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Glutamine promotes the generation of B10⁺ cells *via* the mTOR/GSK3 pathway

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List of abbreviations : **α-KG:** α-ketoglutarate; **B10⁺ cells:** IL-10-producing B cells; **GSL1:** Glutaminase; **GSK3:** Glycogen synthase kinase 3; **IQR:** interquartile range 25-75; **IL:** Interleukine; **mTOR:** mammalian target of rapamycin; **pS6:** phospho-S6 Ribosomal Protein ; **TLR:** Toll-like receptor; **TNFα:** Tumor Necrosis Factor α; **Tregs:** regulatory T cells

Abstract

Alterations in cell metabolism can shift the differentiation of immune cells towards a regulatory or inflammatory phenotype, thus opening up new therapeutic opportunities for immune-related diseases. Indeed, growing knowledge on T cell metabolism has revealed differences in the metabolic programs of suppressive regulatory T cells (Tregs) as compared to inflammatory Th1 and Th17 cells. In addition to Tregs, IL-10-producing regulatory B cells are crucial for maintaining tolerance, inhibiting inflammation and autoimmunity. Yet, the metabolic networks regulating diverse B lymphocyte responses are not well known. Here, we show that glutaminase blockade decreased downstream mTOR activation and attenuated IL-10 secretion. Direct suppression of mTOR activity by rapamycin selectively impaired IL-10 production by B cells whereas secretion was restored upon GSK3 inhibition. Mechanistically, we found mTORC1 activation leads to GSK3 inhibition, identifying a key signalling pathway regulating IL-10 secretion by B lymphocytes. Thus, our results identify glutaminolysis and the mTOR/GSK3 signalling axis, as critical regulators of the generation of IL-10 producing B cells with regulatory functions.

Introduction

B cells contribute to adaptive immunity by secreting antibodies, presenting antigens to T cells, and secreting cytokines, thus enhancing the inflammatory process. However, B cells can also act as regulators, dampening inflammation by skewing T cell differentiation towards a regulatory T cell fate (Blair et al., 2010; Daien et al., 2014; Flores-Borja et al., 2013; Simon et al., 2015). B cell regulatory functions have largely been attributed to their ability to secrete the anti-inflammatory cytokine IL-10 (Das et al., 2012; Liu et al., 2006; Mielle et al., 2018; Stanic et al., 2015; van der Vlugt et al., 2014). Targeting regulatory B cell (Bregs) to restore the balance between tolerogenicity and immunogenicity, has gained interest in the fields of autoimmunity, transplantation and cancer (Alhabbab et al., 2019; Cerqueira et al., 2019; Mauri and Menon, 2017; Sarvaria et al., 2017). Despite considerable efforts to identify lineage-specific markers for Bregs and characterize the signalling pathways that drive their tolerogenic functions (Rosser and Mauri, 2015), no specific marker common to all Bregs has been discovered thus far. Instead, it has been suggested that any B cell, in a specific environment, can activate programs that allow IL-10 production, thereby becoming a B10⁺ cell with regulatory functions (Daien et al., 2014; Rosser and Mauri, 2015; Tedder, 2015).

Rapid and specific metabolic reprogramming is crucial in promoting the responses of immune cells to diverse signals, resulting in their activation, migration and differentiation. Lymphocytes must fine-tune their metabolic programs to meet specific and immediate needs and indeed, each T cell subset displays a distinct metabolic signature that can be manipulated to promote or constrain its development. While the expansion of pro-inflammatory Th1 and Th17 subsets is associated with an increase in glutaminolysis and glycolysis, regulatory T cells (Tregs) require fatty acid oxidation and their emergence is promoted in glutamine-deprived conditions (Johnson et al., 2018; Klysz et al., 2015; Macintyre et al., 2014; Michalek et al., 2011; Nakaya et al., 2014). Enhanced mTOR signalling, a key signalling pathway for cellular metabolic programs, is notably crucial for Th1 and Th17 development, whereas its inhibition promotes Treg development and functions (Delgoffe et al., 2009, 2011; Geltink et al., 2018). In comparison to T cells, data on B cell metabolic requirements are only beginning to emerge. Upon antigen encounter, B cells increase their consumption of extracellular glucose and glutamine to fuel both aerobic glycolysis and oxidative phosphorylation in a balanced manner (Caro-Maldonado et al., 2014; Doughty et al., 2006). To date, only a few studies have investigated how glucose and glutamine metabolism condition the fate and functions of activated B cells and much of this work has focused on plasma cells (Caro-Maldonado et al., 2014; Dufort et al., 2014; Daien et al., 2021). Indeed, both glycolysis and glutaminolysis were shown to be crucial for plasma cell differentiation and antibody production. In line with this, mTOR activation was crucial for plasmablast differentiation (Benhamron et al., 2015; Torigoe et al., 2017, 2019). However, how and which metabolic pathway drives Bregs, or more specifically IL-10-producing B cells (B10⁺ cells) differentiation remain elusive.

GSK3, a serine/threonine protein kinase can integrate signalling through the mTOR pathway, and has a central role in B cells in the control of cell growth, metabolic activity, and proliferation (Beurel et al., 2010; Jellusova and Rickert, 2017; Jellusova et al., 2017; Varano et al., 2017). GSK3 inhibition is known to trigger IL-10 production in many immune cells, such as monocytes and T cells

and has recently been shown to contribute to IL-10 secretion by LPS-activated memory B cells (Antoniv and Ivashkiv, 2011; Hill et al., 2015; Li et al., 2020; Martin et al., 2005; Ohtani et al., 2012; Wang et al., 2011; Weichhart et al., 2008).

Here, we investigated the role of glucose and glutamine metabolic pathways in the generation of B10⁺ cells. Our data reveal the importance of glutamine -and especially glutaminolysis- in promoting B10⁺ cells. Indeed, decreased glutaminolysis attenuated IL-10 secretion through an mTOR-dependent mechanism. We identify mTOR-dependent GSK3 inactivation as a key signalling event regulating IL-10 secretion by B lymphocytes.

Results

B10⁺ cells express higher levels of glutamine transporters as compared to glucose transporters than B10^{neg} cells.

