Human neonatal B cell immunity differs from the adult version by conserved Ig repertoires and rapid, but transient response dynamics

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SUMMARY

The human infant B cell system is considered premature or impaired. Here we show that neonates have mature and fully functional B cell subsets as seen in adults, albeit with distinct transcriptional programs providing accelerated responsiveness to T cell independent and T cell dependent stimulation and facilitated IgA class switching. Stimulation drives extensive differentiation into antibody secreting cells, thereby presumably limiting memory B cell formation. The neonatal Ig-repertoire is highly variable, but conserved, showing 8% recurrent B cell receptor (BCR) clonotypes shared between neonates. Our study demonstrates that neonatal B cells are neither premature nor impaired, but differ from their adult counterpart in a conserved BCR repertoire and rapid but transient response dynamics. These properties may account for the sensitivity of neonates to infections and limited effectivity of vaccination strategies. Finally, our findings have implications for a limited comparability of mouse models to human infant B cell immunity.

INTRODUCTION

Neonates and infants suffer from enhanced susceptibility to infections (Adkins et al., 2004) and four out of ten childhood deaths world-wide are due to infectious diseases (Kollmann et al., 2017; Siegrist and Aspinall, 2009). Birth is a dramatic transition for the neonate, exposing it to an environment with a high microbial burden compared to that *in utero*, and the infant immune system itself is considered immature to meet these challenges (Adkins et al., 2004; Levy, 2007; Siegrist and Aspinall, 2009). Low neutralizing antibody titers are a critical limitation in infant immunity, and current vaccination strategies aim at generating protective seroconversion (Pollard et al., 2009; Siegrist and Aspinall, 2009). T cell-independent (TI) polysaccharide vaccines either fail (Smith et al., 1973) or elicit short-lived responses with quickly waning IgG titers (Blanchard-Rohner et al., 2009) and do not induce B cell memory (Kelly et al., 2006), but rather hyperresponsiveness (immune exhaustion) (Mitchell et al., 2014). Similarly, T cell-dependent (TD) conjugate vaccinations are generally of low magnitude and do not persist well in early infancy (Booy et al., 1992; Pollard et al., 2009). Immature infant B cell immunity is associated with imbalances in T cell support (Debock and Flamand, 2014; Levy, 2007), and further evident by the importance of maternal-derived antibodies in neonatal immunity (Adkins et al., 2004). Several reasons for infant B cell immaturity have been described. Compared to adults, human neonatal-derived B cells (“neonatal B cells”) were reported to express lower levels of important surface molecules (e.g. CD21, CD40, CD80) which are all associated with lower magnitude of B cell responses (Griffioen et al., 1993; Kaur et al., 2007; Pettengill and Levy, 2016; Tasker and Marshall-Clarke, 2003; Viemann et al., 2000). Differences in TLR expression influence innate and adaptive immunity of human neonates (Pettengill et al., 2016), and B cell receptor (BCR) signal transduction may differ significantly from adult-derived B cells (“adult B cells”), depending on the stimulus (Glaesener et al., 2018; Viemann et al., 2000). In

neonatal B cells, IgV gene mutations are infrequent (Kruetzmann et al., 2003; Weller et al., 2008), affinity maturation is delayed (Siegrist and Aspinall, 2009), and IgG/IgA class switching is impaired (Glaesener et al., 2018), even upon co-stimulation with IL-21 (Pettengill et al., 2016).

Besides these limitations, multiple immune factors contribute to infant vulnerability and futility of vaccination (Pollard et al., 2009; Siegrist and Aspinall, 2009). In mice, the interaction of neonatal lymphocytes and dendritic cells (DC) is immature, resulting in delayed maturation of follicular DC networks (Pihlgren et al., 2003). Human infants also show limited germinal centre (GC) reactions (Blanchard Rohner et al., 2008; Kruschinski et al., 2004). Newly generated plasma cells (PC) compete for limited access to survival niches (Belnoue et al., 2008; Marchant et al., 2006; Pihlgren et al., 2001), and limited survival signals (Pihlgren et al., 2006). Finally, single vaccine doses at birth can fail to elicit seroconversion while priming for secondary responses (Pollard et al., 2009), and the low IgV gene mutation load in neonatal B cells was proposed to favor memory B cell over PC differentiation (Siegrist and Aspinall, 2009; Smith et al., 1997). In light of these multiple limitations of infant immunity, B cell-intrinsic differences are considered unlikely by themselves to provide an insurmountable obstacle to strong responses (Adkins et al., 2004).

Current knowledge on the infant B cell system is mostly derived from vaccination studies or is inferred from mouse models. In mice, neonatal B cells include developmentally and functionally distinct B cell lineage (B-1 cells)(Hayakawa et al., 1985) whereas adult hematopoiesis produces conventional B-2 cells (Hamilton and Kearney, 1994). CD5 is used to distinguish murine B-1a ($CD5^+$) from B-1b ($CD5^-$) cells. B-1 and B-2 cell transcriptome patterns diverge (Mabbott and Gray, 2014; Vilagos et al., 2012; Xu et al., 2011). BCR specificity and signaling strength influence fetal B cell development (Lam and Rajewsky, 1999), and can instruct B-1 phenotypes in adult B cells (Graf et al., 2019). Fetal, but not adult B-1 cell Ig repertoires show a proximal-biased V-gene usage (Perlmutter et al., 1985), and limited addition of non-germline-encoded (N) nucleotides (Carlsson and Holmberg, 1990; Kantor et al., 1997). Neonate mice exhibit pre-immune clonal B-1 cell expansions (Yang et al., 2015). B-1 cells display a primitive, innate-like functionality and produce natural antibodies, independent of T cell help (Hayakawa et al., 1983). In humans, the existence of a B-1 cell counterpart is debated (Baumgarth, 2011; Caligaris-Cappio et al., 1982; Ehrhardt et al., 2005; Griffin et al., 2011; Kreslavsky et al., 2018; Kruetzmann et al., 2003; Perez-Andres et al., 2011; Rakhmanov et al., 2009; Reynaud and Weill, 2012; Wardemann et al., 2002; Weller et al., 2008), CD5 expression is not confined to a fetal B cell lineage (DiLillo et al., 2010; Seifert et al., 2012; Sims et al., 2005; Wortis et al., 1995) and human neonatal B cells share only few of the defects observed in mice (Adkins et al., 2004; Tasker and Marshall-Clarke, 2003).

Our understanding of human infant B cell immunity is limited. The current view implies neonatal B cell functional immaturity in responsiveness and differentiation, particularly under TD stimulation, and their interaction with other immune cells is considered less developed.

For a comprehensive characterization of human neonatal mature B cells, we analyzed their Ig repertoires, molecular patterns and functional capacities. We show here that neonatal B cells are phenotypically similar, but highly distinct in development and function from adult B cells. In contrast to current belief, the vast majority of neonatal B cells consists of mature and fully functional B lymphocytes that respond faster and more efficient to autologous T cells, but also to innate stimulation than adult B cells. Neonatal B cells show strong IgA switching capacity and their differentiation potential is mostly confined to antibody secreting cell (ASC) fate. Their molecular and functional distinctness is supported by a conserved neonatal Ig repertoire as up to 8% of BCR clonotypes among 180,000 BCR rearrangements analyzed are identical between individual neonates. Finally, we show both similarities and differences between human neonatal B cells and murine B-1 cells, suggesting limited comparability of their respective molecular patterns between both species.

RESULTS

A Major Fraction of Human Umbilical Cord Blood B Cells is Mature

Human neonates were reported to have a high frequency of $CD5^{+}CD10^{+}CD24^{+}CD38^{high}$ transitional B cells with immature functionality (Blanco et al., 2018; Sims et al., 2005). To investigate whether human neonatal B cell responses are limited by mature B cell numbers, we determined the average number of mature and transitional B cells in a cohort of 43 umbilical cord blood (UCB) samples. Our analysis showed that on average, neonates have similar proportions of B cells among lymphocytes as adults (3% to 18%, average 10% of mononuclear cells, compared to 4% to 20%, average 11% in peripheral blood (PB) of adults > 20 years). Among UCB B lymphocytes, a fraction of 48% to 79% (average 64%) shows a mature phenotype ($CD19^{+}CD10^{-}CD24^{-}CD38^{low}$ $IgM^{+}IgD^{+}$) (Figures 1A and B and S1A and B). Our initial staining panel indicated that the B cell pool in UCB includes a major fraction of IgM expressing (likely foreign antigen-naïve) mature B cells. To allow for comparison of age-related changes (and to assess similarities to murine B-1a and B-1b cells) we analyzed mature $CD5^{-}$ ($CD5^{-}CD23^{+}CD27^{-}CD38^{low}IgD^{+}$) and $CD5^{+}$ ($CD5^{+}CD23^{+}CD27^{-}CD38^{low}IgD^{+}$) B cells from human neonates and adults separately. On average neonatal mature B cells are composed of equal-sized fractions of mature $CD5^{-}$ and $CD5^{+}$ B cells (Figures 1A and S1C), the $CD5^{+}$ fraction decreasing with age (Figure 1C).

We further quantified memory B cells ($CD27^{+}$), plasma blasts ($CD27^{high}CD43^{+}$) and the recently proposed $CD27^{+}CD43^{+}$ human B-1 cells (Figures 1A and S1D) (Griffin et al., 2011).

Surface IgM and IgD expression was investigated to estimate the number of naïve ($IgD^{high}IgM^{low}$), IgM memory ($IgM^{+}IgD^{+/-}CD27^{+}$) and class switched ($IgD^{neg}IgM^{neg}$) B cells (Figure 1A and data not shown). Mature neonatal B cells barely include memory B cells and plasmablasts, whose numbers increase with age until adulthood, or $CD27^{+}CD43^{+}$ B cells (Figure S1D), in line with previous publications (Blanco et al., 2018; Morbach et al., 2010). We conclude that a major fraction of human umbilical cord blood B cells is mature.

Neonatal and Adult Mature B Cells have Distinct Transcriptomes

Transcriptome-wide comparison revealed different functional capacities among human mature $CD5^{-}$ and $CD5^{+}$ B cell subsets (Seifert et al., 2015; Seifert et al., 2012). We generated RNAseq profiles of sort-purified (Figure S1B) neonatal $CD5^{-}$ ($CD5^{-}CD23^{+}CD27^{-}CD38^{low}IgD^{+}$) and $CD5^{+}$ ($CD5^{+}CD23^{+}CD27^{-}CD38^{low}IgD^{+}$) mature B cells from UCB, and adult naive $CD5^{-}$ ($CD5^{-}CD23^{+}CD27^{-}CD38^{low}IgD^{+}$) and $CD5^{+}$ ($CD5^{+}CD23^{+}CD27^{-}CD38^{low}IgD^{+}$) mature B cells for comparison. Human adult IgM memory ($CD5^{-}CD23^{-}CD27^{+}CD38^{low}IgM^{+}IgD^{+}$) and IgA or IgG class switched memory ($CD5^{-}CD23^{-}CD27^{+}CD38^{low}IgG^{+}$ or IgA^{+}) B cells from PB and tonsil served as analytical root for a robust determination of the relative similarity between adult and neonatal subsets.

