL-6 and type I interferons (IFN-I) as well as pattern recognition receptors (PRRs) including toll-like receptors (TLRs) and C-type lectin receptors (CLRs) as preferentially important for MZ B cells during chronic schistosome infections. This analysis thus suggests that inflammatory signals, including pro-inflammatory cytokines and the ligation of PRRs preferentially activate MZ B cells, and provides several leads for further studies.

INTRODUCTION

B cells with regulatory properties, called regulatory B (Breg) cells, are a subset of B cells with immunomodulatory capacity. Breg cells suppress immunity in a range of different contexts, including autoimmunity [1], allergy [2] and cancer [3]. Breg cells are most often characterized by their production of the regulatory cytokine interleukin (IL-)10, although various other mechanisms of suppression have been described [4-6]. Breg cells are moreover a heterogeneous group of cells, and apart from their suppressive function as defining characteristic, various different subsets have been described based on origin and phenotype [6, 7]. Amongst others, marginal zone (MZ) B cells of the spleen and their precursors are well known to acquire regulatory functions [8-10]. Breg cells have been described in a range of infectious diseases [11], including experimental [12-15] and human [12, 16] helminth infections.

Breg cells expand and acquire increased suppressive activity in the context of infection and autoimmunity [1, 6, 17]. The signals for Breg cell development and activation that have been most widely studied are ligation of the B cell receptor (BCR) [18-20], CD40 [10, 18, 21, 22] and toll-like receptors (TLRs), especially TLR4 [23-25], TLR7 [26] and TLR9 [8, 23]. Additionally, a range of cytokines, including IL-1β and IL-6 [27], IL-15 [28], IL-21 [29], IL-35 [30, 31], the tumour-necrosis factor (TNF) family members B-cell-activating factor (BAFF) [32] and a proliferation-inducing ligand (APRIL) [33], as well as type I interferons (IFN-I) [34, 35] have been described to support Breg cell activation. The variety of signals described in Breg cell induction suggests different sources of activating signals. An inflammatory environment seems central and is a shared feature of infection and autoimmunity. The role of BCR- and TLR-derived signals shows that in infection, pathogen-derived molecules are candidates for Breg cell-activating signals. Interestingly, it has recently been shown that pathogen-derived signals that induce Breg cells can also stem from the microbiota, as microbiota-derived signals induced splenic and mesenteric lymph node Breg cells in an IL-1β- and IL-6-dependent manner in arthritis [27], and multiple studies have shown that alterations of the gut microbiota affect the Breg cell compartment [27, 36, 37].

We and others have previously demonstrated that chronic infection with the helminth *Schistosoma* (*S.*) *mansoni* induces IL-10-producing Breg cells in both humans and mice. Most of these cells are found within the human $CD1d^{+}$ B cell subset and the murine $CD1d^{+}CD21^{+}CD23^{lo}$ MZ B cell subset [12, 14]. The number of identified, *S. mansoni*-specific signals that induce Breg cells is however very limited. Breg cells can be induced by schistosomal egg antigens [38, 39], but this is notably less potent than the induction of Breg cells during chronic infection. The *S. mansoni* egg glycoprotein IPSE/alpha-1 is the only single helminth-derived molecule that has been shown to directly interact with splenic B cells and induce IL-10 production, but this has so far only been demonstrated *in vitro* [39]. Chronic *S. mansoni* infection induces a multitude of different immune responses in the host, and it is thus likely that multiple signals synergize *in vivo* to induce optimal Breg cell induction. Herein, we therefore studied the transcriptome of splenic MZ B cells isolated from chronically *S. mansoni*-infected mice.

The transcriptome of certain murine Breg cell subsets has been studied previously. Through such studies, CD9 has been identified as a marker of murine IL-10-competent, $CD19^{+}$ $CD1d^{hi}$ $CD5^{+}$ Breg cells induced by *in vitro* polyclonal stimulation [40]. In addition, by filtering a microarray library for secreted factors produced by splenic B cells activated with LPS and anti-CD40 agonistic antibodies *in vitro*, IL-35 was identified as suppressive cytokine produced by these Breg cells [30]. Interestingly, IL-35 production was confined to $CD138^{hi}$ $CD22^{-}$ regulatory plasma cells described in the context of *Salmonella* infection [30]. A further transcriptomics analysis of a subset of these natural regulatory plasma cells characterized as $LAG\text{-}3^{+}$ $CD138^{+}$ has revealed a distinct expression profile for several transcriptional regulators, including *Klf4*, *Fos*, *Junb*, *Irf8* and *Foxm1* [41]. For Breg cells in the context of acute helminth infections, microarray analysis identified TLR7 as overexpressed in *S. mansoni*-induced $CD19^{+}$ $CD1d^{hi}$ Breg cells compared to $CD19^{+}$ $CD1d^{lo}$ B cells. *In vitro* TLR7 stimulation of splenic B cells from naïve mice promoted Breg cell activation and IL-10 production [26], but it remains unclear from this study whether TLR7 ligands are present and whether TLR7 ligation is central to Breg cell expansion during *S. mansoni* infection.

In this study, we aimed to identify the similarities and differences between MZ and classical follicular (FO) B cells in their response to chronic *S. mansoni* infection, in order to better understand signals, receptors and pathways that lead to the preferential development of MZ B cells into Breg cells. We have compared the transcriptome of MZ B cells and FO B cells from chronically *S. mansoni*-infected and -uninfected control mice.

RESULTS

Chronic *S. mansoni* infection induces Breg cells within the splenic MZ B cell pool

In order to study the transcriptome of splenic MZ and FO B cells, we first isolated total splenic $CD19^+$ B cells and confirmed the capacity of these cells to produce IL-10 by *in vitro* restimulation with *S. mansoni* soluble egg antigen (SEA). As expected, splenic B cells isolated from chronically *S. mansoni*-infected mice secreted significantly more IL-10 protein, but only low levels of IL-6 protein, compared to uninfected controls (**Figure 1A**). The frequency of B cells with intracellular IL-10 expression was also significantly increased (**Figure 1B**). Total non-restimulated splenic $CD19^+$ B cells were sorted into $CD21^+$ $CD23^{lo}$ MZ B cells and the major splenic B cell subset, $CD21^{lo}$ $CD23^{hi}$ FO B cells, for subsequent RNA isolation and RNAseq analysis (**Figure 1C**).

To investigate transcriptional changes that are associated with the response of splenic B cells to *S. mansoni*, we compared the gene expression profile in MZ B cells and FO B cells from chronically *S. mansoni*-infected and uninfected control mice in a principal component analysis (PCA; **Figure 1D**). Biological replicates of all four experimental groups clustered well. MZ and FO B cells were segregated across the first principal component (PC1), representing the cell type difference and accounting for 66.8% of the total data variability. The second principal component (PC2) represented the treatment effect, accounting for 12% of the total data variability. Notably, while PC1 separates MZ and FO B cells to a similar degree irrespective of treatment, PC2 separates FO B cells from *S. mansoni*-infected (FO. Inf) mice and from control (FO.PBS) mice stronger than MZ B cells from *S. mansoni*-infected (MZ.Inf) mice and from control (MZ.PBS) mice. PC3, which does not clearly separate by cell type or treatment condition, only accounts for 6.2% of total variability.