Since the first step in cellular metabolism consists in entry of nutrients into cells by specific transporters, their abundance provides information on the metabolic pathways used by the cell. Thus, we first investigated whether IL-10-producing B cells (B10⁺ cells) had a specific nutrient transporter profile compared to IL-10 non-producing B cells (B10^{neg} cells). We used here the TLR9 agonist CpG to activate B cells as it is one of the most commonly used stimulus to induce IL-10 production in B cells (Bankó et al., 2017; Iwata et al., 2011), and chose to assess B10⁺ cell generation after 24 hours, as previously set up (Daien et al., 2014). It has been shown that B cell activation leads to an increased consumption of glutamine and glucose via their respective transporters (Caro-Maldonado et al., 2014; Jiang et al., 2018). We therefore measured by flow cytometry cell surface expression of ASCT2 (glutamine -neutral amino acid transporter) and GLUT1 (glucose transporter) in CpG activated B10⁺ and B10^{neg} cells. We found that cell-surface ASCT2 was more expressed on B10⁺ cells (delta MFI [IQR]; 22.84 [14.02, 35.63]) than on B10^{neg} cells (14.92 [10.98, 24.33]; n=12; p= 0.003) (Fig. 1A and S1A). Conversely, cell-surface GLUT1 expression was lower on B10⁺ cells (37.75 [20.40, 53.22]) than on B10^{neg} cells (52.91 [27.50, 74.64]; n=12; p= 0.0005) (Fig. 1B). This result could suggest that 24 hours post CpG activation B10⁺ cells preferentially uptake glutamine over glucose.

Extracellular availability of glutamine but not of glucose determines cytokine secretion by B cells

On the basis of these results, we then assessed whether the relative availability of glutamine and glucose in the extracellular medium influenced regulatory B10⁺ cell frequency. For this purpose, we cultured CpG- activated B cells in glucose or glutamine-deprived media and measured B10⁺ frequency after 24 hours. Importantly, neither glucose nor glutamine deprivation from the media did affect cell viability or cell count (Supporting information Fig. S2A). We observed a nearly three-fold decrease in B10⁺ cell frequency in the glutamine-deprived condition (Fold change [IQR]: 0.37 [0.24, 0.53], n=8) whereas glucose restriction had no significant impact (Fig. 1C, 1D Supporting information Fig. S2B). To determine whether glutamine deprivation specifically suppressed IL-10 secretion by B cells, we then measured in the culture supernatants the concentration of IL-10 and of two pro-inflammatory cytokines: IL-6 and TNF α . Glutamine deprivation, in contrast with glucose deprivation, was associated with reduced IL-10 but also IL-6 and TNF α secretion (Fold change to full media [IQR]: 0.22 [0.16, 0.35] for IL-10; 0.41 [0.31, 0.54] for IL-6; 0.23 [0.15, 0.38] for TNF α , n=13 to 14) (Fig. 1E and Supporting information Fig. S2C).

Glutaminolysis blockade preferentially prevents the secretion of interleukin 10 by inhibiting mTOR.

Given the importance of glutamine in regulating B cell cytokine secretion, we then explored the involvement of the different steps of its metabolism (Fig. 2A). As mitochondrial catabolism of glutamine starts with its conversion into glutamate by glutaminase (GLS), we first tested the impact of GLS1 blockade on B cell cytokine production. Chemical inhibition of GLS1 by BPTES led to a

significant decrease in B10⁺ cells (Fold change compared to control condition without inhibitor (FC) [IQR]: 0.72 [0.56, 0.80], n=12) (Fig. 2B). In addition, IL-10 secretion was significantly decreased by GLS1 blockade (FC: 0.77 [0.64, 0.80], n=15). This effect was specific for IL-10 as neither IL-6 nor TNF α were impacted by BPTES (FC: 1.02 [0.86, 1.11] and FC: 0.94 [0.87, 1.15], respectively for IL-6 and TNF α , n=15). In the next step of glutamine metabolism, glutamate enters the TCA in the form of α -ketoglutarate (α -KG). To address whether α -ketoglutarate production was directly involved in IL-10 production by B cell, BPTES-treated B cells were supplemented with a cell-permeable analogue of α -KG (DMK), a glutamine metabolite entering the TCA. DMK supplementation could increase B10⁺ cell frequency (Fold change compared the condition with BPTES: 1.5 [1.28, 2.25], n=6) as well as IL-10 secretion (FC: 1.51 [1.40, 1.79], n=11) (Fig. 2D, 2E and Supporting information Fig. S3A-S3B). These results strongly suggest the implication of glutaminolysis in B10⁺ cell generation.

Both glutamine and glutaminolysis activate mTORC1 (Durán et al., 2012; Nicklin et al., 2009), As expected, S6 phosphorylation, downstream of mTORC1, was reduced when glutaminolysis was altered by either glutamine deprivation or BPTES treatment (Fig. 2F). Importantly pS6 levels were rescued in conditions where DMK was added to the media with BPTES (FC: 0.67 [0.63, 0.74] vs FC: 0.96 [0.80, 1.04]; n=5) (Fig. 2F, right). To confirm a direct implication of mTOR in regulating IL-10 secretion, we then measured cytokine secretion by B cells treated with rapamycin, an mTOR inhibitor. mTOR inhibition, confirmed by a significant decrease in pS6 levels (Supporting information Fig. S3C), drastically impaired B10⁺ cell generation (FC: 0.25 [0.15, 0.29], n=13) (Fig. 2G). Moreover, while IL-6 and TNF α secretions were also decreased by rapamycin treatment (FC: 0.55 [0.48, 0.60] and FC: 0.67 [0.60, 0.79], n=16, IL-6 and TNF α respectively), IL-10 secretion (FC: 0.133 [0.09, 0.18]) was more severely impacted (p < 0.0001 compared to IL-6 and TNF α) (Fig. 2H).

These data suggest that glutamine catabolism preferentially induces IL-10 secretion in B cells *via* mTOR activation.