According to a t-SNE analysis of the top 10,000 expressed genes of each subset analyzed (Figure 2A), and a hierarchical clustering of the 4,400 most variable transcripts (Figure 2B), human neonatal B cell transcriptomes are unique and deviate from adult B cell samples. Main components of the enriched transcription patterns represent “signaling by the BCR”, “MHC-I mediated antigen presentation” and “pre-mRNA processing” as determined by Reactome pathway enrichment analysis (Figure S2C). Gene set enrichment analysis (GSEA) revealed numerous surface receptors and signaling pathways enriched in neonatal versus adult B cells (Table S1). Enriched signatures included multiple gene sets associated with mitogen responses, BCR- and CD40 signaling (Figure 2C), suggesting that neonatal B cells are transcriptionally prepared to respond to TI-I, TI-II and TD stimulation, respectively. Moreover, TGFβ signaling pathway as prerequisite for IgA switching (Cerutti, 2008), and multiple gene sets associated with TNF-receptor-, interleukin-, and chemokine-signaling suggested that neonatal B cells have broad response capabilities in both TI and TD immunity (Table S1 and Figure 2C). The deviant transcriptional character of neonatal and adult B cells is not only reflected by coherent gene signatures, but also evident on single molecule level. Figure 2D shows a supervised selection of central molecules in BCR signaling, B cell activation or T cell-B cell-interaction, spanning up to four-fold change in expression.

CD5 expression discriminates adult and neonatal subsets only marginally on transcriptional level, by a differential expression of 87 (out of 17,000) transcripts (Figures S2A

and B). However, differences between $CD5^+$ and $CD5^-$ subset are mostly mild, and their biological relevance is thus, questionable.

Arid3a and Bhlhe41 expression are characteristic for murine B-1 cells (Kreslavsky et al., 2017; Yuan et al., 2012; Zhou et al., 2015). However, enrichment of such motifs was not detectable. Single cell analysis of *BHLHE41* expression by RNA flow cytometry revealed low levels in neonatal B cells, while higher expression was largely restricted to the adult memory compartment (Figure S2E). Moreover, the RNAseq profiles of neonatal B cells did not show a consistent enrichment of typical murine B-1 cell expression patterns (Figures S2D and F) (Mabbott and Gray, 2014; Vilagos et al., 2012; Xu et al., 2011).

We conclude that $CD5^+$ and $CD5^-$ human neonatal B cells show unique transcriptome patterns, suggesting efficient responsiveness to both TI and TD stimulation, but barely share signatures with murine B-1 cells.

Neonatal and Adult B Cells are Phenotypically Similar, but Respond Differently

Human neonatal B cells were reported to express lower levels of CD21 (Griffioen et al., 1993), TNFRSF13B (Kaur et al., 2007), CD73 (Pettengill and Levy, 2016), CD22 (Viemann et al., 2000), CD62L and CCR7 (Tasker and Marshall-Clarke, 2003), CD40, CD80, and CD86 (Kaur et al., 2007), all of them associated with lower magnitude of B cell responses. Impaired TLR2- and TLR7- but enhanced TLR9-responses (Pettengill et al., 2016), and decreased responsiveness to IL-10 and IL-21 was described among neonatal B cells (Kaur et al., 2007; Pihlgren et al., 2003). Our transcriptome analysis further supported the idea of a large number of differentially expressed genes with high impact on B cell responsiveness. To validate differential expression, we selected 29 surface molecules and performed flow cytometric analysis on a total of 30 UCB and 21 adult PB samples.

Flowcytometric analysis did neither reflect the transcriptional differences nor support the previously reported lower levels in T cell-B cell-interaction molecules or CD21 expression on resting neonatal B cells (Figure 3A and compare e.g. CD79b, ICOSLG, CD86, CD21, IL-4R in Figure 2D). In contrast, we observed high phenotypical similarity between adult and neonatal subsets (Figures 3A and S3). Typical surface expression patterns of murine B-1 cells (Kantor and Herzenberg, 1993) (CD11b/CD18, CD43 or low level of IgD, CD21 or CD23) were not detectable (Figures 3 and S3).

We next investigated the responsiveness of neonatal and adult B cells *in vitro*, stimulated with CpG (TI-I), anti-Ig (TI-II), and anti-Ig plus CD40L (TD). Compared to steady state, we observed often dramatic changes in surface expression upon stimulation, unidirectional (e.g. CD69, CD86, FCGR2A, CD21) as well as differential (e.g. ITGAM, ICOSLG, OX40L, FCER2, IFNGR, IL4R) between neonatal and adult B cells. The type of stimulus often had a strong impact, mostly on response intensity, with a few exceptions

(FCER2, IFNAR2), (Figures 3B and S3). Note that in this analysis $CD5^+$ and $CD5^-$ B cells were not sort-purified, thus CD5 up- or downregulation during culture cannot be estimated. However, $CD5^+$ and $CD5^-$ B cells usually shifted homogenously. We conclude that with few exceptions, neonatal and adult mature B cells are phenotypically similar in the resting state, but have different response potentials.

Human Neonatal B Cell Responses are Rapid but Transient

We analyzed neonatal and adult B cell responses at different time points *in vitro* and observed qualitative and quantitative differences. Neonatal B cells proliferated earlier, as the first cell division was already detectable on day 2 upon TI-I or TD stimulation, whereas adult B cells proliferated at the earliest on day 4 (Figure 4A). TI-II stimulation showed weak induction of proliferation in general, in contrast to TD stimulation, particularly in combination with IL-4 and IL-21 (Figures 4B and S4A, respectively). In contrast, adult B cell division was more delayed and barely detectable upon TI stimulation (Figuress 4A and B). With regard to the known differences in neonatal and adult T cell composition (Kollmann et al., 2017; Levy, 2007), we performed coculture assays of mature B cells and autologous T helper cells ($CD4^+CD25^-$), stimulated with anti-CD2/anti-CD3/anti-CD28 beads. The coculture assays confirmed a TD-proliferation potential among both neonatal and adult B cells, although neonatal B cell division was slower than by stimulation with selected ligands (Figure 4B).

ASC differentiation is highly relevant in neonatal immunity. We investigated Ig secretion potentials by ELISpot and showed that neonatal B cells are fully capable of secreting Ig earlier than adult B cells (Figure 4C). Please note, that the ELISpot membrane covered with IgM-capture antibodies likely generates BCR-stimulating signals during the incubation time. Earlier Ig secretion was in line with an increased IgM production rate (Figure 4D), and (in total) accelerated ASC differentiation as determined by $FSC/SSC^{high}CD38^{high}$ phenotype (Figures 4E and F and data not shown), PRDM1 induction (Figure S4B), ratio of secreted vs. membrane IgM transcripts (Figure S4C), and cell polarization ("IgM capping") after 30 minutes of stimulation (Figures 4I and S4F and G) in neonatal vs. adult B cells. In the first four days neonatal B cells homogenously showed ASC differentiation fate, independent of *in vitro* stimulation conditions (Figures 4C, E and F), whereas only a fraction of adult B cells differentiated. This result was consistent with autologous T cell coculture (Figures 4B, D and F). Neither *in vitro* stimulation nor T cell coculture induced preferential survival among neonatal over adult B cells, but rather increased apoptosis over time (Figure S4E), supporting the transient nature of neonatal B cell responses.

Finally, as suggested by efficient upregulation of TGFBR1 (Figure 3B), we observed enhanced IgA class switching among neonatal, but not adult B cells treated with anti-Ig and TGFb, already at day 3 (Figures 4G and H and S4D). In contrast, anti-Ig, CD40L and IL-4

mediated class switching to IgG was more efficient among adult naïve B cells, yet delayed until day 5 (Figure S4D).

Our data suggest that neonatal B cells are not impaired, but show rapid TI and TD responsiveness, whereas adult B cell proliferation is mostly confined to TD stimulation. Second, ASC differentiation is the predominant fate of neonatal B cells. These unique dynamics favour transient responses, which may cause the observed quickly waning Ig titres and hyperresponsiveness *in vivo*. The quick and preferential response to TI stimulation, efficient IgM secretion and enhanced IgA class switching is also central to murine B-1 cells (Baumgarth, 2011; Kantor and Herzenberg, 1993; Macpherson et al., 2000).

The Human Neonatal BCR Repertoire is Variable, but Conserved

We performed BCR repertoire deep sequencing of mature $CD5^+$ and $CD5^-$ B cells from four neonates and three adults. We sequenced rearranged IGHV genes of at least 100,000 B cells per population, split into two biological replicates, and used unique molecular identifiers to distinguish clonal expansions from Ig sequence amplification during library generation. The vast majority of BCR rearrangements were unique and unmutated, demonstrating that the infant and adult naïve BCR repertoires are similarly variable (Figure 5A). However, whereas clonal expansions were largely absent in adults, they were more frequent in neonates, with almost 2% repeatedly identified sequences on average, and up to 7.3% in one sample (Figures 5A and B). The clonal expansions among both neonatal and adult B cells were devoid of Ig mutations, indicating GC-independent generation. The neonatal-derived clones were larger, up to 40 members, with an average of 4 members per clone (Figure 5C). The comparison of $CD5^+$ and $CD5^-$ B cell populations within an individual neonate or adult revealed that both subsets were highly related. In any given sample, the majority of clonal expansions were distributed between both subsets, and whereas clones derived from $CD5^+$ B cells alone were frequent – particularly in neonates – clones that consisted only of $CD5^-$ B cell-derived sequences were barely detectable (Figures S5A and B). This indicates that neonates have GC-independent BCR selection mechanisms, which are reduced in adults, and that CD5 is not a stable marker within a clone.

We sought to compare the neonatal BCR repertoire on an inter-individual level and therefore, analysed the distribution of clonotypes, i.e. BCR rearrangements with identical *IGHV* gene and 80% to 100% identical amino acid CDRIII sequence between individuals (Figure S5C). At 90% or more CDRIII identity, neonatal samples (n=4) included 3 to 5% (Ig unmutated) clonotypes and adult naïve B cells (n=3) showed none. In total, 4 to 8% of all BCR rearrangements from four neonates were clonotypic (Figure 5D), suggesting that neonatal B lymphopoiesis includes the selection of conserved specificities. We also observed that adult mature B cells – albeit way less frequent – can show neonatal-like BCR specificities, and these

clonotypes are significantly enriched among the mature $CD5^+$ subset (Figure 5E). Clones and clonotypes barely overlapped.

Finally, with regard to murine B-1 cells, the Ig repertoire of human neonatal B cells did not show an enrichment of proximal vs. distal IgV genes, or major biases in IgV gene usage. However, we confirmed a significantly reduced amount of N-nucleotide insertions at D_H-J_H-junctions among neonatal B cells (Figure S5D), in line with previous publications (Hong et al., 2018).

We conclude that the human neonatal mature B cell repertoire is selected to a large extent by inherent mechanisms, which may be similar, but not identical to rodents.

DISCUSSION

Neonates suffer from enhanced susceptibility to infections, and short-lived and quickly waning titers of neutralizing antibody represent a critical factor, thereby also limiting vaccination approaches. Repeated vaccine challenges applied too early in life even bear the risk of hyperresponsiveness, i.e. excess responses consume the already limited pool of reactive B cells or antibodies. Multiple reasons for this have been described, including immaturity or limited support of immune cells or lymphoid tissues. Also, neonatal B cells were – in contrast to adult B cells - reported to frequently show transitional phenotypes, show limited responsiveness, reduced expression of important receptors, or suffer from immature immune interactions. These notions have led to the concept that the infant B cell system is underdeveloped or even defective (Adkins et al., 2004; Kollmann et al., 2017; Levy, 2007; Pollard et al., 2009; Siegrist and Aspinall, 2009).