Both MZ and FO B cells from control and *S. mansoni*-infected mice constitutively expressed the B cell markers *Cd19*, *Cd22*, *Cd79a*/*Cd79b* and *Ptprc* (B220) along with other B cell-associated markers including *Tnfrsf13b* (TACI) and *Tnfrsf13c* (BAFF-R). MZ B cells expressed lower levels of *Sdc1* (coding for CD138) than FO B cells (**Figure 1E**), and *Lag3* was not detectable in the dataset, indicating that the B cells assessed here are phenotypically different from the natural regulatory plasma cells recently described [41]. T cell genes including *Tcra*, *Tcrb*, *Cd3e*, *Cd4*, *Cd8a* and *Cd28* were also not detected in the dataset, showing that the isolated B cell populations were not contaminated with T cells. MZ B cells strongly overexpressed *Cd1d1* and *CD1d2* (together CD1d), *Cd9*, and to a lesser extent also *Cd5, Cd24a*, *Cd27* and *Cd38*, all markers characteristic of a Breg cell phenotype (**Figure 1E**). The strong overexpression of *Cd9* in MZ B cells is in line with previous reports on this marker as identifier of murine IL-10 competent, $CD19^+$ $CD1d^{hi}$ $CD5^+$ Breg cells [40]. With respect to cytokines, MZ B cells strongly overexpressed *Il10*, confirming that this splenic B cell subset is IL-10-competent. MZ B cells also mildly overexpressed *Ebi3*, in addition to expressing *Il12a*, which together form the regulatory cytokine IL-35 described to be produced by certain Breg cell subsets [30] (**Figure 1E**). In chronically *S.mansoni*-infected animals, both B cell subsets moreover strongly overexpressed *Ighe* and *Ighg1* compared to steady-state (**Figure 1E**). B cells from all experimental groups constitutively expressed the B cell lineage transcription factor (TF) *Pax5*, while FO B cells overexpressed *Bcl6* exclusively expressed in germinal centers and MZ B cells overexpressed *Prdm1* (Blimp-1) which drives B cell differentiation into plasma cells. The TFs IRF4, NFAT and STAT1/STAT3 have previously been suggested to be important for B cell IL-10 expression [20, 34, 35, 42], but expression of these genes did not differ between MZ and FO B cells or undergo changes with

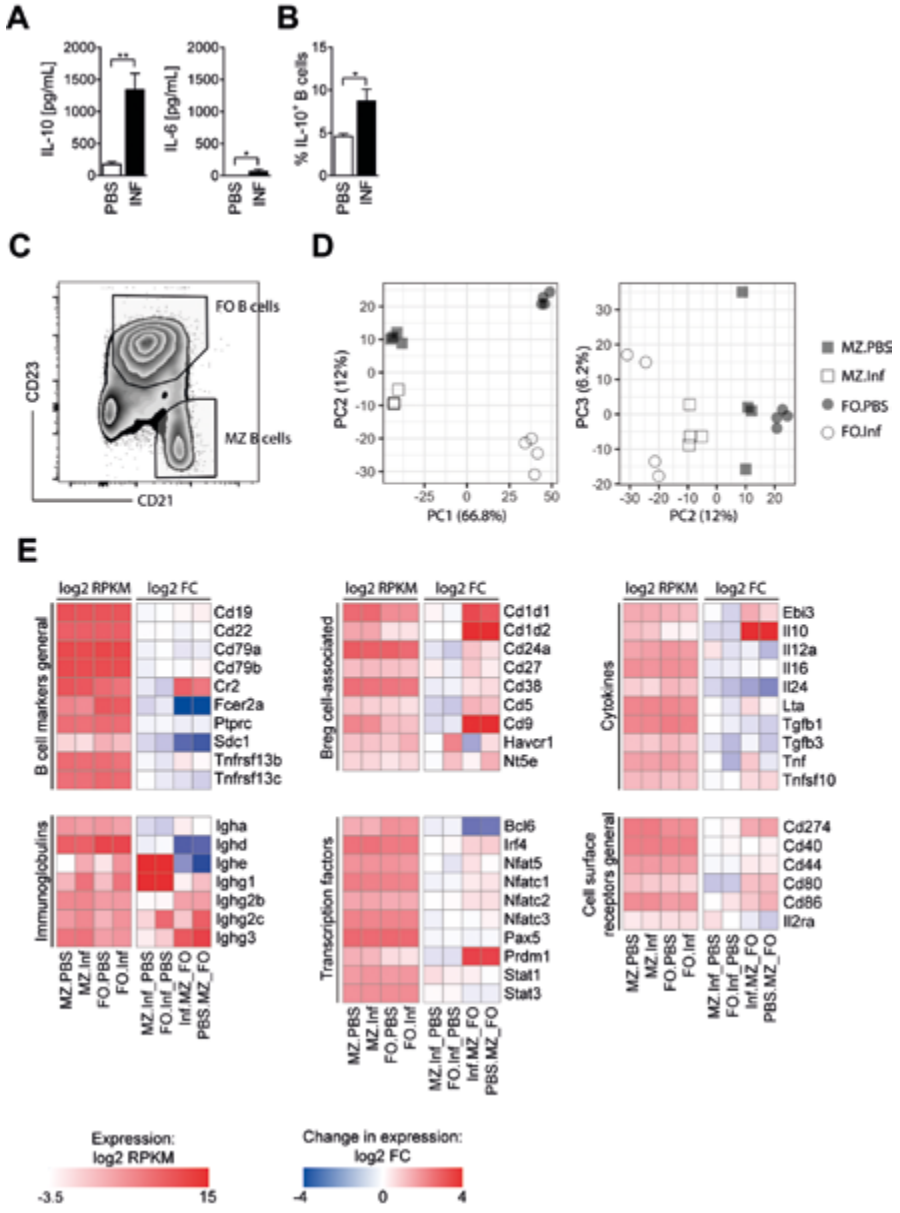

Figure 1. Chronic *S. mansoni* infection induces Breg cells within the splenic MZ B cell pool. Splenic B cells from chronically (14 weeks) *S. mansoni*-infected mice and uninfected control mice were isolated by CD19 MACS isolation. (**A-C**) Cells were restimulated with SEA (20µg/ml) for 2 days, n=5-6/group. Statistical significant differences were determined by unpaired t-test and are indicated by * $p < 0.05$, ** $p < 0.01$. (**A**) Cytokine concentrations in culture supernatant determined by ELISA. (**B**) Intracellular IL-10 expression after addition of Brefeldin A during the last 4 hours of culture, determined by FACS. (**C**) Representative FACS plot depicting the gating strategy to identify MZ B cells ($CD21^{+}CD23^{lo}$) and FO B cells ($CD21^{lo}CD23^{hi}$) applied during sorting. (**D-E**) Sorted MZ and FO B cells were subjected to RNA isolation and RNAseq analysis. (**D**) Principal component analysis (PCA) including all 16 samples. (**E**) Heatmaps depicting the level of expression in reads per kilobase of transcript per million mapped reads (RPKM) for each experimental group, and the expression fold change (FC) between pair-wise comparisons.

infection (**Figure 1E**). B cells from all experimental groups moreover constitutively expressed *Cd40*, whereas *Cd274* (PD-L1), *Cd80* and *Cd86* were slightly overexpressed in MZ B cells (**Figure 1E**).

MZ and FO B cells display distinct transcriptional profiles and undergo transcriptional changes that are partially cell-type-specific in response to chronic *S. mansoni* infection

To investigate the genes and signalling pathways associated with B cell responses to *S. mansoni*, we performed a differential expression analysis either comparing *S. mansoni* infection and control condition for both B cell subsets (denoted as MZ.Inf_PBS and FO.Inf_PBS, respectively), or comparing MZ and FO B cells in uninfected and *S. mansoni*-infected mice (denoted PBS.MZ_FO and Inf.MZ_FO, respectively). Chronic infection with *S. mansoni* resulted in transcriptional changes in both MZ and FO B cells, albeit to a different degree. MZ B cells displayed less differentially expressed genes (DEGs) than FO B cells in response to infection (**Figure 2A**, upper panel). The transcriptional profile strongly differed between MZ and FO B cells, both after chronic *S. mansoni* infection and in control mice (**Figure 2A**, lower panel). A list of all DEGs per condition is provided as **suppl. Table S1**.