GSK3 inhibition following mTOR activation specifically triggers IL-10 secretion in B cells

Numerous studies have highlighted an antagonistic relationship between mTORC1 and GSK3 (Wang et al., 2011b). However, while the inhibition of mTORC1 by GSK3 *via* phosphorylation of TSC2 was first demonstrated, mTORC1 was also able to inactivate GSK3 through the inhibitory phosphorylation at serine^{9/21}, which decreases its catalytic activity. Conversely, mTORC1 inhibition by rapamycin lead to GSK3 activation (He et al., 2018; Zhang et al., 2006). This implies a bidirectional control of GSK3 and mTORC1 activities, depending on cell type and context. To determine whether GSK3 was a downstream target of mTORC1 in CpG-stimulated B cells, we quantified β -catenin accumulation, as it is a readout for the decrease in GSK3 global activity in the presence or absence of rapamycin (Doble and Woodgett, 2003; van Noort et al., 2002; Yost et al., 1996). First, we found that CpG stimulation led to GSK3 α phosphorylation at serine 21 and β -catenin accumulation in B cells (Supporting information Fig. S4 A-C). Despite the fact that no significant impact on GSK3 α phosphorylation could be detected (Supporting information Fig. S5A and S5B), mTOR inhibition by rapamycin significantly counteracted β -catenin accumulation (Fig. 3A). We also found that mTORC1 activity, measured by pS6 level was not increased upon GSK3 inhibition (Fig. 3B). This strongly

suggests that in CpG activated B cells, GSK3 is a downstream target of mTORC1 and that GSK3 inhibition do not activate mTORC1. Interestingly, glutamine deprivation also reduced β -catenin accumulation whereas BPTES treatment had no significant impact (Supporting information Fig. S5C and S5D). This differential impact on GSK3 inhibition may be explained by a reduced impact of BPTES treatment on mTORC1 activation as compared to glutamine deprivation (Supporting information Fig. S5E).

As GSK3 suppression promotes the secretion of IL-10 in several human immune cells including monocytes, macrophages, T cells and dendritic cells (Antoniv and Ivashkiv, 2011; Hill et al., 2015; Martin et al., 2005; Wang et al., 2011), we thought then to determine whether GSK3 was involved in mTOR-dependent regulation of IL-10 secretion in B cells. To address this hypothesis, we first evaluated the efficacy of the SB216763 and CHIR99021 GSK3 inhibitors and found that they induced a similar accumulation of β -catenin in B lymphocytes (Supporting information Fig. S6A). We therefore then assessed whether these two inhibitors could counteract the effect of rapamycin on B10⁺ cell frequency and cytokine secretion. We found that B10⁺ cell frequency and IL-10 secretion were restored when adding the GSK3 inhibitors SB216763 (FC: 1.42 [0.77, 1.99], n=11 for B10⁺ frequency and 1.07 [0.88, 1.2], n=15 for IL-10 secretion) or CHIR99021 (0.80 [0.63, 0.90], n=11 for B10⁺ frequency and 0.99 [0.74, 1.18], n=15 for IL-10 secretion) to the rapamycin condition (Fig. 3D and Supporting information Fig. S6B). Of note, IL-6 secretion was only partially restored by SB216763 treatment while TNF α secretion was even increased by the addition of CHIR99021 in the presence of rapamycin (Supporting information Fig. S6C). However, GSK3 inhibitors were not sufficient to fully compensate for the negative impact of glutamine deprivation on IL-10 secretion, probably because of more global effect of glutamine deprivation on B cells (Supporting information Fig. S6D and S6E). Overall, these data suggest that in B cells, mTORC1-induced inactivation of GSK3 promotes IL-10.

GSK3 inhibition drives B cell selective secretion of IL-10 and ability to induce Type 1 regulatory T cells (Tr1).

We then assessed the direct effect of GSK3 inhibitors on B10⁺ cells. Both B10⁺ cell frequency and IL-10 production were increased fold when B cells were stimulated by CpG in the presence of SB216763 (1.96 [1.20, 5.33], n=10 for B10⁺ and 1.38 [1.27, 1.48], n=17 for IL-10) or CHIR99021 (2.72 [1.97, 4.12], n=10 for B10⁺ and 1.68 [1.32, 2.03], n=12 for IL-10) (Fig. 4A and 4B). In contrast, IL-6 and TNF α secretions were not significantly modified (Fig. 4B and Supporting information Fig. S7A-D), showing that GSK3 inhibitors specifically increased IL-10 secretion in B cells.

As B cells do not spontaneously produce IL-10 *in vitro*, stimulating compounds such as the TLR9 activator CpG are commonly used for the study of B10 cells. However, we thought here to determine whether GSK3 suppression was sufficient to trigger IL-10 secretion even in the absence CpG. Interestingly, GSK3 inhibition could also drive a five-fold increase in B10⁺ cell frequency without CpG stimulation (5.88 [3.40, 12.57] for SB216763 and 4.95 [3.73, 13.93] for CHIR99021) (Fig. 4A and 4B and Supporting information Fig. S7A). Similarly, IL-10 secretion was increased by five-fold under conditions where GSK3 was inhibited (5.95 [4.37, 8.01] for SB216763 and 5.50 [4.68, 7.72] for CHIR99021, n=10-17) while IL-6 and TNF α secretions were similar to the control condition (Fig. 4A

and 4B and Supporting information Fig. S7B-D). In addition, treatment with CHIR99021 induced IL-10 secretion in rapamycin-treated B cells (Fig. 4C), in accordance with the fact that, GSK3 was a downstream target of mTOR. Altogether, GSK3 inhibitors can specifically increase IL-10 secretion in B cells, independently of mTOR activity.

We then wanted to test whether B cells whose IL-10 secretion was triggered by a GSK3 inhibitor had regulatory functions. We previously showed that B10 cells can increase the differentiation of CD4⁺CD25⁻ T cells into IL-10-producing type 1 regulatory T cells (Tr1). We therefore investigated the ability of B cells treated with GSK3 inhibitors to induce Tr1 (Mielle et al., 2018). To do so, SB216-treated, rapamycin-treated, or untreated B cells were co-cultured with autologous CD4⁺CD25⁻ cells T cells under CD3 activation (Fig. 4D). The frequency of IL-10-producing type 1 regulatory T cells (Tr1) generated in these different conditions was then assessed by flow cytometry. We found that inhibition of GSK3 with SB216 could increase the ability of B cells to generate Tr1 (2.04 [1.10, 3.15], n=8, p<0.05) while mTOR inhibition with rapamycin had opposite effects (FC: 0.62 [0.44, 0.87], n=8) (Fig. 4D, 4E and Supporting information Fig. S7E). Overall we showed that GSK3 can increase the generation of B10⁺ cells with regulatory functions, as they promote Tr1 differentiation.