Our study aimed at a comprehensive characterization of human neonatal B cells to reveal putative limitations by phenotypical, molecular and functional analysis. Our results contrasted with the current perception, that neonatal B cells are premature or impaired.

According to our analysis about 60% of the neonatal B cell pool is mature, expressing the typical receptor profile that is detectable on adult B cells in the resting state. On transcriptional level, however, a major distinctness can be observed, spanning thousands of differentially expressed transcripts and gene sets, suggesting that neonatal B cell responses differ from those of adult B cells, particularly in their responsiveness to TI versus TD stimulation.

Indeed, functional analyses revealed that neonatal B cells are highly sensitive to TI and TD stimulation, whereas adult B cells required T cell help for efficient responses. TI-I stimulation was mimicked by CpG, but neonatal B cells also responded efficiently to other mitogens (R848, Pam2CSK4, Pam3CSK4, data not shown), in line with their expression of several pattern recognition receptors. Neonatal B cell responses revealed their full capability

of ASC differentiation, antibody production and secretion, as shown by several approaches *in vitro*. Considering that neonatal T cells may have lower (or different) stimulating capacities, we also confirmed efficient B cell responsiveness to autologous T cell stimulation. The reported reduction in class switching (Glaesener et al., 2018; Pettengill et al., 2016) is putatively limited by cytokine levels in neonates (Nesin and Cunningham-Rundles, 2000), but not by neonatal B cells itself, as these showed fast and efficient CSR in particular to IgA. We suggest that the high propensity to IgA (over IgG) class switching supports the quick generation of mucosal immunity in neonates.

The human neonatal BCR repertoire is unmutated, but highly variable, and no major differences to the adult repertoire in composition, VDJ-usage or CDRIII diversity could be observed. The previously reported reduction in N2-non-germline encoded nucleotides in 30% of the IGHV rearrangements, argues for a reduced junctional diversity in neonatal early pro B cells (Hong et al., 2018). Moreover, we observed clonal expansions lacking intraclonal diversification, indicating that early in life selection processes exist, that do not require GC diversification. Such clonal expansions were also observed among adult B cells, and then always related to the peculiar mature $CD5^+$ B cell subset, showing autoimmune specificities (Hardy et al., 1987; Herve et al., 2005), associated with autoimmune disorders (Burastero et al., 1988; Dauphinee et al., 1988), and chronic lymphocytic leukemia pathogenesis (Seifert et al., 2012).

It was previously reported that neonates show few similar Ig rearrangements (clonotypes) among three individuals analyzed (Soto et al., 2019). Our data set of four neonatal mature B cell samples, contained on average 8% of the BCR rearrangements with identical *IGHV* genes and at least 90% CDRIII amino acid sequence identity being shared between individuals. Thus, we propose that the neonatal mature B cell repertoire is substantially selected by inherent, evolutionary conserved mechanisms. These neonatal clonotypes are very rare among adults, but highly enriched in mature $CD5^+$ B cells (and their few clonally related $CD5^-$ B cells). This is similar to the connection of GC-independent clonal expansions to adult $CD5^+$ B cells, and it is tempting to speculate on selection of autoantigenic specificities during neonatal B lymphopoiesis, similar to mouse models (Hayakawa et al., 2003) and potentially contributing to removal of cell debris in the periphery of human neonates (Baumgarth, 2011). Note that our study design lacks Ig light chain information, preventing direct proof of this point. Moreover, the rarity of neonatal clonotypes among adult B cells further precludes the conclusion whether these rare clones are long-lived or adult-derived, and whether *de-novo* generated BCR specificities instruct a neonatal B cell fate (Graf et al., 2019).

Besides a broader responsiveness, we observed that neonatal B cells differ essentially from their adult counterparts in their response dynamics. Neonatal B lymphocytes respond sensitive and fast to stimulation, showing first cell polarizing events already after 30 minutes,

secreting Ig within hours and entering cell cycle within the first day after stimulation thereby outpacing adult memory B cell responses (Engels and Wienands, 2018; Seifert et al., 2015; Shi et al., 2003). The ELISpot analysis indicated that neonatal B cells (and to a lesser extent also adult $CD5^+$ B cells) secrete IgM efficiently, already upon short-term contact with the capture antibody.

The second – and immunologically likely most relevant – distinctness in neonatal B cell response dynamics is that ASC differentiation is global, i.e. the vast majority of the cells responds to stimulation with quickly induced expansion and efficient Ig production. Thereby, considering the limited infrastructure in B cell follicles (Blanchard Rohner et al., 2008; Kruschinski et al., 2004; Pihlgren et al., 2003), the rarity of PC survival niches in the bone marrow (Belnoue et al., 2008; Marchant et al., 2006), and the deprivation of survival signals (Pihlgren et al., 2006), it is easily anticipated why the rapid neonatal B cell responses are transient and wane quickly (Pollard et al., 2009; Siegrist and Aspinall, 2009). In line with this view, we observed enhanced apoptosis and cell death among neonatal B cells after four days of stimulation *in vitro*, as compared to adult naïve B cells that underwent the same number of cell divisions on that day. This effect is for sure also influenced by e.g. nutrition matters ("all B cells die in culture"). However, after sort-purification and stimulation induction, a fraction of adult B cells survives and proliferates repeatedly, whereas among the neonatal B cell culture cell division and subsequent cell death is more closely connected.

Taken together, we did not confirm the previously reported neonatal B cell limitations in surface receptor expression, reduced responsiveness to interleukins or limited class switching capacity. Discrepancies to previous studies may be due to the fact that transitional cells were not excluded, that different culture conditions or stimuli were used or simply, that B cell responses were not measured over time. Instead, we observed that neonatal B cell responses show "flash in the pan" characteristics, favoring ASC fates. These neonatal responses are likely transient, given by the limited accessibility of PC survival niches and decreased survival signals in young infants (Belnoue et al., 2008; Marchant et al., 2006; Pihlgren et al., 2006; Pihlgren et al., 2001).

We anticipate that these dynamics are the primary cause for the *in vivo* observed quickly waning Ig titers and hyperresponsiveness upon vaccination strategies too early in life (Kollmann et al., 2017; Pollard et al., 2009; Siegrist and Aspinall, 2009). Our study shows that the neonatal B cell system has a BCR repertoire as seen in adults and the available B cells respond fast and comprehensively with highly avid antibody titers, but this comes at the expense of prolonged immunity in the shape of memory B cells and long-lived ASCs, and thus, inability to cope with high pathogen burdens, and prolonged or repeated infections.

This implies that vaccinations aiming at the generation of immune memory are futile or even dangerous when applied too early in life. Consequently, systematic vaccination strategies

encompassing the neonate's environment (elder siblings and insufficiently immunized adults) are mandatory to generate herd immunity. Moreover, passive immunization and treatment strategies to sustain ASC survival should be taken into research focus.

Finally, many aspects of our knowledge on neonatal B cell immunity and susceptibility to infection are based on mouse models. However, a human B-1 lineage counterpart is a long-standing matter of controversy, thereby raising the question of a general comparability of human neonatal B cells to the murine B-1 lineage. We studied human mature $CD5^+$ and $CD5^-$ B cells to allow comparison of our findings to ontogenetically early B-1a and B-1b, and adult B2 cells from animal models.

We observed similarities in function, e.g. the fast and high responsiveness to TI stimulation of human neonatal B cells is reminiscent of the innate-like behavior of B-1 cells in mice. Moreover, the fast and efficient IgM secretion of neonatal B cells further argues for overlapping functions. However, the highly specific developmental regulation and homeostasis of murine B-1 cells, as well as transcriptional patterns were only marginally similar, or even absent in human B cells. Moreover, the human neonatal BCR repertoire, albeit indicating peculiar selection mechanisms, did not show the biased Ig gene usage observed in mice. Thus, human neonatal B cells can only partially be explained by murine B-1 lineage characteristics. However, we speculate that the functional similarities of B-1 cells and neonatal B cells give insight into essential, evolutionary conserved immune mechanisms in infants. This includes broadly reactive B cell types that respond quickly with a main focus on antibody generation. Early specificities seem to be polyreactive, i.e., autoantigenic stimulation likely influences B cell selection positively also in humans. Notably, IgA class switching is a central feature in mice and humans, thereby suggesting that after birth, mucosal infections are the primary challenge.

We conclude that human neonatal and adult B cells are phenotypically similar, but differ markedly in their response potentials. This major distinctness exists in mice and humans, suggesting that a fast and independent IgM and IgA antibody response early in life proved beneficial in evolution. However, the molecular patterns underlying these neonatal B cell responses seem to vary between species. Our study contrasts with many aspects of the current view on human neonatal B cell responses. This has important implications not only for our understanding of infant vulnerability to microbes, but also suggests that vaccination strategies aiming directly at neonatal B cells rather cause hyperresponsiveness than long-lasting protection.

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AUTHOR CONTRIBUTIONS

M.S. developed the concept and prepared the manuscript. B.B., A.K. M.B., and E.H. designed and carried out most of the experiments and contributed to manuscript preparation. B.B. developed and performed bioinformatical and statistical analysis. K.B., J. A. R., A.G., M.A.W., J.D., T.K., S.V., F.M., P.J., A-C.L. and A.R. contributed experimental work and data interpretation. P.R., D.A. and P.K. contributed samples. M.L., W.H., J.D., and B.G. designed experiments and contributed valuable discussion. R.K., C.C.O., M.A.W., B.B., D.H., B.G., A-C.L. and A.R. reviewed the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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MAIN FIGURE TITLES AND LEGENDS

Figure 1. Quantification of Human Neonatal and Adult Mature B Cells.

(A) Gating strategy for neonatal and adult mature B cell subsets.

(B) Proportions of mature ($CD5^+$ and $CD5^-$) B cells and transitional B cells in 43 randomly selected neonates (UCB).

(C) Age-dependent changes in the proportion of mature $CD5^+$ B cells from 45 donors aged 0 – 90 years. Line of fit (bold line) and 95% confidence interval (shaded area) were calculated by R smooth.

Figure 2: Transcriptome-wide Comparison of Human Neonatal and Adult Mature B Cells.

(A) T-SNE plot (R-package Rtsne and standard settings, https://CRAN.R-project.org/package=Rtsne) showing the distribution of different B cell subsets based on the top 10,000 expressed genes.

(B) Hierarchical clustering and heat map showing the 4,400 most variable transcripts (MANOVA, $q<0.05$) of neonatal and adult PB mature $CD5^+$ and $CD5^-$ B cells, and adult PB IgM memory B cells. The colouring spans 4-fold differences, row-wise normalized (legend).

(C) Gene set enrichment analysis of hallmark, C2, and C3 gene sets. Shown are gene sets enriched (black) or depleted (red) in neonatal versus adult B cells with a p-adjusted value below 0.05 with their normalized enrichment score (NES) (R package fgsea, doi: 10.1101/060012). This selection focusses on immune signalling, for the total list, see Table S1.

(D) Detailed heatmap for selected genes involved in BCR signalling, B cell activation/modulation, B cell/T cell interaction, and B-1 cell development among neonatal and adult mature $CD5^+$ and $CD5^-$ B cells, and adult IgM memory B cells. The colouring depicts row-wise normalized fold-changes.

Figure 3: Flow Cytometric Characterization of Neonatal and Adult Mature B Cells in Resting State and Upon *in vitro* Stimulation.