A total of 438 DEGs were shared between MZ and FO B cells in response to infection, of which 19 DEGs were differentially expressed in opposing direction. A total of 252 DEGs (37%) in MZ B cells and 1982 DEGs (82%) in FO B cells were uniquely regulated for the respective B cell subset in response to infection (**Figure 2B**, upper panel). The transcriptional profile of MZ and FO B cells clearly differs, both after chronic *S. mansoni* infection and in control mice, with the majority of DEGs (4492 DEGs; 79% and 75%, respectively) being shared in both conditions (**Figure 2B**, lower panel).

Canonical Pathways differentially activated in MZ and FO B cells of chronically *S. mansoni*-infected animals

To evaluate the response of MZ and FO B cells to *S. mansoni* infection more broadly, we used the Canonical Pathway function of Ingenuity Pathway Analysis (IPA) [43] to search for common and discordant signalling pathways in MZ and FO B cells. To identify pathways that are more related to MZ or FO cells, respectively, we made use of the activation z-score calculated by IPA, which is a statistical measure to infer the likely activation state of the pathway, with z-scores of > 2 or < -2 suggesting significant activation or inhibition, respectively. Both MZ and FO B cells proliferate in infected mice, as apparent from cell cycle-related pathways being significantly activated in both cell types in response to infection (**Figure 3**). Interestingly, immune-related functions are found in the list of pathways significantly downregulated after infection for both cell types, which may suggest that these pathways are negatively regulated following previous activation. The list of pathways found to be significantly inhibited in the comparison MZ.Inf_PBS but not in FO.Inf_PBS (**Figure 3**, left panel) contained 'Toll-like Receptor Signaling', 'STAT3 Pathway', 'B Cell Activating Factor Signaling' (B-cell activating factor = BAFF) and 'April Mediated Signaling', which is in line with previous reports suggesting TLR-, STAT3-, BAFF- and APRIL-signalling to be associated with Breg cells [23, 32, 35, 44]. The pathways 'p38 MAPK Signaling', which is downstream of receptors for inflammatory cytokines, FasL, TGF-β, G-protein coupled receptors as well as DNA damage signals [45], and 'Acute Phase Response Signaling', which is a rapid inflammatory response pathway triggered by pro-inflammatory cytokines [46], likely reflect the response of MZ B cells to the inflammatory environment in chronic *S. mansoni* infection. Pathways found to be significantly inhibited in the comparison FO.Inf_PBS but not in MZ.Inf_PBS (**Figure 3**, right panel) contained 'IL-6 Signaling', 'IL-8 Signlling' and 'HMGB1 Signaling'. In human infection and cancer, B cells have been suggested to express the IL-6 receptor [47, 48], whereas little is known of B cell responses to IL-8. HMGB1 is secreted by immune cells, and mediates inflammatory responses via interaction with TLR4 [49, 50].

We did not only identify Canonical Pathways for the pairwise comparisons of cellular responses to infection, but also for cell type differences both at steady-state and after chronic *S. mansoni*

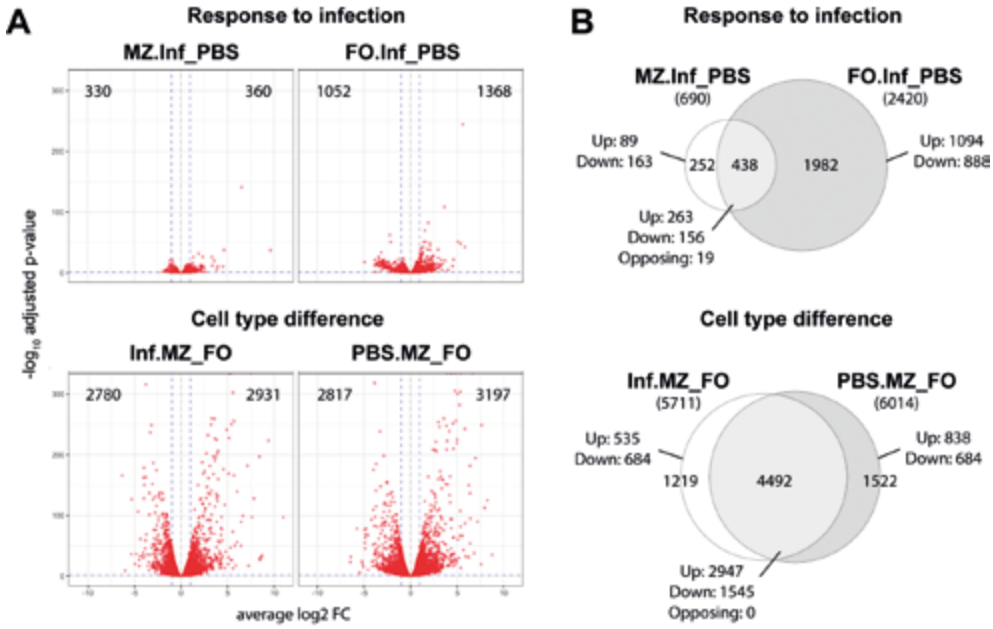

Figure 2. MZ and FO B cells display distinct transcriptional profiles and undergo transcriptional changes that are partially cell-type-specific in response to chronic *S. mansoni* infection. (**A**) Volcano plots comparing differential gene expression for the response of MZ and FO B cells to infection, and we as for cell type difference in control and infected condition. The number of differential up- and downregulated genes for each comparison is depicted in the plot. avgLog2FC = average log2 fold change; minusLog10AdjPVal = -log10 adjusted p-value. (**B**) Venn diagrams depicting the overlap of differentially expressed genes (DEGs) for the response of MZ versus FO B cells to infection, and for the cell type difference after infection versus at steady-state. The number of DEGs that are unique or shared, and whether they were upregulated ('up'), downregulated ('down'), or opposing for shared DEGs ('opposing') is indicated.

infection. A list of significantly activated or inhibited pathways for these comparisons is provided as **suppl. Figure S1**.

Predicted Upstream Regulators with differential activation status in MZ and FO B cells of chronically *S. mansoni*-infected animals

The Upstream Regulator analysis of IPA [43] examines the presence of known targets and their direction of change in the dataset compared to what is expected based on the literature in order to predict likely relevant upstream signals that explain the observed gene expression changes in the dataset. These Upstream Regulators do not have to be part of the dataset itself. As many Upstream Regulators passed the regular z-score threshold, we applied a more stringent cut-off of z-scores > 4 or < -4 to identify highly activated or inhibited Upstream Regulators (**suppl. Figure S2**). These lists still contained many hits of a range of different molecule types, which is why we focussed on cytokines and cell surface receptors as most informative molecule types with respect to identifying extracellular signals important for Breg cell activation in chronic *S. mansoni* infection. The list of significant (z-score > 2 or < -2) cytokines predicted as Upstream Regulators that are unique to the response of MZ B cells to infection includes IL-1β as IL-6 as strongly activated and highly significant hits (**Figure 4A**, left panel). In contrast, the list of Upstream Regulators that are unique to the response of FO B cells to infection