Discussion

The aim of the present study was to determine whether a specific metabolic pathway was necessary for IL-10 secretion in B cells. Our data revealed that extracellular glutamine availability conditioned IL-10, but also IL-6 and TNF α secretion in CpG stimulated B cells. However, we found here that IL-10 secretion was preferentially decreased when mTOR signalling was attenuated following GLS1 blockade or rapamycin treatment, suggesting that IL-10 secretion required activation of mTOR. In addition, we identify GSK3, downstream of mTORC1, as a key metabolic regulator of IL-10 secretion in B cells. Indeed, we found that GSK3 inhibition selectively increased IL-10 production but not TNF α or IL-6 by B cells, and was able to rescue IL-10 production in condition where mTOR activity was abrogated by rapamycin treatment. Furthermore, GSK3 inhibition selectively increased IL-10 production even in the absence of TLR9 activation, leading to an increase in B cell ability to induce Tr1 in co-culture experiments. Altogether, we identified the mTOR/GSK3 pathway, which can be impacted by glutaminolysis, as a key inducer of functional B10⁺ cells.

We report here that B10⁺ cells expressed more ASCT2 than B10^{neg} cells, confirming at the protein level the microarray analysis performed on isolated human B10⁺ and B10^{neg} cells from Van de Veen *et al.* Indeed, they found that among only 0.7% of genes differentially expressed between the two B cell populations, ASCT2 gene expression was higher in B10⁺ cells compared to B10^{neg} cells (van de Veen *et al.* 2013).

It is interesting to note that GLS1 blockade and glutamine deprivation had different effects on cytokine secretion. Indeed, glutamine deprivation led to a decrease in IL-10, IL-6 and TNF α secretions whereas glutaminolysis blockade had a specific, but lower impact on IL-10 production. Before being converted to glutamate by GLS1, glutamine can be used in various metabolic pathways such as the co-transport of essential amino acids by glutamine, nucleotide synthesis, glutathione synthesis and protein N-glycosylation. Importantly, a recent study from Torigoe *et al.* showed that leucine entry through the transporter LAT1 (SCL7a5), in exchange for glutamine efflux, is increased in human B cells following CpG stimulation and is necessary for IL-10, IL-6 and TNF α secretion (Torigoe *et al.*, 2019). This may explain why glutamine deprivation, by negatively impacting leucine transport into B cells by LAT1, causes a greater inhibition of mTOR and has a greater effect on cytokine secretion than BPTES. We can thus speculate that in CpG-activated B cells, part of the glutamine pool is used for glutaminolysis which specifically leads to the secretion of IL-10 while the other part is used for other pathways controlling global cytokine secretion.

We found here that IL-10 secretion is specifically decreased by mTOR inhibition while it is increased by GSK3 inhibition. Such role of mTOR activation or GSK3 inhibition in regulating selectively IL-10 production over pro-inflammatory cytokines has been evidenced in numerous studies in monocytes, macrophages, DCs and T cells (Antoniv and Ivashkiv, 2011; Hill *et al.*, 2015; Martin *et al.*, 2005; Ohtani *et al.*, 2012; Wang *et al.*, 2011; Weichhart *et al.*, 2008). However data on B cells are sparse. It has been showed that *in vitro* treatment of primary human B cells with rapamycin led to a decrease in IL-10 secretion with significantly less impact on IL-6 or TNF α production, in accordance with our findings (Torigoe *et al.*, 2019; Ziegler *et al.*, 2014). Noteworthy,

this involvement of the mTOR pathway in regulating B-cell IL-10 secretion is supported by *in vivo* data, as in kidney transplant patients, rapamycin treatment decreased B10⁺ cells cell count compared to untreated healthy controls however the role of glutamine was not investigated in this study (Latorre et al., 2016). The data presented in this study extend our understanding of the pathways regulating IL-10 secretion and suggest that inhibition of GSK3 selectively controls the generation of this cytokine following B cell activation.

Although our results suggest that mTOR is upstream of GSK3 in CpG-treated B cells, the connection between mTOR and GSK3 could be indirect. Indeed, although we showed that GSK3 activity, as assessed by β -catenin accumulation, is affected by rapamycin treatment, we failed to show a clear change in GSK3 phosphorylation at Ser^{9/21}. Other mechanisms may also modulate GSK3 activity, including its expression level, phosphorylation of other residues (eg, GSK-3 α -pTyr-279, and GSK-3 β -pTyr-216 or others), its acetylation, subcellular localization, or suppression of interaction with its substrates. Thus, inhibition of GSK3 activity within the β -catenin destruction complex may be dependent on the phosphorylation status of GSK3 or on other mechanisms (Sarikhani et al., 2018; Thornton et al., 2008, 2016; Beurel et al., 2011).

The present study focused on glutamine and glucose metabolism but regulatory B cell metabolic signature might be way more complex. Indeed, a recent study from Bibby *et al* has showed that cholesterol metabolism could control IL-10 secretion by CpG-induced human B cells (Bibby et al., 2020). Interestingly they also found that GSK3 inhibition was involved in this pathway. Our findings are also supported by a recent study from Li *et al* showing that GSK3 blockade also increased IL10 secretion in human LPS- activated B cells (Li et al., 2020). These LPS activated B cells treated with SB216763 had a greater ability to decrease CD8⁺ T cell proliferation. Although B10⁺ metabolism is not yet fully elucidated, which may be partly attributable to their heterogeneous phenotype (Jansen et al., 2021), GSK3 appears to be a master regulator of IL-10 secretion in B cells, whose activity could be influenced by different metabolic pathways.