This meta-FACS-analysis shows average MFI values (A) of 29 selected surface markers expressed on neonatal and adult CD19-enriched B cells in the resting state, grey bars mark significant differences between neonate and adult ($p < 0.05$, Wilcoxon test).

(B) MFI fold-changes after o/n stimulation under TI-I, TI-II or TD conditions. Dot size and colour code reflect the average MFI value (green, A) or fold-change versus unstimulated condition (yellow for increase, blue for decrease, B) for three to seven samples per subset and condition. Black ($p < 0.05$, Wilcoxon test) or grey ($p < 0.1$, Wilcoxon test) circles show the certainty of increase or decrease compared to resting state.

Figure 4: Functional Analysis of Neonatal and Adult Mature B Cells.

(A) Example histograms of the cell proliferation dye eFluor™ 670 in sort-purified neonatal $CD5^+$ (red) and $CD5^-$ B cells (orange) and adult mature $CD5^+$ (blue) and $CD5^-$ (mint) B cells until day two (d2) or until day four (d4) upon stimulation with TI-I stimulus [CpG] (left), and TD [anti-Ig + CD40L] (right).

(B) Summary of five individual experiments as shown in (A), including TI-II [αIg] stimulation and coculture with activated autologous T cells ($CD4^+CD25^-$).

(C) IgM Elispot assays of neonatal and adult mature $CD5^+$ and $CD5^-$ B cells unstimulated (top) or upon stimulation with TI-I [CpG] (bottom). Plus and minus indicate positive (IgM memory B cells plus R848) and negative controls (no B cells), respectively.

(D) IgM production rate (intracellular IgM accumulation within 4h with GolgiStop™ and GolgiPlug™) of neonatal and adult mature $CD5^+$ and $CD5^-$ B cells, stimulating conditions as in (B).

(E) ASC differentiation as determined by flow cytometry (increasing FSC / SSC) among neonatal and adult B cell samples upon stimulation on day 2.

(F) Summary of SSC increase among at least five neonatal or adult B cell samples on d0 to d4 after stimulation (TI-I [CpG], autologous T cells).

(G) IgA (ordinate) and IgG (abscissa) class switching of human neonatal B cells on d3 upon stimulation with TGFb and αIg.

(H) Summary of three independent IgA class switching experiments of human neonatal ($CD5^+$ and $CD5^-$ B cells were not separated for the sake of sufficient cell numbers) and adult $CD5^+$ and $CD5^-$ B cells, experimental conditions as in (G).

(I) Cell polarization (IgM capping) of neonatal mature B cells after 30 minutes of stimulation with CpG. DAPI (blue), CD5 (red), IgM (green). *$p < 0.05$, **$p < 0.01$, *** $p < 0.001$, Wilcoxon rank sum test.

Figure 5: NGS Analysis of the Neonatal and Adult Mature B Cell Ig Repertoire. Top: Sequences that were repeatedly identified in biological replicates or among different cell samples from the same donor were classified as (int*ra*-individual) clones.

(A) Fraction of clonal sequences within one selected sample, and (B) all samples (four neonates and three adults).

(C) Clone sizes detected in all samples. The median clone size among adults is 3 with a maximum of 26 members, whereas the median for neonatal clones is 4 with clone sizes up to 40 members.

Bottom: Clonotypes (inte*r*-individual): Sequences were classified as clonotypic when bearing the same IGHV gene and at least 90% CDRIII amino acid sequence identity among different donors.

(D) The percentage of clonotypes among total neonatal and adult mature $CD5^+$ and $CD5^-$ B cell derived sequences, and among all neonatal sequences.
(E) Number of clonotypes identified in neonatal samples among adult B cell subsets at 100% total amino acid sequence identity. Wilcoxon test was used to determine p-values (four neonates and three adults).

STAR Methods

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Marc Seifert (marc.seifert@uni-due.de).
This study did not generate new unique reagents.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human samples

PB samples from healthy adults were collected at the Institute of Transfusion Medicine, University Hospital Essen. UCB samples from healthy term neonates (n > 200, ratio male / female 0.97) were collected at the University Hospital Essen at the Clinic for Obstetrics and Gynaecology, University Hospital Essen. All samples were obtained after informed consent was given according to the Declaration of Helsinki, as well as approval by the ethics committee of the Medical Faculty of the University of Duisburg-Essen, Germany. For samples from PB of children aged 1.5 to 6 years, the study was approved by the Human Research Ethics Committee of the Medical Faculty at University of Gothenburg, Sweden. Informed written consent was obtained from the parents of the children.

METHOD DETAILS

Magnetic cell separation

B lymphocytes from different tissues were isolated by Ficoll gradient density centrifugation (density 1.077 g/ml, Pan BioTech, Aidenbach, Germany) followed by selection of CD19-or CD3expressing cells by magnetic cell separation (MACS) (Miltenyi Biotec, Bergisch Gladbach, Germany) or untouched magnetic selection (EasySep, STEMCELL Technologies, Cologne, Germany). Hematopoietic precursor cells were obtained by Ficoll gradient density centrifugation and selection of CD34-expressing cells by MACS. CD5-expressing B cells were separated from conventional B cells by untouched selection of B cells followed by positive selection of $CD5^+$ B cells using anti-CD5-APC (BD Biosciences, Heidelberg), followed by either

counterstaining with anti-APC beads (Miltenyi) and MACS enrichment or by fluorescence-activated cell sorting (FACS).

Flow cytometry

Lymphocytes were analysed for the expression of distinct cell surface molecules after staining with fluorescence-conjugated antibodies. Stained cells were either analysed on a Cytoflex flow cytometer (Beckman Coulter) using the Kaluza software (Beckman Coulter) or sort-purified on a FACSAria III cell sorter (BD Biosciences) / FACSAria Fusion cell sorter (BD Bioscience) equipped with BD FACSDiva software (BD Biosciences). If not indicated otherwise, mature $CD5^+$ B cells were defined by the marker constellation $CD5^+CD23^+CD27^-CD38^{low}IgD^+$ and $CD5^-$ (naïve) B cells as $CD5^-CD23^+CD27^-CD38^{low}IgD^+$ cells. Autologous T cells were isolated from CD19-MACS flow through by sort-purification of $CD4^+CD25^-$ lymphocytes. Dead cells were excluded by 7-AAD (Beckman Coulter) staining.

ELISpot assays

ELISpot membranes (MabTech, Macleod, Australia) were coated with anti-IgM antibodies as recommended by the manufacturer. B cells were sort-purified and distributed at 5,000 cells in 100 µl RPMI1640 (Pan BioTech) per well. After 16 hours of incubation (37°C and 5% CO_2), cells were discarded and secreted IgM was visualized as recommended by the manufacturer (MabTech, Macleod). The developed spots were quantified by an ELISpot reader (ELISpot Reader System, Autoimmun Diagnostika GmbH, Strassberg, Germany).

In vitro functional assays

For functional studies, mature B cell subsets were isolated from human PB or UCB and cultured in RPMI 1640 medium with 20% foetal bovine serum (Pan Biotech), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C and 5% CO_2. All populations were left unstimulated or were activated. TD stimulation was executed using 0.03 µg/µl anti-immunoglobulin (anti-Ig, Jackson ImmunoResearch, Newmarket, UK) and 1 µg/ml CD40-ligand-HA with 5 ng/ml anti-HA antibodies (R&D Systems, Minneapolis, USA), and separately in combination with IL-4 and IL-21 (100 IU each). T cell independent type 1 (TI-I) stimulation was mimicked by incubation with CpG OND Type B or R848 (InvivoGen, San Diego, USA). TI type 2 (TI-II) simulation was performed by 0.03 µg/µl anti-Ig treatment. For coculture experiments, B cells and autologous T cells were co-cultivated in equal numbers (250,000 cells). T cells were left unstimulated or activated via anti-CD2/anti-CD3/anti-CD28 beads (Treg Suppression Inspector, Miltenyi) according to the manufacturer's instructions.

Survival and proliferation

B cells were cultivated under TD, TD+IL-4+IL-21, TI-I or TI-II stimulating conditions, activated autologous T cells, or left untreated (see above). Survival was determined by assessing the fraction of vital cells at defined time points during culture by flow cytometric exclusion of propidium iodide (PI) (Merck, Darmstadt, Germany) and Annexin V-APC (BD Biosciences) positive cells. To determine proliferation, isolated B cells were stained with Cell Proliferation Dye eFluor™ 670 (5 µM, Invitrogen) and measured instantly and after 48 h and 96 h.

Confocal microscopy

For each stimulation condition, 1 x 10^6 $CD3^-CD10^-$ cells (anti-CD3-APC (BD Biosciences) and anti-CD10-FITC (BD Biosciences)) were sort-purified and stimulated with either TI-II or CpG at 37°C and 5% CO_2 for 30 min, and subsequently stained with anti-CD5-PE-Cy5 (BD Biosciences, Heidelberg). The stained cells were fixed with Fixation/Permeabilization Solution (BD Biosciences) according to manufacturer instructions, followed by intracellular staining with anti-IgM FITC (BD Biosciences, Heidelberg) and Hoechst 33342 (Thermo Fisher). Confocal microscopy was performed with a Leica SP8 gSTED Super Resolution Microscope at the Imaging Center Essen (IMCES).

Transcriptome analysis

IgM memory ($CD5^-CD23^-CD27^+CD38^{low}IgM^+IgD^{low}$) and IgA or IgG class switched memory ($CD5^-CD23^-CD27^+CD38^{low}IgG^+$ or IgA^+) B cells from PB of adult donors, and $CD5^-$ ($CD5^-CD23^+CD27^-CD38^{low}IgD^+$) and $CD5^+$ ($CD5^+CD23^+CD27^-CD38^{low}IgD^+$) B cells from UCB and adult PB, were isolated by flow cytometry (purity >99%) from 6 adult donors and 6 UCB. RNA was isolated by the RNeasy Micro Kit (Qiagen, Hilden, Germany). For the generation of RNAseq libraries, the NuGEN Trio RNA-Seq System (NuGEN, Redwood City, California, USA) was used. Samples were split equally and processed in independent sequencing steps to allow for correction of batch effects. Sequencing was performed with paired-end sequencing and two times 100 bp length. Sequences were aligned with HiSAT2 (version 2.1.0. Kim D, Langmead B and Salzberg SL. HISAT: a fast-spliced aligner with low memory requirements. *Nature Methods* 2015) to the human genome hg38 and analysed with DESeq2,(Love et al., 2014) and R (www.R-project.org/). Hierarchical tree analysis was performed using Genespring GX 11 (Agilent Technologies, Waldbronn, Germany).

Real Time Quantitative (qRT-)PCR

qRT-PCR was performed using commercial TaqMan probes (Applied Biosystems) targeting PRDM1 in 9x10^4 neonatal and adult mature B cell subsets per condition after stimulation for 48 h (unstimulated control, CpG, α-Ig, TD), as described in the *in vitro* functional assay paragraph. Total RNA was extracted using RNeasy Micro Kit (Qiagen) and cDNA synthesis

was performed according to manufacturer's instructions using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The qRT-PCR conditions consisted of 2 min at 50°C, 10 min at 95°C and 45 cycles of 15 s at 55°C and 1 min at 60°C. Every experiment was performed three times and the samples were tested in triplicate. The average of ΔCT values for the amplicon of interest was normalized to that of GAPDH.