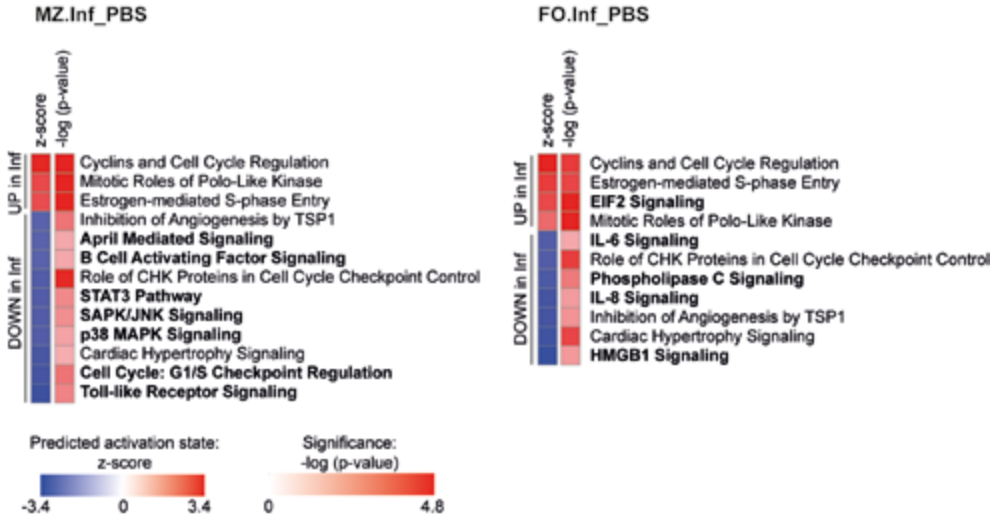

Figure 3. Canonical Pathways differentially activated in MZ and FO B cells of chronically *S. mansoni* infected animals. IPA software was used to identify Canonical Pathways significantly activated (z-score > 2; -log(p-value) > 1.3) or significantly inhibited (z-score < -2; log(p-value) > 1.3) for the response of MZ B cells to infection and of FO B cells to infection. Canonical Pathways that are not shared between the two pair-wise comparisons are indicated in bold font. Canonical Pathways significant for the comparison of MZ and FO B cells at steady-state and after infection is given in **suppl. Figure S1**.

includes amongst others the Th2 cytokines IL-4 and TSLP as activated Upstream Regulators (**Figure 4A**, right panel). In comparison of MZ and FO B cells at steady-state and after infection, it becomes apparent that the list of Upstream Regulators predicted to be more activated in MZ B cells contains several interferons, including members of the IFN-I family and IFNγ as type II interferon (**Figure 4B**). Notably, Upstream Regulators are signals solely predicted by IPA to cause the observed transcriptional pattern, irrespective of whether e.g. corresponding receptors are expressed. We therefore evaluated the expression of receptors for predicted Upstream Regulators in our dataset. The IL-6R subunit *Il6ra* and the IL-1R accessory protein *Il1rap* were both expressed in both B cell subsets, with *Il1rap* being slightly overexpressed in MZ B cells and *Il6ra* being slightly overexpressed in FO B cells. Moreover, the IFNAR subunits *Ifnar1* and *Ifnar2* are both clearly expressed in MZ and FO B cells irrespective of infection status (**Figure 4C**). Collectively, these data might indicate that, whereas FO B cells respond to the Th2 environment to undergo a germinal center reaction and differentiate into long-lived plasma and memory B cells, MZ B cells might be more responsive to pro-inflammatory, non-Th2-related cytokines such as IL-1β, IL-6 and IFN-I.

With respect to surface receptors, only few Upstream Regulators were predicted as significantly activated or inhibited for the response of MZ and FO B cells to infection, respectively (**Figure 4D**). The comparison of both cells types at steady-state and after infection yielded TNFRSF17 and TLR4 as significantly more activated in MZ than FO B cells at steady-state, and TLR9 as well as TLR7 as significantly more activated in MZ compared to FO B cells after infection (**Figure 4E**). These data suggest that TNFRSF17 (BCMA), which binds APRIL with high affinity and BAFF with low affinity, as well as the toll-like receptors TLR4, TLR7 and TLR9 are more important for the activation of MZ than FO B cells. This goes hand in hand with the fact that these receptors are known to be important for the induction of Breg cells [8, 23, 24, 26, 32]. In our dataset, *Tlr4* and *Tlr9* were constitutively expressed across all conditions with little changes between cell types or in response to infection, whereas *Tlr7*

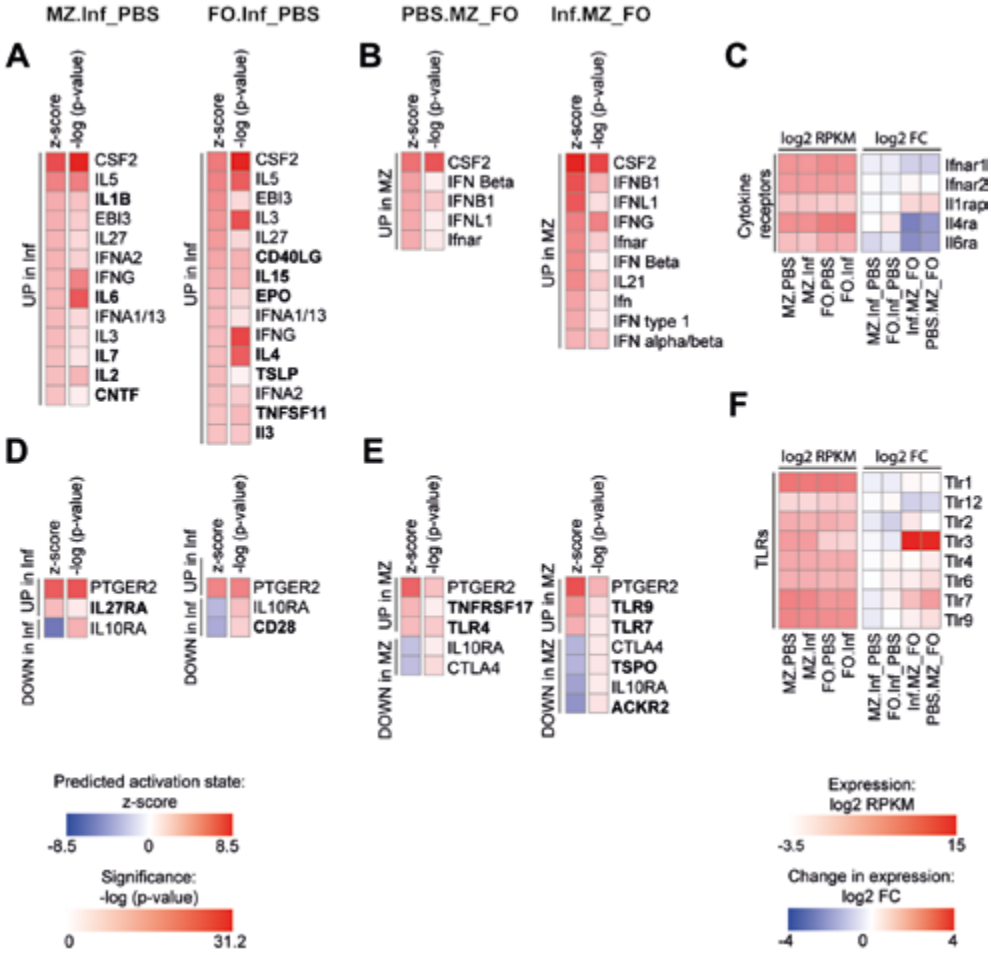

Figure 4. Predicted Upstream Regulators with differential activation status in MZ and FO B cells of chronically *S. mansoni*-infected animals. IPA software was used to identify predicted Upstream Regulators significantly activated (z-score > 2; p-value < 0.05) or significantly inhibited (z-score < -2; p-value < 0.05). An overview over all identified Upstream Regulators is given in **suppl. Figure S2**. Upstream regulators were filtered either for 'cytokines' manually including cytokines listed under molecule type 'group' (**A**, **B**), or for 'transmembrane receptor' and 'G-protein coupled receptor' to select for cell surface receptors (**D**, **E**). (**A**, **D**) Upstream Regulators for the response of MZ and FO B cells to infection. (**B**, **E**) Upstream Regulators for the cell type difference at steady-state and after infection. Upstream Regulators that are not shared between pair-wise comparisons for cellular response to infection and cell type difference are indicated in bold font. (**C**, **F**) Heatmaps depicting the level of expression in reads per kilobase of transcript per million mapped reads (RPKM) for each experimental group, and the expression fold change (FC) between pair-wise comparisons.