Importantly, our data suggest that B10 generation can be achieved by inhibiting GSK3, without using the TLR9 agonist CpG. This result would offer new alternative for the development of B10⁺ cell-based therapy. Indeed, the classical stimuli to trigger IL-10 secretion in B cells are bacterial products (LPS, CpG) which also induce the release of pro-inflammatory cytokines. Therefore, they are not suitable for *in vivo* nor *ex vivo* expansion of B10⁺ cells (Lighaam et al., 2018; Mauri and Menon, 2017; Mielle et al., 2018). Conversely, GSK3 inhibitors, which activate selectively IL-10 secretion in B cells, would be of great interest for the *ex vivo* expansion of regulatory B cells and their subsequent reinjection, in line with what is being done for Treg therapies (Marek-Trzonkowska et al., 2014; Mathew et al., 2018; Romano et al., 2019). Moreover, GSK3 inhibitors have also been shown to restrain T cell activation (Muralidharan et al., 2011), to potentiate Treg cell suppressive functions (Graham et al., 2010), to block Th17 differentiation (Beurel et al., 2011), and to decrease pro-inflammatory cytokine release by rheumatoid arthritis synoviocytes (Kwon et al., 2014). *In vivo* inhibition of GSK3 has therefore been beneficial in different pre-clinical model of inflammatory and auto-immune diseases such as solid organ transplantation, experimental autoimmune

encephalomyelitis or collagen-induced arthritis (Beurel et al., 2011; Graham et al., 2010; Kwon et al., 2014; Martin et al., 2005) and could thus also be considered for *in vivo* expansion of B10⁺ cells.

We have shown here that glutaminolysis modulations coupled with inhibition of the GSK3 pathway provide unique opportunities to generate B10 compatible with therapeutic use. Future work should aim to unravel the interplay between metabolic pathways, IL10 programs and regulatory functions in B lymphocytes.

Methods

Subjects

For nutrient transporter measurement, blood was obtained from either blood donors or patients seen in the department of Rheumatology (Teaching hospital, Montpellier) for mild osteoarthritis or mechanical pain with no general pathology or infection and receiving no immunomodulatory drugs. All subjects signed a written informed consent for the study in accordance with the 2013 Declaration of Helsinki and as approved by the Medical Ethics Committee of Nimes hospital, France (CPP_2014-A01585-42). Alternatively, blood was obtained from anonymous healthy donors from the Etablissement Français du Sang (French Blood Bank) under approval number 21PLER2019/0002.

Cell isolation and activation

PBMCs were isolated by Ficoll gradient density centrifugation or B cells were isolated using RosetteSep human B enrichment (StemCell, Grenoble France) followed by Ficoll separation. B cells were cultured in filtered complete media RPMI 1640 with glucose and stable glutamine (Sigma), with 10% fetal calf serum (FCS) and penicillin (100 UI/ml)/streptomycin (100 UI/ml)) on plate-bound CD40L (1 µg/mL, Peprotech). Cells were stimulated 24 hours on complete media with CpG (10 µg/mL, ODN 2006, InvivoGen, Toulouse, France). For nutrient deprivation experiment, B cells were cultured in media RPMI 1640 w/o glutamine, w/o glucose (PAN biotech) but supplemented with 2,5% foetal calf serum (FCS) to minimize glutamine and glucose supply, while ensuring cell viability. As compared to full media containing glucose (11 mM), and L-glutamine (2mM) conditions of deprivation resulted in final of glucose and glutamine concentrations of 87 µM and 27 µM respectively.

For inhibition experiments, B cells were pre-treated 30 min with BPTES (10 µM, Sigma Aldrich), rapamycin (10nM, Sigma Aldrich), SB216763 (10µM, Sigma Aldrich) or CHIR99021 (2µM, Tocris), in filtered complete media before CpG stimulation. For rescue experiment, DMK (3.5mM) was added to the culture media.

Flow cytometry stainings

Surface GLUT1 and ASCT2 expression were monitored by binding to their respective retroviral envelope ligands fused to eGFP and rFc (Metafora biosystems) at room temperature and for rFc-coupled ligand, binding was revealed with an Alexa Fluor 647-conjugated anti-mouse IgG (Invitrogen) (Manel et al., 2003, 2005; Oburoglu et al., 2014).

For intracellular IL-10 assessment by FACS, ionomycin (0.2 µg/mL, BioVision) and Phorbol 12-myristate 13-acetate (PMA) (0.1 µg/mL, BioVision, Nanterre, France) were added for the last 4 hours and brefeldin A (BFA)(10 µg/mL, Sigma Aldrich) for the last 2 hours of culture. B10⁺ cells were stained using BV450-conjugated anti-CD19 (Clone HIB19, BD-bioscience) or FITC-conjugated anti-CD19 (HIB19, BD-biosciences) before fixation (cytofix/cytoperm buffer BD biosciences) and permeabilization (Perm wash buffer, BD Pharmingen). Intracellular IL-10 staining was performed with IL10-PE (JES3-9D7, ebioscience, Thermofisher, USA) or APC-conjugated anti-IL-10 (JES3-19F1-

BD-biosciences) or Vio515-conjugated anti-IL-10 (JES3-9D7, Miltenyi Biotec). Dead cells were excluded using a fixable viability dye efluor506 discriminant (Thermofisher), used according to the manufacturer's instructions.

For phosphorylated- ribosomal protein S6 measurement (pS6), cells were stimulated with 24 hours CpG on CD40L-coated plate, without ionomycin/PMA/BFA addition. Cells were then fixed in 4% paraformaldehyde and permeabilized with transcription factor permeabilization buffer (Miltenyi). Staining was performed with an anti-pS6 antibody (clone 91B2, Cell Signalling) and revealed with a secondary Alexa Fluor 647-anti-rabbit immunoglobulin G (Life Technologies).

For β -catenin staining, cells were stimulated with 20 hours CpG on CD40L-coated plate, without ionomycin/PMA/BFA addition. B cells were stained using BV450-conjugated anti-CD19 (Clone HIB19, BD-bioscience) and fixable viability dye efluor506 discriminant (Thermofisher). Cells were then fixed in cytofix/cytoperm buffer (BD Pharmingen) and permeabilized with Perm wash buffer (BD Pharmingen). Staining was performed with an anti- β -catenin (clone 14/Beta-Catenin, BD Pharmingen).

Flow cytometry analyses were performed in adherence to the guidelines for the use of flow cytometry and cell sorting in immunological studies (Cossarizza et al., 2021). Most of flow cytometry data were acquired on BD FACSCanto II (BD Biosciences) or alternatively -for β -catenin staining- on NovoCyte ACEA (Agilent), and analyzed with FlowJo software (TreeStar).

Cytokine assessment in the supernatant

After 24 hours of culture, supernatants were collected to measure IL-10, IL-6 and TNF α secretion by ELISA (Human ELISA MAX Deluxe, Biolegend, San Diego, USA) according to the manufacturer's instructions. As the addition of ionomycin and PMA stimulation was shown to modify cell metabolism, notably by increasing glutamine catabolism and mTORC1 activation (Belouski et al., 2010; Wu, 1996), we chose to analyse cytokine profile without adding ionomycin nor PMA.