Semi-quantitative PCR

IgM transcripts from total RNA extracts (RNeasy Micro Kit, Qiagen) were reversely transcribed (High-Capacity cDNA Reverse Transcription Kit, Applied Biosystems) with IgM constant-specific primer 5´-GCCCTGCCCAACAGGGTCA-3´. IgM transcript splice variants for membrane expression or secretion were amplified with 5´-AGCAAAGCAGTGTGGGGTAGA-3´ and 5´-ACACACAGAGCGGCCAGC-3´ primers (all IDT, Leuven, Belgium), respectively. After agarose gel-electrophoresis, products were visualized by GelRed (Biotium, Fremont, USA) and product intensity measured by Software *ImageJ (PMID* 22930834) and normalized to ACTIN amplicon products (5´-GACGACATGGAGAAAATCTG-3´ and 5´-ATGATCTGGGTCATCTTCTC-3´, Sigma-Aldrich, St. Louis, Missouri, USA).

RNA flow cytometry

To assess *BHLHE41* expression in human PB- and UCB-derived B cell subsets, the PrimeFlow RNA assay (Thermo Fisher) was performed with high sensitivity Alexa Fluor 647 probe targeting human *BHLHE41* according to the manufacturer's instructions. *CD8A* probe was used as a negative control.

QUANTIFICATION AND STATISTICAL ANALSYSIS

IGHV gene rearrangement analysis by high throughput sequencing

Genomic DNA was extracted from sorted B cell populations (Gentra Puregene Core Kit, Qiagen). Rearranged *IGHV* genes of B cells sort-purified in duplicates were sequenced by massive parallel sequencing. By PCR using IGHV1, 3 and 4 subgroup primers for *IGHV* framework region 2 and a mixture of all *IGHJ* primers (Supplementary Table 2), DNA was amplified to generate deep sequencing libraries on the MiSeq Illumina platform (Illumina, San Diego, California, USA). In the first two PCR cycles the template (rearranged *IGVH* gene) is copied only once, while 12 nucleotides *unique molecular identifier* (UMI) are introduced into the amplicon by the first set of primers. Following removal of those primers, the constructs are amplified by a second set of primers, which in turn tag the construct with sequences essential for hybridization on the flow cell. Sequencing was performed with custom primers and the rapid run program with paired-end sequencing and two times 300 bp length. Reads generated by MiSeq were included only when the average quality was ≥ 25. Ambiguities between forward

and reverse reads were replaced by “N”. Sequences with identical UMI were classified as PCR duplicates and reduced to the longest detected sequence. Each N in a given sequence was replaced by the most frequent (majority vote) nucleotide. Only in-frame sequences detected more than once were further processed. Differences in the amount of sequences between mature $CD5^+$ and naive B cells were balanced by random drawing from the population with higher amount of sequences. Sequences were considered clonally related when using the same *IGHV* gene and sharing 100% complementarity determining region III nucleotide sequence identity. All statistical and bioinformatical evaluations were performed in R (www.R-project.org/) and based on the international ImMunoGeneTics information system (IMGT) database (www.imgt.org/). Mutation frequencies were calculated based on the number or relative position of nucleotide exchanges in the *IGHV* region of each sequence in comparison with the most similar allelic variant present in the respective donor (determined from unmutated sequences). *IGHV* identification was carried out with BLAST. *IGHD* and *IGHJ* identification was performed by calculating the best letter-wise matching gene. For IGH gene usage, the fraction of a given IGH gene among total sequences (all subgroups) detected was calculated. Every nucleotide between and including the first and the last non-germline encoded nucleotide between *IGHV* and *IGHD* or *IGHD* and *IGHJ* gene, with highest germline identity was counted as N1 (V-D) or N2 (D-J) non-germline encoded nucleotide, respectively. IGHV usage was determined by R-package iggeneusage (DOI: 10.18129/B9.bioc.IgGeneUsage).

Statistical Analysis

Statistical parameters including the description of each data point (n value), the number of mice or human samples per experiment, the number of replicates, the meaning of bars and the statistical tests used are contained in the figure legends. Statistics were calculated using R (www.R-project.org/). Other R packages or software used are given in the related section. For comparisons between two groups a two-tailed paired t test or two-sided paired Wilcoxon-test was used. For comparison between more than two groups statistical analysis was performed using a one-way ANOVA with the post hoc Tukey test. In the figures, asterisks stand for p-values: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

DATA AND SOFTWARE AVAILABILITY

The *IGHV* gene sequences have been deposited in the Genbank Sequence Read Archive under accession number SRP142713. RNA Sequencing data is available from the GEO database under accession no. GSEXXXX.

SUPPLEMENTAL INFORMATION

Supplemental Figure 1. Quantification of Human Neonatal and Adult Mature B Cells.

(A) Representative flow cytometry of CD38 and CD10 expression on total $CD19^+$ B cells from neonate (UCB) and adult PB.

(B) Gating strategy for neonatal and adult mature $CD5^+$ and $CD5^-$ B cell subsets.

(C) Proportions of mature $CD5^+$ and $CD5^-$ B cells among 43 randomly selected neonates (UCB).

(D) Age dependency of mature $CD5^-$ (naïve) B cells, $CD27^+$ memory and $CD27^+CD43^+$ B cells from 45 donors aged 0 – 90 years. Line of fit (bold line) and 95% confidence interval (shaded area) were calculated by R smooth.

Supplemental Figure 2. Transcriptome Wide Comparison of Neonatal and Adult B Cells.

(A) T-SNE plot showing the distribution of neonatal and adult mature $CD5^+$ and $CD5^-$ B cell and adult IgM memory B cell subsets, based on 87 significantly different expressed genes (MANOVA, $p < 0.001$, two-fold difference) between $CD5^+$ or $CD5^-$ mature B cells, regardless of age status (R-package Rtsne with standard settings).

(B) Detailed heatmap of the 87 genes shown in (A).

(C) Reactome pathway map ("Immune System") of enriched gene sets ($q < 0.15$) between neonatal and adult B cell subsets.

(D) Heatmap for selected genes involved in murine B-1 cell development, expressed among neonatal and adult mature $CD5^+$ and $CD5^-$ B cells, and adult IgM memory B cells. The colouring depicts row-wise normalized fold-changes.

(E) *BHLHE41* expression in human adult and neonatal B cell subsets as determined by PrimeFlow RNA assay.

(F) GSEA of published murine B-1 or B-2 cell specific gene sets among neonatal versus adult B cells ($CD5^+$ and $CD5^-$ pooled).

Supplemental Figure 3. Examples for Flow Cytometry of Neonatal and Adult Mature B Cells in Resting State and Under *in vitro* Stimulation.

This Figure shows representative flow cytometric data for selected surface molecules underlying the meta-FACS-analysis shown in Figure 3. All flow cytometric data shown in Figures 3 and S3 are based on unstimulated (d0) or stimulated (o/n) samples, that were not sort-purified. Due to the multiple conditions and limited cell numbers per donor, the data shown in each row are not always derived from the same donor. In some instances, contaminating T cells are detectable ($CD5^{high}$), which were excluded by gating strategy. Mean fluorescence intensity (MFI) values are shown in Figure 3, also when in some donors a biphasic distribution

was detectable among mature $CD5^+$ B cells ($CD5^{dim}$ and $CD5^{high}$) (see e.g. CD79b or IFNGR1). (A) Gating strategy, (B-M) marker panel.

Supplemental Figure 4. Functional Analysis of Neonatal and Adult Mature B Cells.

(A) Summary of five samples each of neonatal and adult mature $CD5^+$ and $CD5^-$ B cells, proliferating upon TD plus IL-4 and IL-21 stimulation.

(B) Delta-Ct values of PRDM1 transcription upon stimulation on d3, normalized to GAPDH. PRDM1 transcription was not detectable under TI-II stimulation.

(C) Ratio of membrane-IgM and secreted-IgM transcript levels upon stimulation on d3, as determined by semi-quantitative PCR.

(D) Summary of three to five independent class switching experiments of human neonatal ($CD5^+$ and $CD5^-$ B cells were not separated for the sake of sufficient cell numbers) and adult $CD5^+$ and $CD5^-$ B cells, stimulated with TGFb and anti-Ig (IgA switching) or stimulated with IL-4, IL-10 and TD conditions.

(E) Apoptosis (top) and cell death (bottom) of neonatal (left) and adult (right) B cells under TI-I stimulation on d4.

(F and G) Cell polarization (IgM capping) of neonatal (F) or adult (G) mature (naïve) B cells after 30 minutes of stimulation with CpG. DAPI (blue), CD5 (red), IgM (green).

Supplemental Figure 5. NGS Analysis of the Neonatal and Adult Mature B Cell Ig Repertoire.

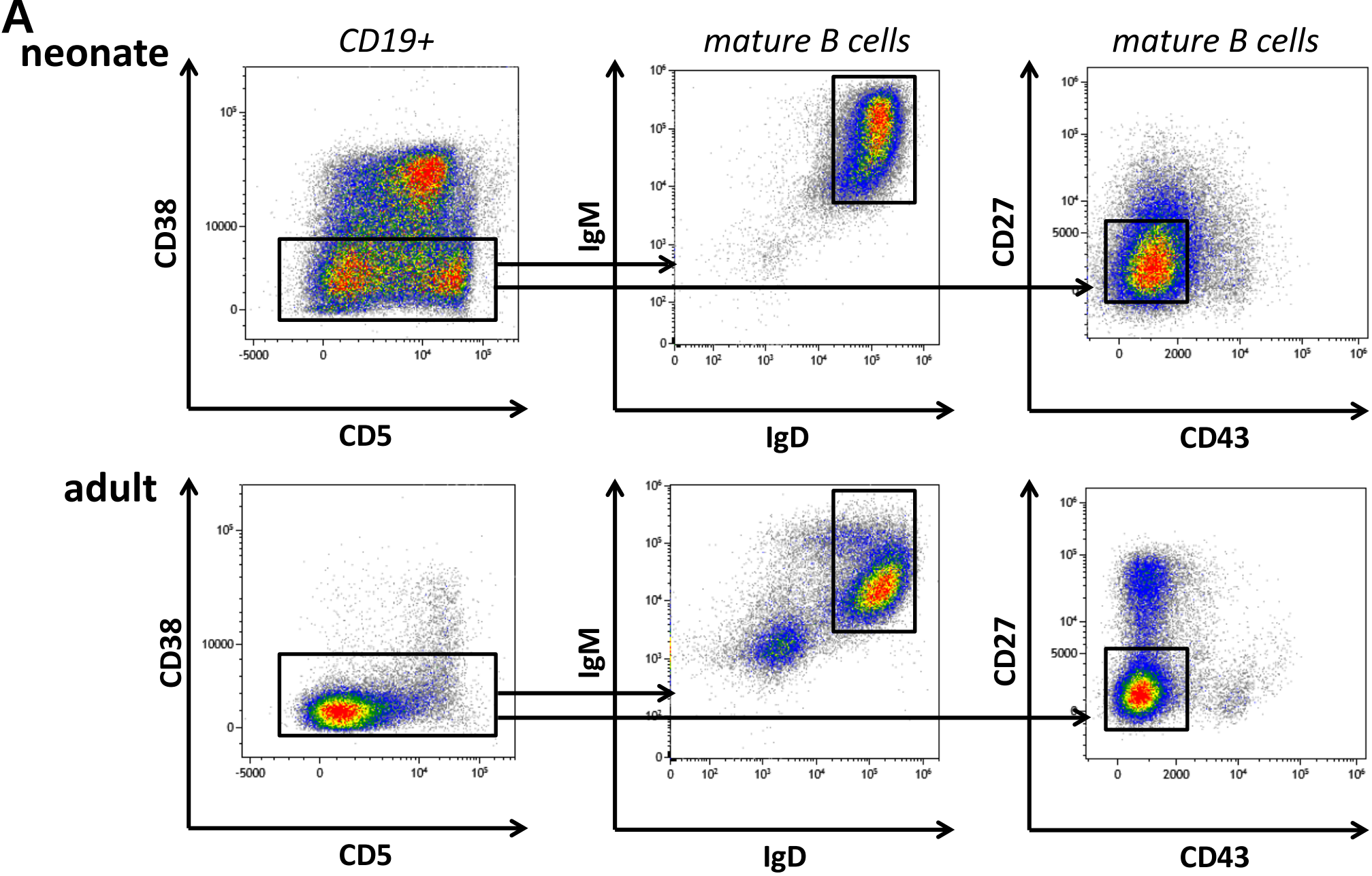
This treemap (https://CRAN.R-project.org/package=treemapify) depicts the total number (1 box per clone) and clone sizes (colour and relative box size) of clonally related sequences, detected in one representative neonate (A) or adult donor (B). The clones are subdivided into those consisting of $CD5^-$ B cell-derived sequences alone, $CD5^+$ B cell-derived sequences alone, or $CD5^+$ and $CD5^-$ B cells-derived sequences in combination.