was overexpressed in MZ B cells. Interestingly, *Tlr3* was found to be strongly overexpressed on MZ B cells (**Figure 4F**), and TLR3 was also predicted by IPA as Upstream Regulator for MZ B cells, although not passing the p-value significance threshold (data not shown). In summary, TLR ligation might thus, in addition to signals provided by cytokines, be important for the response of MZ B cells to *S.mansoni* infection.

A cluster identifies genes with opposing transcriptional changes in MZ and FO B cells

We also compared the DEG lists for the response of MZ and FO B cells to infection, and the cell type difference in control condition and after infection, in plots comparing DEG fold changes between conditions. Interestingly, both plots for cell type difference and for response to infection displayed a distinct cluster of 64 and 71 genes, respectively (lists provided as **suppl. Table S4**). Almost all genes (n=63) were contained in both clusters (**suppl. Figure S3**). These genes are upregulated in MZ B cells, but downregulated in FO B cells, in response to infection (**Figure 5A**), or upregulated in MZ B cells from infected animals, but downregulated in steady-state MZ B cells, compared to the respective FO B cell condition (**Figure 5B**). The cluster contained the genes *C1qa*, *C1qb*, *C1qc* and *C6* coding for the complement factors C1 and C6. The cluster also contained *Clec4a3*, *Clec4n* and *Mrc1*, the latter ones coding for the C-type lectin receptors (CLRs) dectin-2 and mannose receptor (MR; CD206), respectively (**Figure 5C**). Thus, the cluster identified additional signals that might preferentially activate MZ B cells in the context of *S. mansoni* infection.

DISCUSSION

Breg cells are a relatively new member in the network of immune regulatory cells that have only been extensively studied in the recent decades, and many open questions around their origin, induction as well as phenotypical and functional characteristics remain. In this study, we focused on the identification of signals that may drive the preferential development of splenic MZ B cells over FO B cells into IL-10-producers in chronic *S.mansoni* infection in order to identify leads for further investigation.

We and others have previously reported that chronic *S. mansoni* infection induces IL-10$^+$ B cells within the murine and human CD1d$^+$ B cell subset [12, 14], which corresponds to the CD21$^+$ CD23^{lo} MZ B cell subset. In our dataset, MZ B cells as expected overexpressed several markers characteristic for splenic MZ Breg cell subsets, but are phenotypically distinct from regulatory plasma cells. Breg cells are most widely recognized for their production of IL-10 [5, 6]. We found *Il10* to be strongly overexpressed in MZ B cells compared to FO B cells, both at steady-state and after infection, identifying MZ B cells as IL-10-competent. The expression of *Il10* did not further increase after infection in either of the B cell subsets, indicating that optimal IL-10 expression requires an additional *ex vivo* restimulation with a mitogen, as described previously [51].

From the analysis of DEGs it became apparent that MZ and FO B cells display clearly distinct transcriptional profiles, not only after infection but also at steady-state. This difference likely reflects the distinct roles of MZ B cells as innate-like cells that mediate responses to antigens in blood-borne pathogens, and of FO B cells undergoing germinal center reactions to differentiate into long-lived plasma cells producing class-switched antibodies [52]. Both B cell subsets underwent transcriptional changes in response to chronic *S. mansoni* infection, whereby FO B cells displayed about 3.5-times more DEGs than MZ B cells. Again, these differences might be related to the unique function of both cell types rather than a differential activation status of MZ and FO B cells after infection, which is supported by the fact that Canonical Pathways identified by IPA to be highly activated in both cell types in response to infection include proliferation-related pathways. The analysis within IPA also suggested several pathways which have previously been associated with Breg cell induction [23, 32, 44] as more relevant for MZ B cells in response to infection, including TLR-, STAT3- BAFF- and APRIL-signalling. This indicates that these pathways are also induced in the context of *S. mansoni* infection.

The Upstream Regulator analysis performed in IPA identified several regulators as predicted to be more activated in MZ B cells of chronically *S.mansoni* infected mice than in FO B cells. With respect to cytokines, IPA identified IL-1β and IL-6 as potential Upstream Regulators, and their receptors are part of the transcriptome dataset and expressed in MZ and FO B cells. Rosser *et al.* showed that both of these cytokines directly promote suppressive Breg cells, as mice with B cell-specific deficiency in

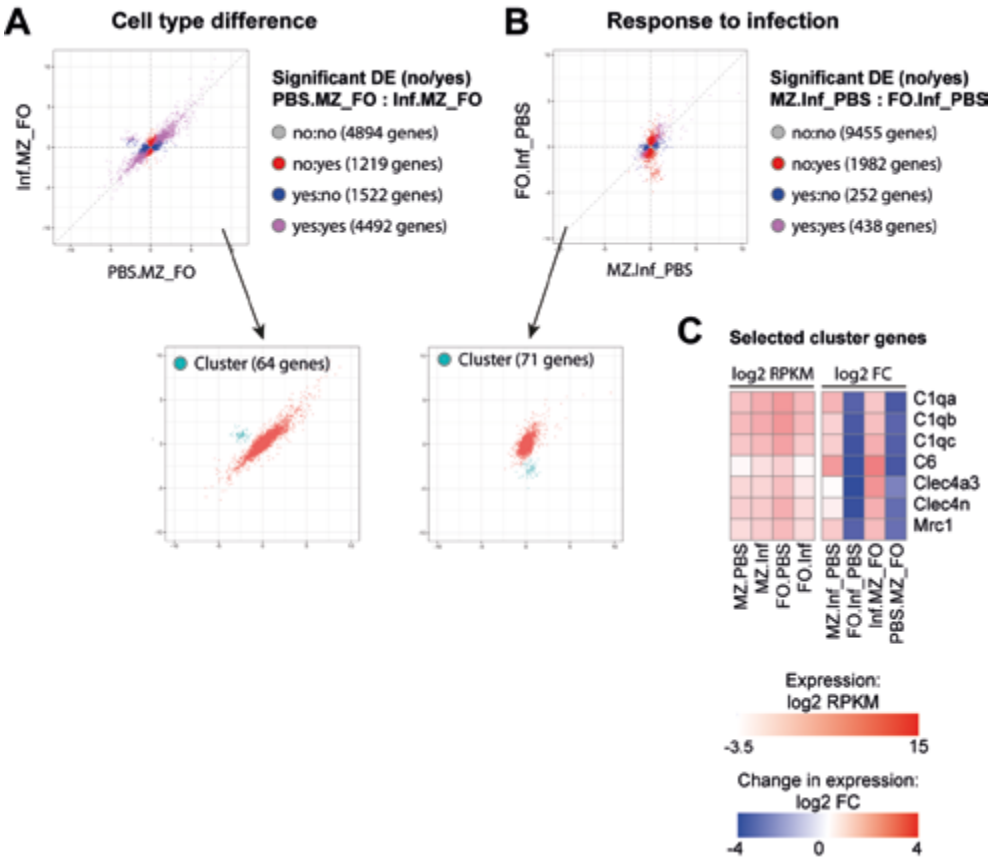

Figure 5. A cluster identifies genes with opposing transcriptional changes in MZ and FO B cells. (**A**, **B**) Plots depict the fold change (FC) of pair-wise comparisons for the cellular response to infection (**A**) and for cell type differences (**B**). (**C**) A heatmap depicting the level of expression in reads per kilobase of transcript per million mapped reads (RPKM) for each experimental group, and the expression fold change (FC) between pairwise comparisons for selected genes included in the identified clusters.