Functional assays

Isolated B cells were cultured on plate-bound CD40L, in filtered complete media with rapamycin (Sigma) or SB216763 (Tocris) or vehicle alone (DMSO). The day after, B cells were washed by RPMI 1640 10% SVF. Autologous naïve T cells (CD4⁺CD25^{neg}) were sorted from PBMCs by FACS Aria. Naïve T cells were stained with FITC-conjugated anti-CD4 (clone RPA-T4, BD Pharmingen), PE-conjugated anti-CD25 (clone BC96, ebioscience, Thermofisher, USA) antibodies. Dead cells were excluded using DAPI staining. B cells and naïve T cells were then co-cultured for 96 hours in filtered complete media (RPMI 1640 with 10% FCS) on anti-CD3-coated plates.

Tr1 intracellular staining was performed after stimulation with ionomycin, PMA (4hours) and BFA (2hours). Cells were then stained with APC conjugated anti-CD3 (clone UCHT1, BD bioscience), V450-conjugated anti-19 (clone HIB19, BD biosciences), vio515-conjugated anti-IL-10 (clone JES3-9D7, Miltenyi). Dead cells were excluded using a fixable viability dye efluor506 discriminant

(Thermofisher), used according to the manufacturer's instructions. Tr1 were defined as CD3⁺IL-10⁺ cells.

Data acquisition and Flow cytometry analysis

Data was acquired by a FACS CANTO II. Gating strategies for flow cytometry analysis were performed using FlowJo software V10 (Treestar, Ashland, OR). First, lymphocytes were gated based on forward scatter (FSC) and side scatter (SSC), followed by exclusion of doublets and dead cells. Gating strategies are shown in Supporting information file S1.

Western Blot analysis

Isolated B cells (7×10^5) were stimulated with CpG at the indicated times and the reaction was stopped on ice. Cells were washed with cold phosphate buffered saline (PBS) and lysed with lysis buffer (50 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 1% β -mercaptoethanol). Cell lysates were migrated onto a NuPAGE™ 4 to 12%, Bis-Tris, 1.5 mm gel, transferred to nitrocellulose membrane and incubated overnight with a phospho-GSK-3 α / β (Ser21/9) Antibody (Cell signaling technology #9331) or phospho-S6 Ribosomal Protein (Ser235/236) (91B2) Rabbit mAb (Cell signaling technology #4857) and incubated one hour with a secondary antibody. Blots were developed and scanned, and densitometric signals were determined by using NIH image 1.63. Data are expressed as arbitrary units after normalization to levels of β -actin.

Statistical analysis

Normality of the data sets was first assessed by Shapiro Wilk Normality test. Comparisons between paired culture conditions were performed using either t-test or one-way ANOVA followed by Tukey's post-hoc test when data were normally distributed or with their non-parametric equivalent -Wilcoxon matched pairs test or Friedman ANOVA with Dun's post-hoc test- data were not normally distributed. Significance was ascribed to p values < 0.05. We expressed data as median \pm interquartile range 25-75. All analyses were performed in Graph Pad Prism 5 (San Diego, California).

Author Contributions

JMi designed and performed the experiments; collected, analyzed, and interpreted the data; and wrote the manuscript. JM, BC obtained financial support for the study and approved the manuscript. JEH and JL performed the experiments. LM, NT, VD gave scientific advice throughout the study and critically read the manuscript. RA obtained financial support, designed and performed the experiments, interpreted the data, and wrote the manuscript. CD obtained financial support, designed the experiments, conducted patient enrolment into the study, interpreted the data, and wrote the manuscript.

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Conflict of Interests

The authors declare no competing interests. The authors received funding (DREAMER Grant) from Novartis France S.A., a commercial company, for this study. This funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Data availability

Data available on request from the authors

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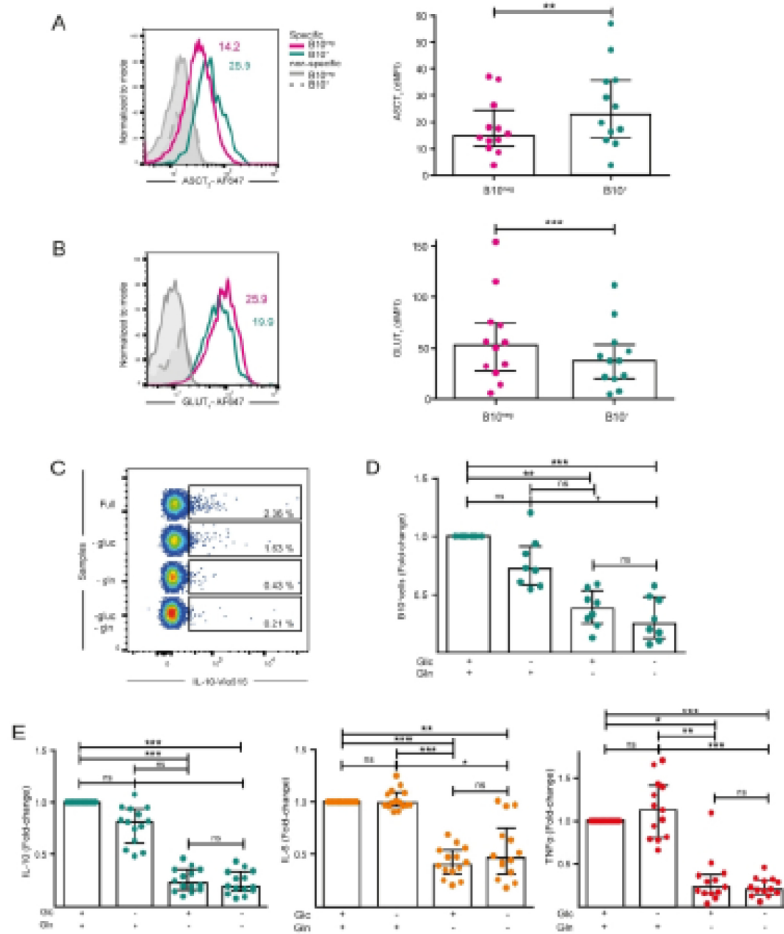


Fig. legends

Fig. 1: Human B cells require glutamine but not glucose for cytokine production.