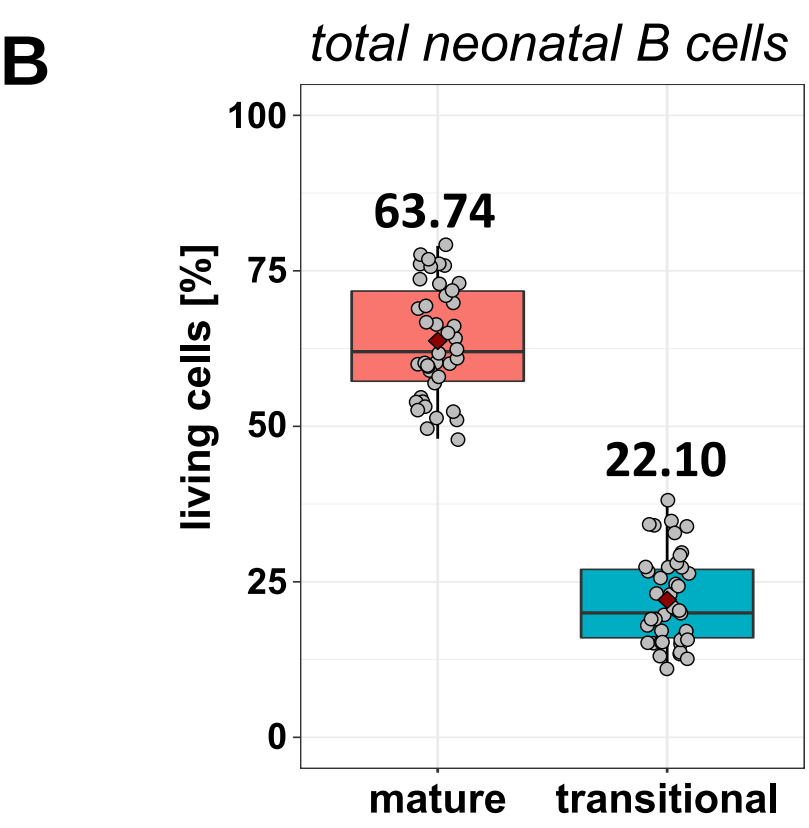
(C) Shown is the frequency of clonotypes among total sequences per subset, according to CDRIII amino acid identity, only fixed values were calculated (vertical lines).

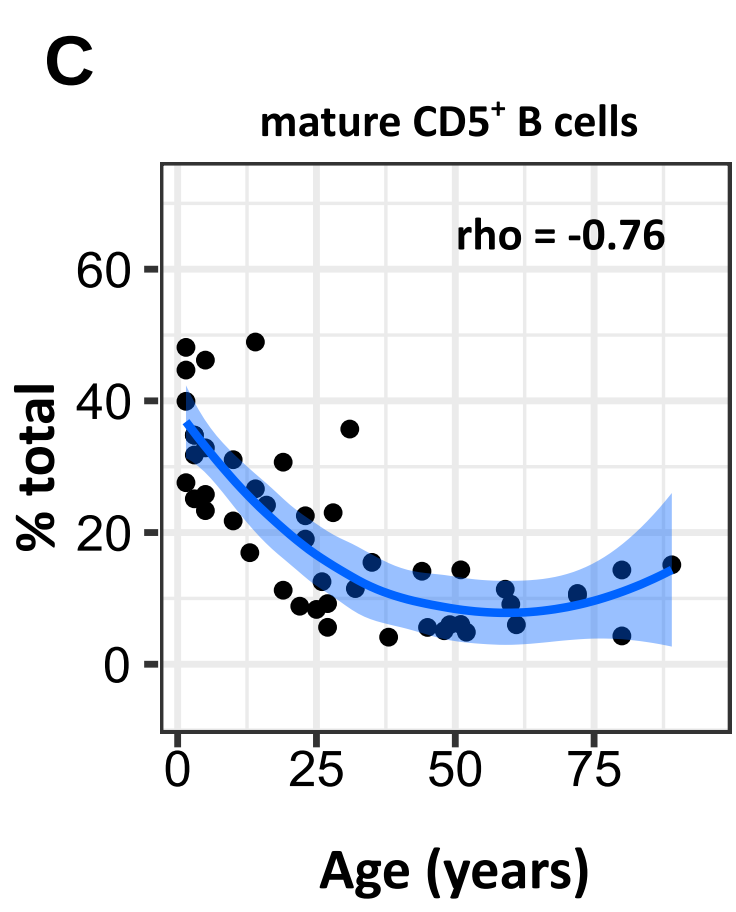
(D) Frequency of N-nucleotide insertion numbers at D_H-J_H-joints in neonatal and adult mature $CD5^+$ and $CD5^-$ B cells. Black bars indicate median values.

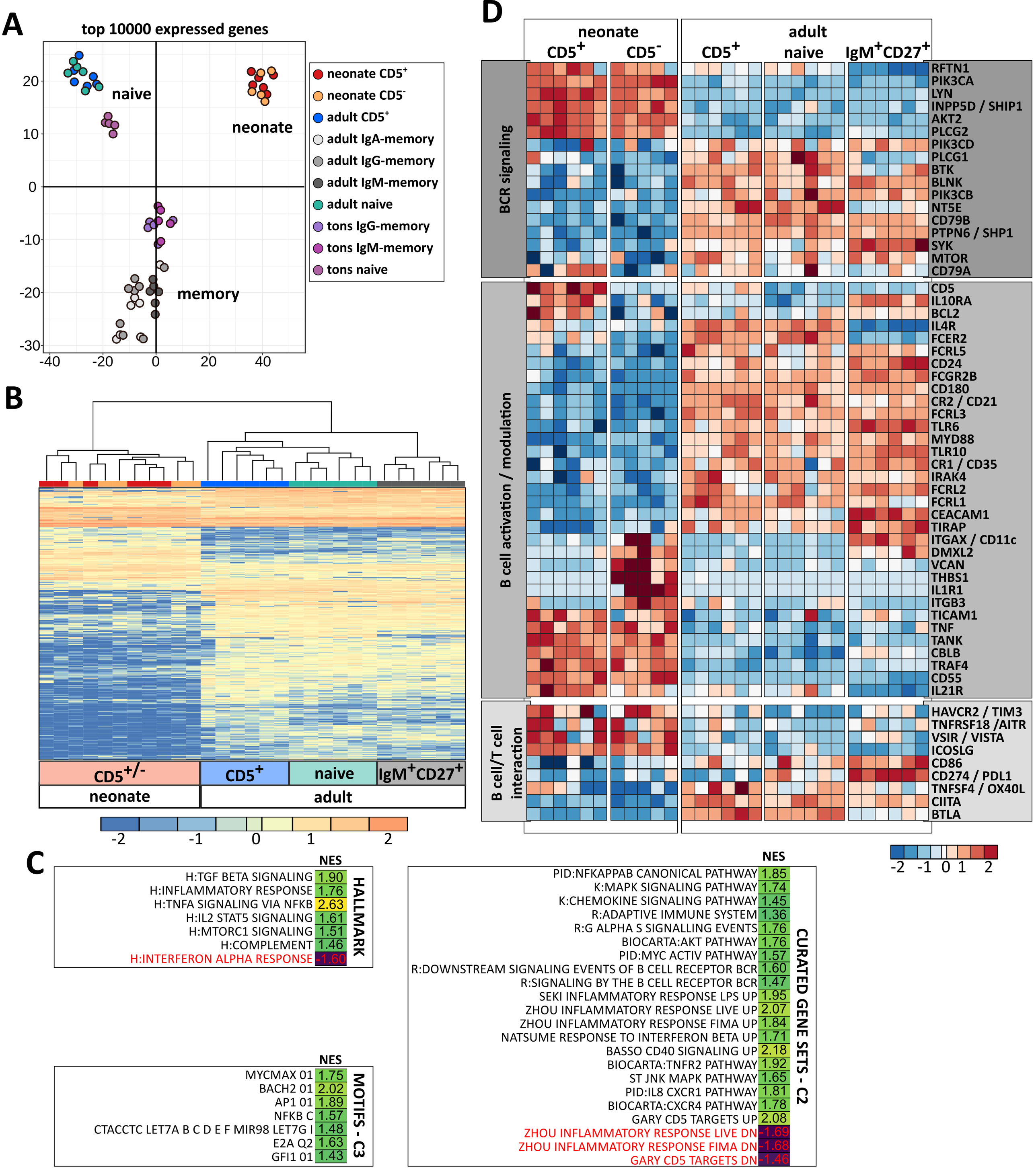
Supplemental Table 1. GSEA of Neonatal Versus Adult B Cell Transcriptomes.