the IL-1- or the IL-6-receptor (IL-1R, IL-6R) develop more severe experimental arthritis. The production of IL-1β and IL-6 by macrophages and DCs was moreover found to be dependent on an intact gut microbiome [27]. With respect to *S. mansoni*, it is tempting to speculate that an infection and especially eggs penetrating the intestinal wall may cause translocation of bacteria across the intestinal wall and systemic exposure to microbial products, potential providing signals for Breg cell induction. In humans, it has already been shown that schistosomiasis can cause high endotoxemia [53].

Apart from IL-1β and IL-6, IPA also identified several members of the IFN-I cytokine family as well as IFNγ as Upstream Regulators predicted to be more activated in MZ B cells compared to FO B cells. Menon *et al.* showed that IFNα as a IFN-I family member induces Breg cells in humans [35]. B cells are widely recognized to express the IFNα/β receptor (IFNAR) and to respond to IFN-I [54-56], and both IFNAR subunits *Ifnar1* and *Ifnar2* are expressed in MZ and FO B cells irrespective of infection status. Moreover, recent reports have highlighted that helminths including *S. mansoni* or their products can induce IFN-I production in mouse models. Gastrointestinal helminths of rodents have been shown to induce IFN-I

in gut and lung in an microbiota-dependent manner [57], and in skin DCs [58], respectively. *S. mansoni* eggs and egg antigens induce an IFN-I signature both in splenic DCs *in vivo* [59] and in bone marrow DCs *in vitro* [60]. Collectively, this analysis suggests the cytokines IL-1β, IL-6 and members of the IFN-I family as Upstream Regulators more important for MZ than FO B cells in *S. mansoni* infection, and the literature provides interesting initial observations that they might be involved in Breg cell induction, making them promising targets for further investigation.

With respect to cell surface receptors, IPA identified TLR7 and TLR9 as Upstream Regulators predicted to be significantly more activated in MZ B cells than FO B cells after infection. Both TLR7 and TLR9 are endosomal TLRs that recognize nucleic acids. TLR7 has been described to be overexpressed in $CD19^+$ $CD1d^{hi}$ B cells compared to $CD19^+$ $CD1d^{lo}$ B cells, and to increase the capacity of $CD19^+$ $CD1d^{hi}$ B cells to produce IL-10 [26]. TLR9 expressed on B cells has been reported to recognize DNA-containing complexes on apoptotic cells, resulting in IL-10 production and protection from experimental autoimmune encephalitis (EAE) [8], whereas another study suggests B cell TLR9 is not required for the recovery from EAE [23]. The notion that TLR9 induces Breg cells is supported by numerous studies using synthetic TLR9 ligands to induce murine and human Breg cells *in vitro* [61, 62], but the role of TLR9 for Breg cell induction has so far not been studied in the context of *S. mansoni*. As chronic *S. mansoni* infection and especially egg migration causes tissue damage, granuloma formation and potentially also systemic exposure to microbial pathogen-associated molecular patterns (PAMPs), it is however likely that endogenous ligands released following apoptosis and/or microbial ligands for nucleic acid-binding TLRs including TLR9 get exposed. In addition to *Tlr7* and *Tlr9*, the third endosomal TLR recognizing nucleic acids, *Tlr3,* is strongly overexpressed in MZ B cells. The Upstream Regulator analysis does however not suggest a differential signalling through TLR3 in MZ and FO B cells in chronic *S. mansoni* infection to be responsible for the differential expression patterns found in these cell types. Collectively, these data suggest that *Tlr3* is predominantly expressed on MZ B cells compared to FO B cells, but that TLR7 and TLR9 rather than TLR3 might play a role in the differential response of both cell types to chronic *S. mansoni* infection.

A cluster of genes with opposing expression pattern in MZ and FO B cells identified complement factors and CLRs as upregulated in MZ B cells, but downregulated in FO B cells, after *S. mansoni* infection. B cells express various complement receptors including complement receptor 2 (CR2 = CD21), but little is known about the expression of complement factors by B cells [63, 64]. CD21 on B cells recognizes complement factor C3d-opsonized microbial products, resulting in enhanced BCR signalling [65]. To our knowledge, the expression of complement factors by B cells has not been described before, and our findings should be supplemented by gene and protein expression data before potentially investigating the role of complement expression by B cells further.

Apart from TLRs, CLRs are a second class of pattern recognition receptors (PRRs). Others have previously reported that B cells express CLRs including dectin-1 and MR [66], and we have found CLRs including dectin-2 and MR to be preferentially expressed by MZ B cells after infection. Notably, *S. mansoni* molecules have been described to ligate CLRs. Omega-1 induces DC Th2 polarization via MR [67], but failed to induce Breg cell development *in vitro* in naïve splenic B cells [39], likely arguing against a potent role for MR in Breg development. SEA induces prostaglandin E_2 synthesis by DCs through dectin-1 and dectin-2, also promoting DC Th2 polarization [68]. Apart from one study suggesting that signalling via dectin-1 induced IgG1 class switching by LPS-activated B cells [69], the role of these dectins in B cell and specifically Breg cell activation has not been addressed.

Collectively, comparing the transcriptome of MZ and FO B cells identified cytokines including IL-1β, IL-6 and IFN-I, as well as PRRs including the TLR7 and TLR9, Dectin-1 and MR as predicted upstream regulators that might cause a differential activation of these B cell subsets in *S. mansoni* infection. Apart from cytokine receptors and TLRs, the BCR and CD40 have been most well-described as cell

surface receptors involved in the induction of Breg cells, at least in the context of autoimmunity [10, 18-22]. The analysis performed herein however suggests that these receptors are not differentially activated in MZ and FO B cells of chronically *S. mansoni* infected animals.

Transcriptomics allow to study the entirety of genes transcribed at a certain time point and potentially allows insight into the breadth of cellular process at interplay. Such a dataset allows to address very different points of interest, including but not limited to predicted upstream signals and pathways that may lead to the observed transcriptional pattern. Others, such as e.g. the identification of cell-type-specific TFs or metabolism-related regulators and pathways were not the focus of this study, albeit the fact that they may be of interest in the context of Breg cells. Transcriptomics as performed in this study also bear limitations. Gene expression does not necessarily reflect the translation into functional protein, and key findings therefore will need to be supplemented e.g. by data on protein expression. It also has to be taken into consideration that this analysis is a snapshot of the splenic MZ and FO B cell transcriptome after 14 weeks of *S. mansoni* infection. At this time point, the peak of Th2 response to egg deposition has passed, and regulatory responses are on the rise. Moreover, the pool of MZ B cells and FO B cells isolated for analysis and carefully sorted into subsets on the basis of expression of selected markers might still comprise a heterogeneous population of cells at different stages of maturation and differentiation. In order to fully understand changes of MZ and FO B cells as the infection progresses from the acute, Th2-dominated to a more chronic state, the transcriptome of cells isolated form different time points of infection should be compared. Making use of single cell RNAseq technologies would also allow to better assess cellular differences.