(A) Expression of the ASCT2 transporter was evaluated 24h following CpG stimulation of freshly isolated human B lymphocytes. A first gating was performed on B cells to separate the B10⁺ cells (defined as CD19⁺IL-10⁺ cells) and B10^{neg} cells (CD19⁺IL10^{neg} cells) (see Fig S1), and then GLUT1 and ASCT2 staining were quantified among these two populations. Representative histograms (left panel) showing staining on B10⁺ (green) and B10^{neg} (pink) lymphocytes are presented together with non-specific staining controls (grey). Quantification of delta mean fluorescence intensity (dMFI) of ASCT2 staining in 12 healthy donors is presented (median+/- IQR) (right panel). (B) GLUT1 surface expression was similarly assessed 24h following CpG stimulation and representative histograms in B10⁺ and B10^{neg} cells are shown (left). Quantification of GLUT1 dMFI in 12 controls (median+/- IQR) is presented (right). Statistical difference was determined by a two-tailed paired t-test. **p < 0.01; ***p < 0.001. (C) B cells were activated by CpG in full media (+Glc+Gln), glucose-deprived (-Glc), glutamine-deprived (-Gln) or glucose/glutamine-deprived (-Glc/-Gln) for 24 hours. Representative dot plot of concatenate samples from one donor and the frequency of B10⁺ cells within the CD19⁺ B cell population are shown. (D) Relative change in B10⁺ cells as a function of the nutrient-replete media (set at 1). (E) IL-10, IL-6 and TNF α secretions were monitored in the indicated conditions at

24h post stimulation and the fold-change, relative to nutrient-replete conditions, are presented. Each point represents an individual donor (n= 13 to 14) and data was obtained in five independent experiments. Medians and IQR are presented. Statistical significance was determined by a one-way Friedman Anova with Dunn's post-hoc test. ns, non-significant; *p <0.05; **p<0.01; ***p<0.001

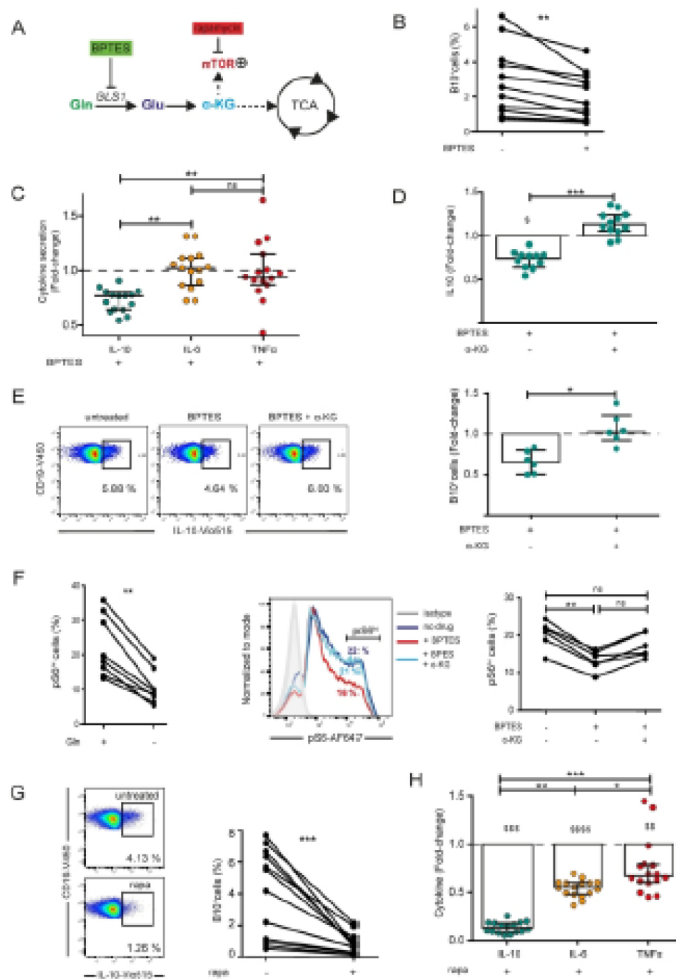


Fig. 2: IL-10 secretion by B cells is dependent on glutaminolysis and downstream of mTOR activation. (A) Schematic illustrating the different steps of glutaminolysis: glutamine (Gln); glutamate (Glu); glutaminase (GLS1); alpha-ketoglutarate (α KG). (B) B cells were activated by CpG in the absence or presence of BPTES (10uM) and B10⁺ cells were assessed by flow cytometry (n=12 donors in 4 independent experiments, two-tailed paired t-test) and (C) cytokine secretion was evaluated at 24h by ELISA (n=15 donors, in 5 independent experiments, one-way ANOVA with Tukey's post-hoc test). Results were expressed as fold change compared to the control condition (no BPTES) and differences analysed by one-way ANOVA with Tukey's post-hoc test. (D) B cells were activated in the presence or absence of BPTES and/or exogenous α KG (dimethyl- α KG, 3.5mM). IL-10 secretion was determined by ELISA and fold changes relative to control conditions (-BPTES/- α KG) are presented (n=12 donors, Friedman ANOVA, p<0.0001, with Dunn's post-hoc test). (E) Dot plots and graph showing the percentages of IL-10-secreting B cells treated with or without BPTES and α KG (n=6 donors, Friedman ANOVA with Dunn's post-hoc tests). (F) Phosphorylation of the S6 ribosomal protein, was monitored by intracellular staining in the indicated conditions, 24h post CpG-stimulation. Representative histograms are shown (middle) with isotype stainings (grey) untreated (dark blue), BPTES-treated (red), and BPTES supplemented with α KG (light blue) Quantification of pS6 fluorescence (dMFI) in the absence of Gln was monitored in 8 independent donors (left) as well

as in the presence or absence of BPTES and α KG (right) is presented (n=6). Statistical differences were monitored by Wilcoxon match-pairs test (left) and Friedman ANOVA, **p= 0.001, with Dunn's post-tests (right). (G) Frequency of B10⁺ cells after rapamycin treatment (10nM) (n=13 donors in 4 independent experiments, Wilcoxon matched-pairs tests). (H) Fold change in 10, IL-6 and TNF α secretion in these conditions (n=16 donors in 6 independent experiments). Wilcoxon or paired t test were used to compare impact of rapamycin to control condition with no drug with \$ p 0.05; \$\$ p<0.01; \$\$\$ p<0.001 and \$\$\$\$ p<0.0001, whereas statistical significance between all cytokines was evaluated by Friedman ANOVA with Dunn's post- hoc tests with * p<0.05, ** p<0.01, *** p<0.001