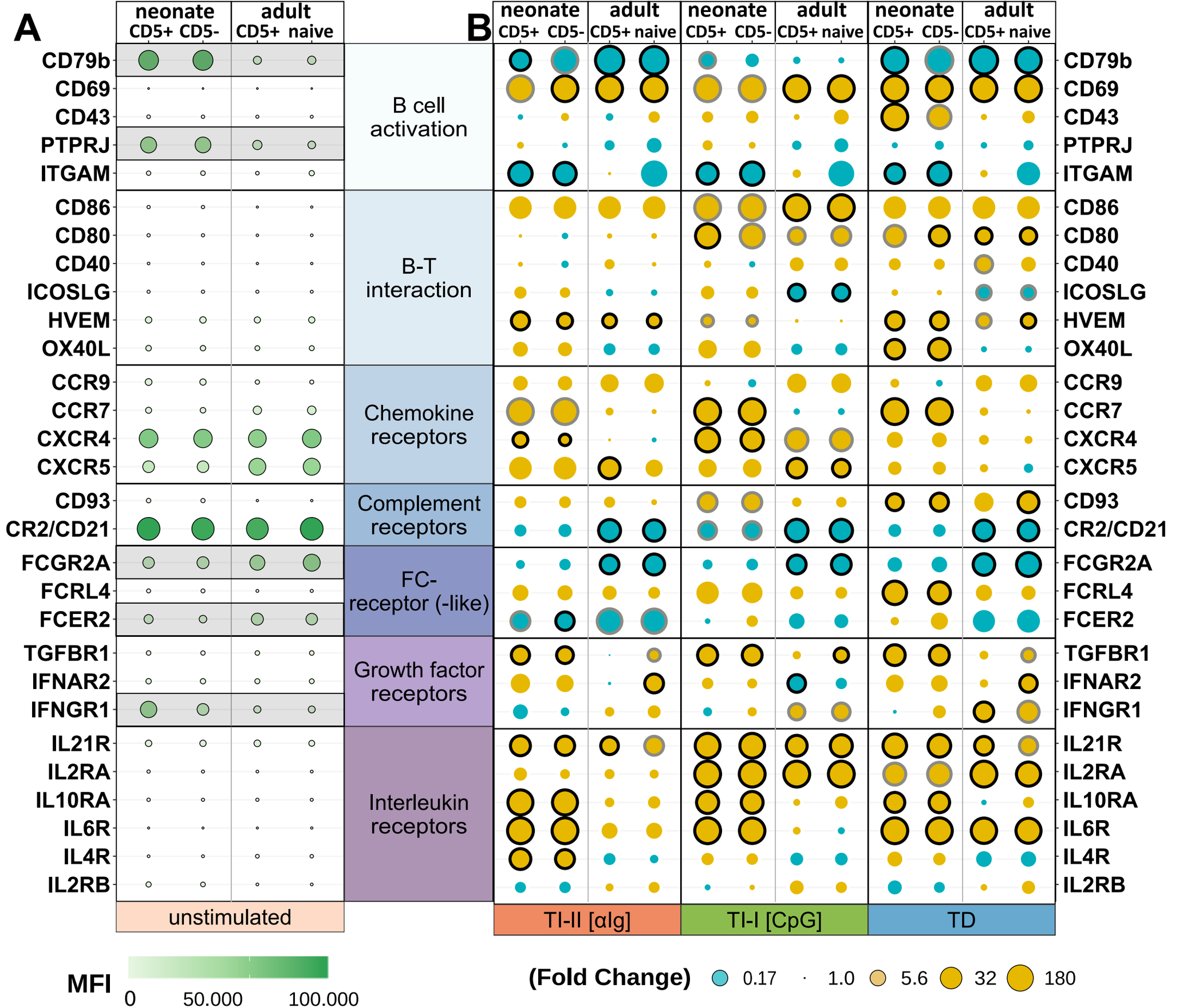
Gene set enrichment analysis of hallmark, C2, and C3 gene sets. Shown are gene sets enriched (positive NES) or depleted (negative NES) in neonatal versus adult B cells (R package fgsea, doi: 10.1101/060012).

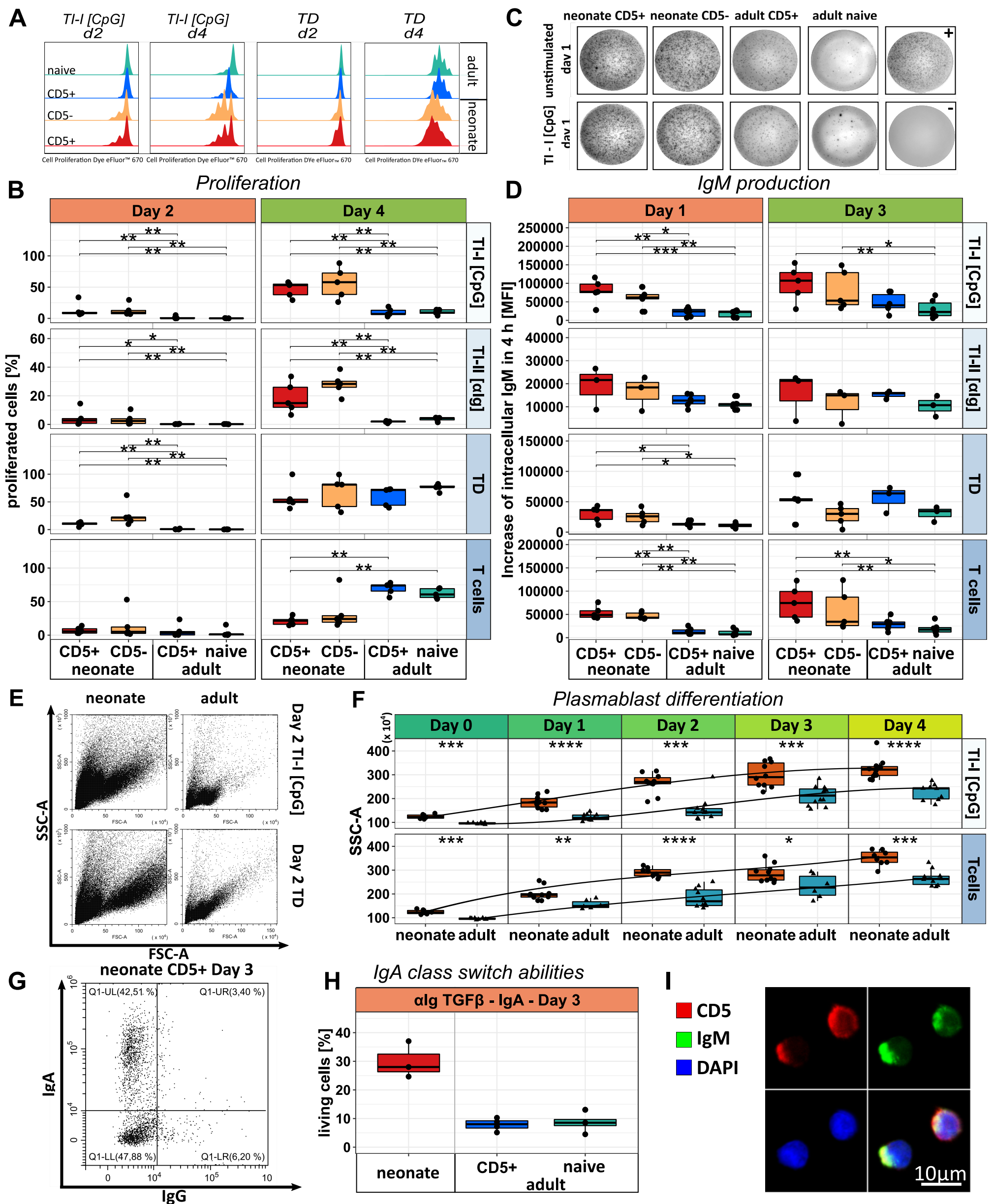

A
neonate
CD19+
mature B cells
mature B cells
CD38
CD5
IgM
IgD
CD27
CD43
adult
CD38
CD5
IgM
IgD
CD27
CD43


B
total neonatal B cells
living cells [%]
63.74
22.10
mature
transitional

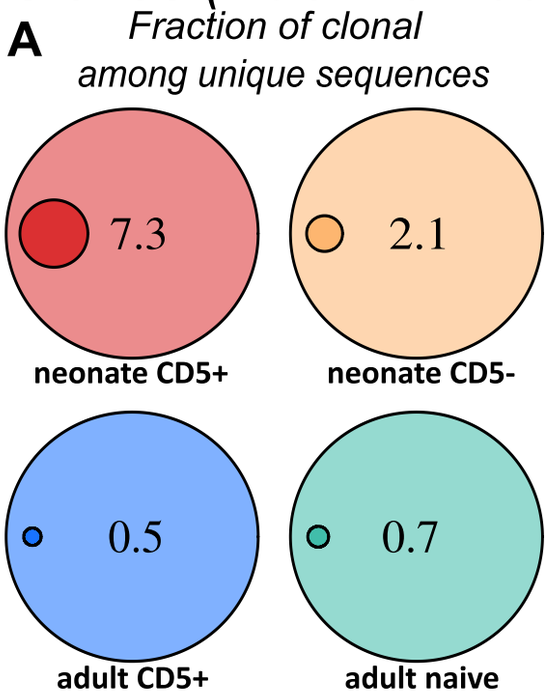

C
mature CD5+ B cells
rho = -0.76
% total
Age (years)

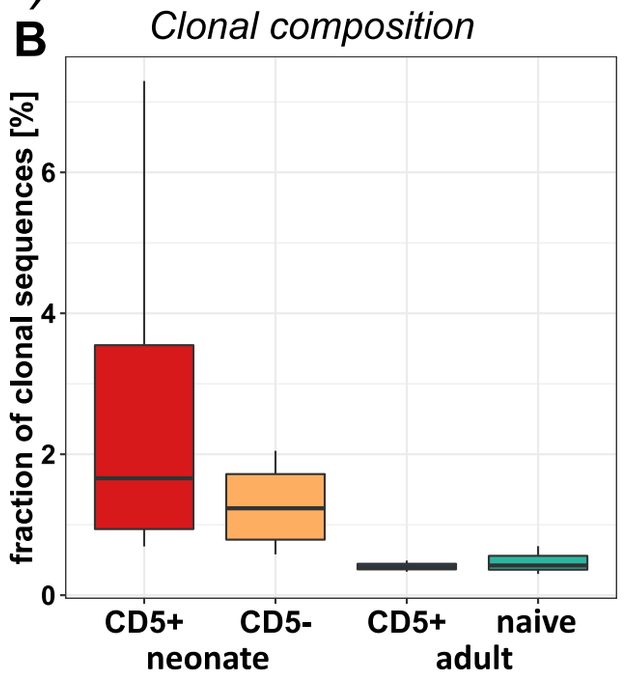

A
top 10000 expressed genes
naive
neonate
memory
neonate CD5+
neonate CD5-
adult CD5+
adult IgA-memory
adult IgG-memory
adult IgM-memory
adult naive
tons IgG-memory
tons IgM-memory
tons naive
B
CD5+/-
CD5+
naive
IgM+CD27+
neonate
adult
C
NES
H:TGF BETA SIGNALING 1.90
H:INFLAMMATORY RESPONSE 1.76
H:TNFA SIGNALING VIA NFKB 2.63
H:IL2 STAT5 SIGNALING 1.61
H:MTORC1 SIGNALING 1.51
H:COMPLEMENT 1.46
H:INTERFERON ALPHA RESPONSE -1.60
HALLMARK
NES
MYCMAX 01 1.75
BACH2 01 2.02
AP1 01 1.89
NFKB C 1.57
CTACCTC LET7A B C D E F MIR98 LET7G I 1.48
E2A Q2 1.63
GFI1 01 1.43
MOTIFS - C3
NES
PID:NFKAPPAB CANONICAL PATHWAY 1.85
K:MAPK SIGNALING PATHWAY 1.74
K:CHEMOKINE SIGNALING PATHWAY 1.45
R:ADAPTIVE IMMUNE SYSTEM 1.36
R:G ALPHA S SIGNALLING EVENTS 1.76
BIOCARTA:AKT PATHWAY 1.76
PID:MYC ACTIV PATHWAY 1.57
R:DOWNSTREAM SIGNALING EVENTS OF B CELL RECEPTOR BCR 1.60
R:SIGNALING BY THE B CELL RECEPTOR BCR 1.47
SEKI INFLAMMATORY RESPONSE LPS UP 1.95
ZHOU INFLAMMATORY RESPONSE LIVE UP 2.07
ZHOU INFLAMMATORY RESPONSE FIMA UP 1.84
NATSUME RESPONSE TO INTERFERON BETA UP 1.71
BASSO CD40 SIGNALING UP 2.18
BIOCARTA:TNFR2 PATHWAY 1.92
ST JNK MAPK PATHWAY 1.65
PID:IL8 CXCR1 PATHWAY 1.81
BIOCARTA:CXCR4 PATHWAY 1.78
GARY CD5 TARGETS UP 2.08
ZHOU INFLAMMATORY RESPONSE LIVE DN -1.69
ZHOU INFLAMMATORY RESPONSE FIMA DN -1.68
GARY CD5 TARGETS DN -1.46
CURATED GENE SETS - C2
D
neonate
CD5+
CD5-
adult
CD5+
naive
IgM+CD27+
BCR signaling
RFTN1
PIK3CA
LYN
INPP5D / SHIP1
AKT2
PLCG2
PIK3CD
PLCG1
BTK
BLNK
PIK3CB
NT5E
CD79B
PTPN6 / SHP1
SYK
MTOR
CD79A
B cell activation / modulation
CD5
IL10RA
BCL2
IL4R
FCER2
FCRL5
CD24
FCGR2B
CD180
CR2 / CD21
FCRL3
TLR6
MYD88
TLR10
CR1 / CD35
IRAK4
FCRL2
FCRL1
CEACAM1
TIRAP
ITGAX / CD11c
DMXL2
VCAN
THBS1
IL1R1
ITGB3
TICAM1
TNF
TANK
CBLB
TRAF4
CD55
IL21R
B cell/T cell interaction
HAVCR2 / TIM3
TNFRSF18 /AITR
VSIR / VISTA
ICOSLG
CD86
CD274 / PDL1
TNFSF4 / OX40L
CIITA
BTLA