In this study, we assessed the transcriptome of B cells during chronic *S. mansoni* infection and gained insight into the global pattern of differential signals and pathways important for MZ and FO B cells, respectively. We identified several inflammatory signals that seem to preferentially drive the activation of MZ B cells over FO B cells, but we cannot dissect from these data whether these signals are *S. mansoni*-specific or the result of the general inflammatory environment caused by chronic infection. A strategy to further address this topic could be to compare this transcriptome of MZ and FO B cells from chronically infected mice to the one of mice injected with *S. mansoni* eggs in the absence of full infection, a model we have previously shown to also induce IL-10^{+} splenic MZ B cells [39]. This model induces less inflammation as a result of tissue damage and pathology, and could therefore potentially allow to distinguish schistosome-specific and general inflammatory signals.

Collectively, this study identified several interesting leads with respect to signals that might differentially activate MZ and FO B cells in *S. mansoni* infection, but follow-up studies will be needed to verify the findings and test functional consequences of individual pathways and regulators for Breg cell induction.

MATERIAL AND METHODS

Animals

Female C57BL/6 mice (Harlan) were housed under SPF conditions in the animal facility of the Leiden University Medical Center (Leiden, The Netherlands). All animals were used for experiments at 6-12 weeks of age. All animal studies were performed in accordance with the Animal Experiments Ethical Committee of the Leiden University Medical Center.

S. mansoni infection & isolation of splenic B cell subsets

Mice were infected percutaneously with 36 cercariae and readouts were performed during the chronic phase of infection (14 weeks p.i.). Spleens were homogenized by passage through a 70μM cell strainer (BD Biosciences) and erythrocytes depleted from the single cell suspension by osmotic lysis. B cells were

purified from splenocytes by anti-CD19 MicroBeads (Miltenyi Biotech) following the manufacturer's instructions. Subsequently, B cells were labelled with fluorescent antibodies against CD21 (clone 7G6) and CD23 (clone B3B4) and sorted into MZ B cells ($CD21^{+}$ $CD23^{lo}$) and FO B cells ($CD21^{lo}$ $CD23^{hi}$). Sorted cells were washed twice with PBS and snap-frozen and transferred to -80°C until further use.

RNA isolation

RNA was isolated from snap-frozen cell pellets by use of NucleoSpin RNA isolation kit (Machery-Nagel). A small aliquot of RNA was used to quantify RNA content using a Qubit Fluorometer (Invitrogen) and to confirm sample quality by Bioanalyzer total RNA pico kit (RIN 7.4-9.2). Library preparation and sequencing were contracted out to ServiceXS BV (now GenomeScan BV; Leiden, The Netherlands). The NEBNext Ultra Directional RNA Library Prep Kit for Illumina was used to process the samples. mRNA was isolated from total RNA using oligo-dT magnetic beads and used for library preparation.

RNA sequencing, read mapping and differential expression analysis

For each of the 4 experimental groups (MZ_PBS, FO_PBS, MZ_Inf, FO_Inf), 4 biological replicates were generated, resulting in a total of 16 samples. Paired-end stranded RNA sequencing was performed on a Illumina HiSeq 2500, and generated approximately 17 million (range: 15.7-40.8 million) paired-end fragments per sample. Consistent across all samples, approximately 75% of the fragments could be aligned to annotated transcripts (UCSC annotation, dated 2014-11-25) over *Mus musculus* mm10 reference genome using GSNAP aligner (version dated 2014-12-23). The Trimmed Means of M values (TMM) method was used to produce normalization factors correcting raw counts for different library sizes. The initial count table (ca. 25.000 genes) was filtered to only include genes which had at least 2 aligned fragments per million of aligned fragments (CPM) in at least 4 out of the 16 samples, resulting in a final count table of ca. 12.000 genes included in further analysis. For each gene, its reads per kilobase of transcript per million mapped reads (RPKM) values have been obtained by normalizing CPM values to the transcript lengths approximated by sums of lengths of all exons of the gene. Analysis of differential gene expression was performed with the edgeR package in the R environment. A design matrix for a model with no baseline and 4 groups has been constructed. Next, a genewise negative binomial generalized linear model, with the design matrix and gene-specific dispersions, was fitted followed by a likelihood ratio tests for 4 contrasts (MZ.Inf_PBS contrasting MZ.Inf vs. MZ.PBS, and similarly: FO.Inf_PBS, Inf.MZ_FO and PBS.MZ_FO). Finally, in order to correct for multiple testing, we used the False Discovery Rate control method of Benjamini–Hochberg at a $p < 0.05$ threshold.

IPA Canonical Pathway and Upstream Regulator analysis

Canonical Pathway and Upstream Regulator analysis were performed using Ingenuity Pathway Analysis (IPA; Qiagen Inc.; [43]). The input were all up- and down-regulated genes using a cut-off for the adjusted p-value of < 0.05. A core analysis was performed for each of the pair-wise comparisons followed by a comparison analysis. For the analysis of Canonical Pathways, the list of available pathways was filtered prior to analysis to contain only the category 'signalling pathways' and exclude 'metabolic pathways'. Canonical Pathways were considered significant if log(p-value) > 0.5 and z-score > 2 or <-2. Upstream Regulators were considered significant if p-value < 0.01 and z-score > 2 or < -2 or highly significant if z-score > 4 or <-4.

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SUPPLEMENTARY MATERIAL

Online supplemental material

Suppl. Table S1 shows a list of all DEGs for each of the four analysed contrasts.

Suppl. Table S2 contains results of the Canonical Pathway analysis performed in IPA.

Suppl. Table S3 contains results of the Upstream Regulator analysis performed in IPA.

Suppl. Table S4 shows a list of DEGs within the identified cluster.

Suppl. Tables S1-S4 are available as Excel files under https://www.dropbox.com/sh/zj1c4b3iaubb3ki/AABiGi6nVT-Qe4tz6syTYsOSa?dl=0.