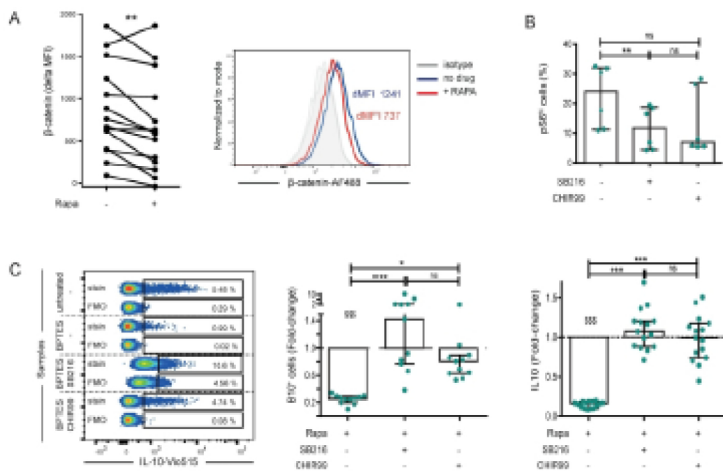


Fig. 3: GSK3 inhibition restores B cell IL-10 secretion under conditions of decreased mTOR activation

(A) β -catenin accumulation in CpG activated B cells was measured by intracellular staining and changes in MFI (β -catenin MFI minus Isotype control MFI) were evaluated after 24 hours of rapamycin treatment ($n=14$, paired t-test). **(B)** S6 phosphorylation levels measured by Flow cytometry in CpG activated B cells in the presence or absence of GSK3 inhibitors. **(C)** The frequency of B10⁺ cells was evaluated in rapamycin-treated B cells in the absence or presence of SB216763 and CHIR99021 ($n=9$ donors in 3 independent experiments, left panel). Due to its yellow color, SB216 induces autofluorescence, therefore FMO staining (all antibodies except IL-10) are also shown to illustrate specific staining. Representative dot plot of concatenate samples from one donor is shown. Statistical significance was determined by a Friedman ANOVA with Dunn's post-hoc tests. IL-10 secretion by these B cells was evaluated by ELISA and data from 15 donors (5 independent experiments) are presented (right panel). Statistical difference was determined by one-way ANOVA plus Tukey's post-hoc test. Wilcoxon or paired t test were used to compare impact of rapamycin to control condition with no drug with \$\$\$ $p < 0.001$

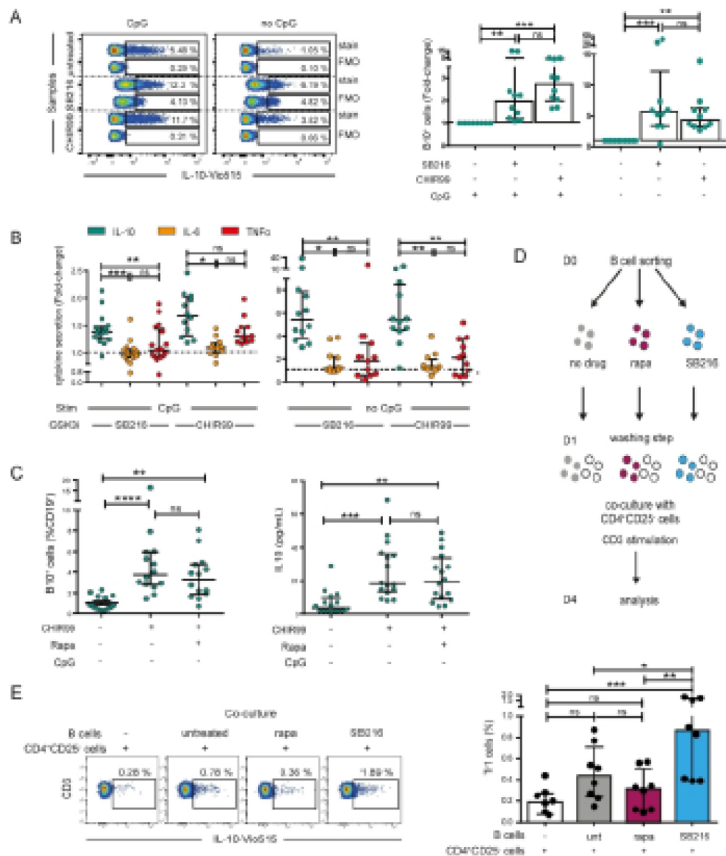


Fig. 4: GSK3 inhibition induces B10 cells with regulatory features.

(A) B cells were cultivated either with or without CpG in the presence or absence of SB216763 and CHIR99021. The frequency of B10⁺ cells was evaluated and both dot plot of concatenate samples from one donor (left) and fold-changes for all donors (right) are presented (n=10). Statistical significance was determined by a Friedman ANOVA with Dunn's post-hoc test. **(B)** B cells were activated as in panel A and secretion of IL-10, IL-6, and TNF α was monitored by ELISA. The fold-change in secretion relative to control conditions is presented (n=12 to 17 donors in 3 to 5 independent experiments). Statistical significance was determined by Friedman ANOVA with Dunn's post-hoc test. *: statistics between the different cytokine secretions with * p<0.05, ** p<0.01, *** p<0.001 **(C)** The frequency of B10⁺ cells and IL-10 secretion was evaluated after rapamycin and CHIR99021 treatment in the absence of CpG (n=14). One-way ANOVA and Tukey's post-hoc tests. **(D)** B cells were cultivated without CpG for 24h in the absence or presence of rapamycin or SB216763, washed and then co-cultured in a 1:1 ratio with autologous sorted naïve CD4⁺CD25^{neg} T cells for 96h in the absence of any exogenous cytokines. **(E)** The frequency of IL-10-producing T cells (Tr1) was evaluated by flow cytometry and representative dot plots (left) and graph (right) are shown. (n=6-8 donors in 3 independent experiments). One-way ANOVA and Tukey's post-hoc tests, with * p<0.05, ** p<0.01, *** p<0.001.