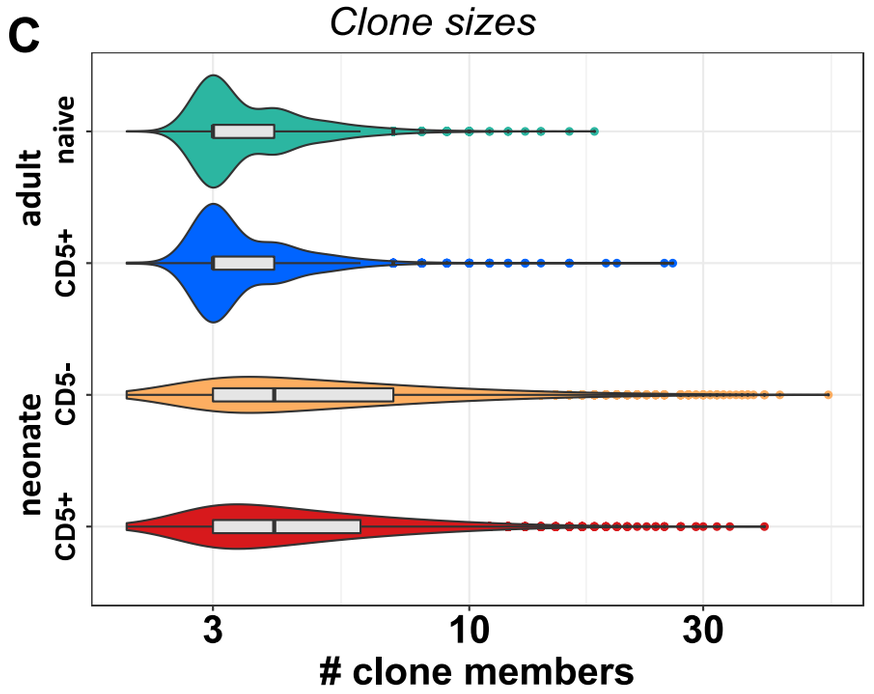
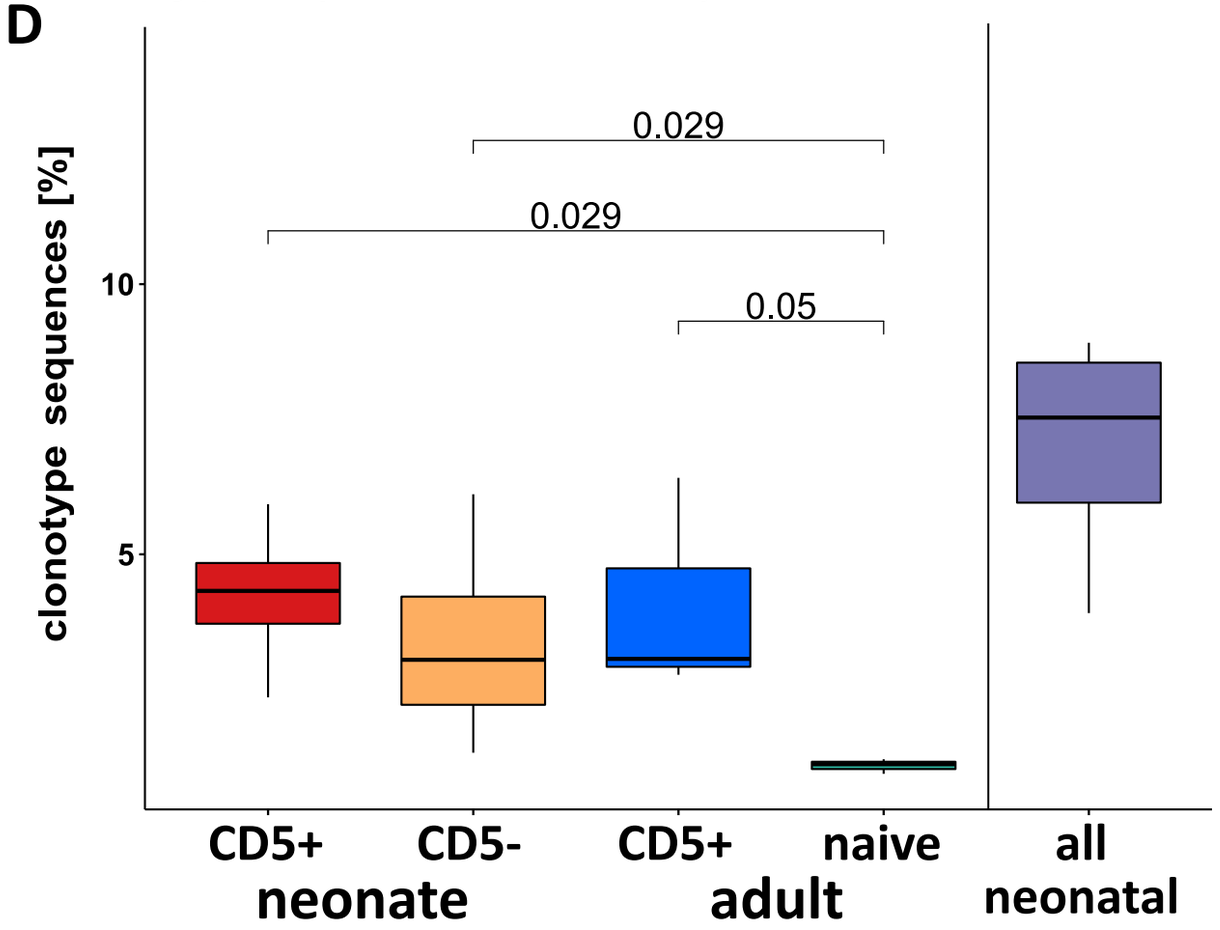
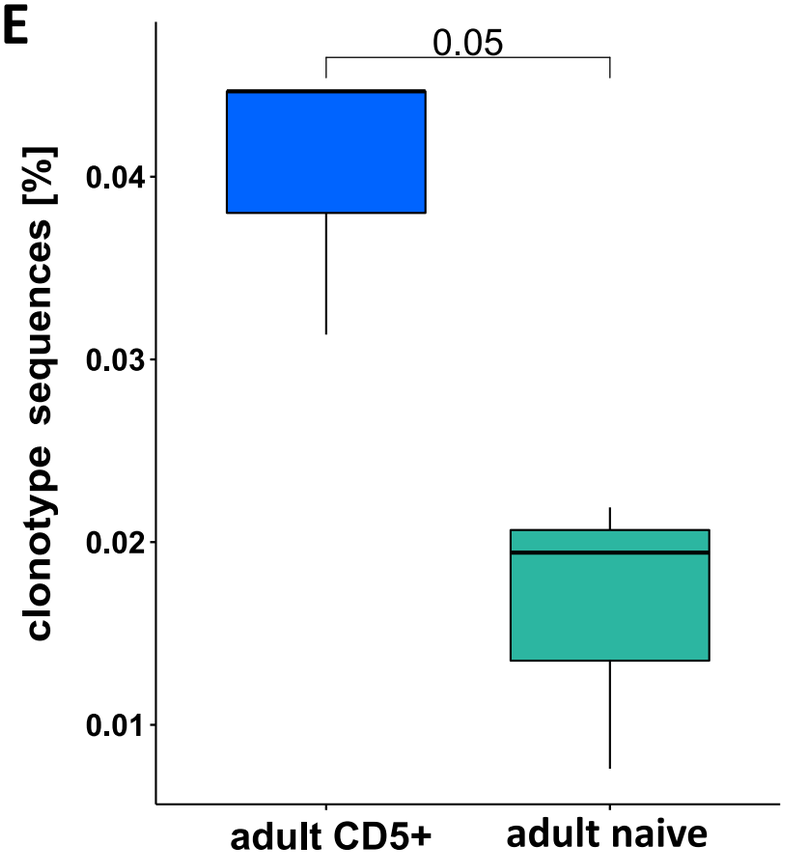

A
neonate
CD5+ CD5-
adult
CD5+ naive
B
neonate
CD5+ CD5-
adult
CD5+ naive
CD79b
CD69
CD43
PTPRJ
ITGAM
B cell activation
CD86
CD80
CD40
ICOSLG
HVEM
OX40L
B-T interaction
CCR9
CCR7
CXCR4
CXCR5
Chemokine receptors
CD93
CR2/CD21
Complement receptors
FCGR2A
FCRL4
FCER2
FC-receptor (-like)
TGFBR1
IFNAR2
IFNGR1
Growth factor receptors
IL21R
IL2RA
IL10RA
IL6R
IL4R
IL2RB
Interleukin receptors
unstimulated
TI-II [αIg]
TI-I [CpG]
TD
MFI
0
50.000
100.000
(Fold Change)
0.17
1.0
5.6
32
180


A
TI-I [CpG] d2
TI-I [CpG] d4
TD d2
TD d4
naive
CD5+
CD5-
CD5+
adult
neonate
Cell Proliferation Dye eFluor™ 670
C
neonate CD5+
neonate CD5-
adult CD5+
adult naive
unstimulated day 1
TI - I [CpG] day 1
B
Proliferation
Day 2
Day 4
TI-I [CpG]
TI-II [αIg]
TD
T cells
proliferated cells [%]
CD5+ CD5- neonate
CD5+ naive adult
D
IgM production
Day 1
Day 3
Increase of intracellular IgM in 4 h [MFI]
E
neonate
adult
Day 2 TI-I [CpG]
Day 2 TD
SSC-A
FSC-A
F
Plasmablast differentiation
Day 0
Day 1
Day 2
Day 3
Day 4
Tcells
neonate adult
G
neonate CD5+ Day 3
Q1-UL(42,51 %)
Q1-UR(3,40 %)
Q1-LL(47,88 %)
Q1-LR(6,20 %)
IgA
IgG
H
IgA class switch abilities
αIg TGFβ - IgA - Day 3
living cells [%]
neonate
CD5+
naive
adult
I
CD5
IgM
DAPI
10µm

Clones (intra-individual)







Clonotypes (inter-individual)