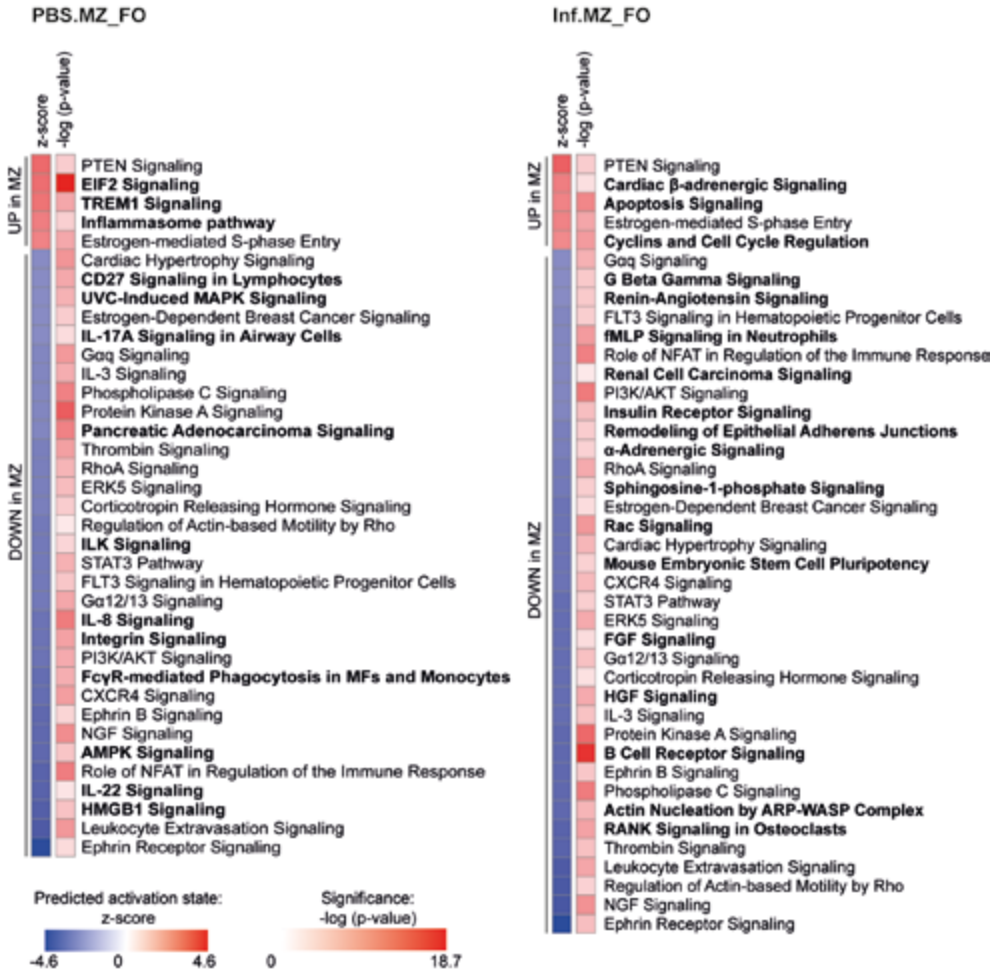

Figure S1. Canonical Pathways significantly activated or inhibited for the difference of MZ and FO B cells at steady-state and after infection. Also see Figure 3.

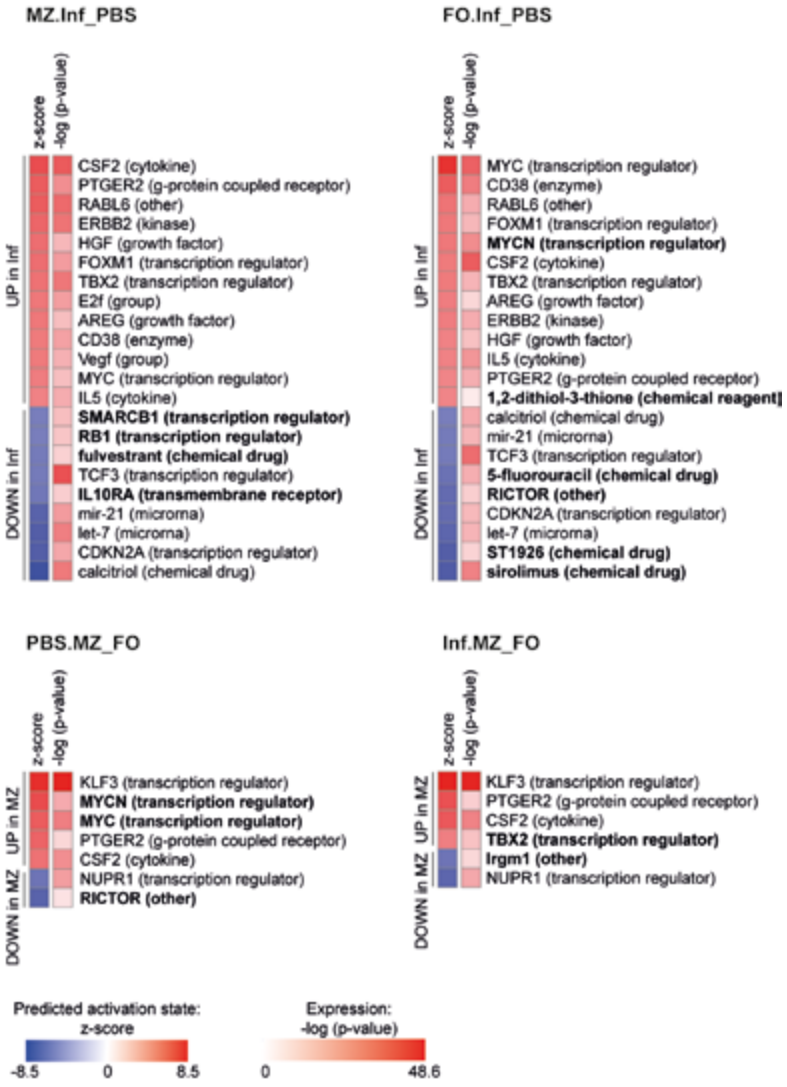

Figure S2. Upstream Regulators significantly activated or inhibited for all pair-wise comparisons and containing all molecule types. Also see Figure 4.

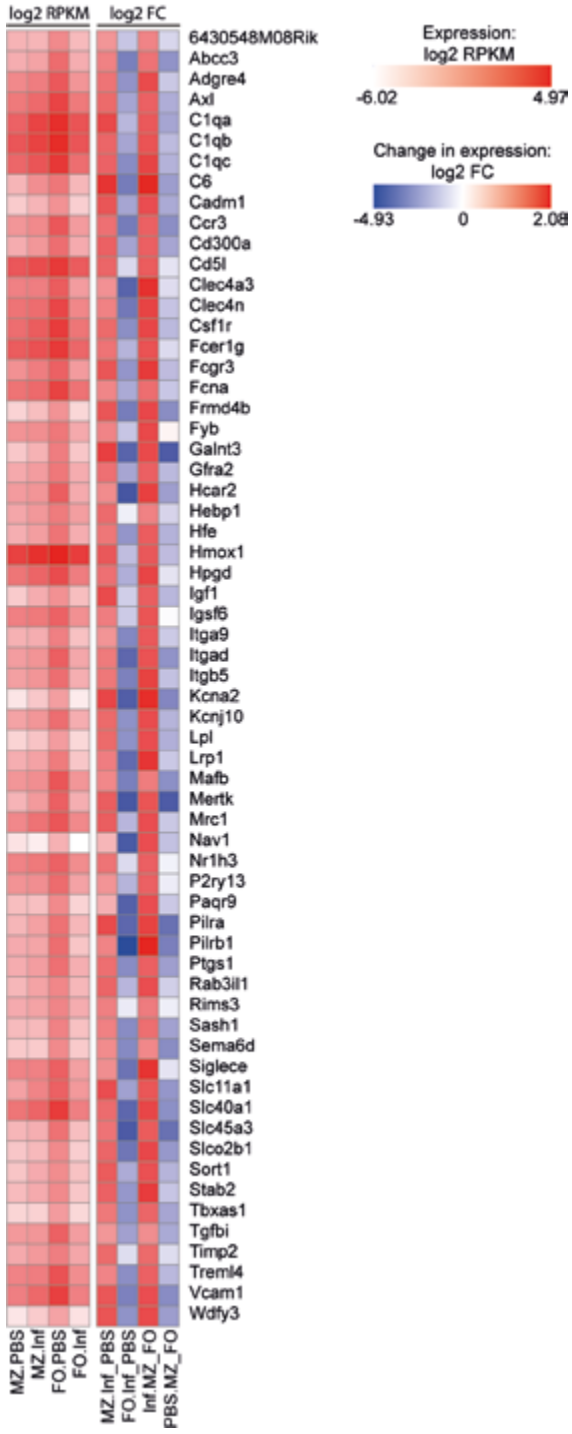

Figure S3. Clusters of genes with opposing fold change for both cell type difference and response to infection comparisons. Also see Figure 5.