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CUTTING EDGE

Cutting Edge: IL-4-Mediated Protection of Primary B Lymphocytes from Apoptosis via Stat6-Dependent Regulation of Glycolytic Metabolism[1]

Fay J. Dufort, Blair F. Bleiman,* Maria R. Gumina,* Derek Blair,* Dean J. Wagner,§ Mary F. Roberts,† Yousef Abu-Amer,‡ and Thomas C. Chiles[2]**

IL-4 prevents the death of naive B lymphocytes through the up-regulation of antiapoptotic proteins such as Bcl-x_L. Despite studies implicating glucose utilization in growth factor-dependent survival of hemopoietic cells, the role of glucose energy metabolism in maintaining B cell viability by IL-4 is unknown. We show that IL-4 triggers glucose uptake, Glut1 expression, and glycolysis in splenic B cells; this is accompanied by increased cellular ATP. Glycolysis inhibition results in apoptosis, even in the presence of IL-4. IL-4-induced glycolysis occurs normally in B cells deficient in insulin receptor substrate-2 or the p85α subunit of PI3K and is not affected by pretreatment with PI3K or MAPK pathway inhibitors. Stat6-deficient B cells exhibit impaired IL-4-induced glycolysis. Cell-permeable, constitutively active Stat6 is effective in restoring IL-4-induced glycolysis in Stat6-deficient B cells. Therefore, besides controlling antiapoptotic proteins, IL-4 mediates B cell survival by regulating glucose energy metabolism via a Stat6-dependent pathway. The Journal of Immunology,* 2007, 179: 4953–4957.

Naive lymphocytes require extrinsic signals to maintain viability and promote growth. Many prosurvival factors prevent death through the up-regulation of antiapoptotic Bcl-2 family proteins (1, 2). These proteins function, in part, to maintain mitochondrial membrane integrity (2, 3). The role of the mitochondria in apoptosis and glucose metabolism suggests that these two cellular processes may be coupled (4–6). Indeed, a growing body of evidence indicates that impaired glycolysis may limit the oxidizable substrate availability needed to maintain mitochondrial membrane potential, thereby triggering apoptosis (2, 3, 5).

IL-4 is a pleiotropic cytokine that acts on naive B cells to prevent cell death (7–9). Binding of IL-4 to its receptor (IL-4R) results in the activation of several signal transduction cascades, including the JAK1 and JAK3 tyrosine kinases (7, 10). JAK1 and JAK3 phosphorylate tyrosine residues in the cytoplasmic domain of the IL-4Rα that serve as docking sites for Stat6 (7, 11). Stat6-deficient B cells undergo apoptosis in the presence of IL-4, which has been linked to impaired Bcl-x_L induction (9).

The IL-4R α-chain recruits insulin receptor substrate-2 (IRS-2),[3] permitting association with the p85α regulatory subunit of PI3K (7, 12). IL-4 enhances survival through recruitment of IRS-2 and PI3K activation; B cells deficient in IRS-2 or p85α undergo apoptosis despite the presence of IL-4 (9, 12–13). Although the cellular targets of IRS-2/PI3K remain to be identified in B cells, studies in hemopoietic cells suggest that PI3K signaling modulates glucose metabolism (3, 14, 15). We demonstrate herein that IL-4 increases glucose transport and glycolysis in B cells. Blocking of IL-4-induced glycolytic flux leads to decreased B cell survival. IRS-2 and PI3K activity are dispensable for IL-4-induced glycolysis, but glycolysis is dependent upon Stat6 in B cells. This is the first report demonstrating an essential role for glucose energy metabolism in the prosurvival activity of IL-4 and implicating Stat6 signaling in the regulation of glucose utilization.

Materials and Methods

Reagents

Anti-Stat6 (Tyr^{641}), anti-phospho-ATF2 (Thr^{71}), and anti-phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) Abs were from Cell Signaling Technology. The anti-Glut1 Ab was from Research Diagnostics. PE-conjugated $F(ab')_2$ of goat anti-rabbit IgG was obtained from Caltag Laboratories. $F(ab')_2$ of goat anti-mouse IgM (anti-Ig) were obtained from Jackson ImmunoResearch Laboratories. Murine IL-4 was from R&D Systems. LPS was from Sigma-Aldrich. All other reagents were from Calbiochem-Novabiochem International. Cellular ATP was determined using the ATP determination kit from Molecular Probes.

B cell isolation

BALB/cByJ and p85α-deficient mice (BALB/cAnNTac-Pik3r1 N12, backcrossed nine times) were obtained from Taconic Farms. Stat6-deficient mice

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[3] Abbreviations used in this paper: IRS-2, insulin receptor substrate-2; 1D-HMQC, one-dimensional heteronuclear multiple quantum correlation; 2DG, 2-deoxy-D-glucose; WT, wild type.

(BALB/cByJ, backcrossed 6 times) were from The Jackson Laboratory (9). IRS-2-deficient mice (B6;129-*Irs2*^{tm1Mfw}/J, backcrossed 13 times) were provided by Dr. M. F. White (Children's Hospital, Harvard Medical School, Boston, MA) (16). Splenic B cells of mice at 8- 12 wk were purified by negative selection using the MACS system (Miltenyi Biotec), and small dense B cells were further isolated following centrifugation through a discontinuous Percoll gradient as described (15).

Flow cytometry

Apoptosis measurements were conducted by TUNEL according to the manufacturer's instructions (BD Biosciences Pharmingen). For Glut1 staining, B cells were washed twice in staining buffer (1 ml of PBS containing 1% FCS and 0.1% NaN_3) and then incubated for 20 min (4°C) with the anti-CD16/CD32 (clone 2.4G2) mAb Fc block reagent (1:500 v/v). Cells were washed twice in staining buffer and incubated for 60 min with 1/500 dilution of anti-Glut1 Ab or isotype control Ab (4°C) followed by incubation with PE-conjugated F(ab′)$_2$ of goat anti-rabbit IgG (1/800) as described (15). Cells were then analyzed on a BD FACSCanto flow cytometer (BD Biosciences).

Glucose utilization measurements

Glucose uptake was measured by monitoring the uptake of ^{3}H-labeled 2-deoxy-D-glucose (2-DG) (Amersham Biosciences) as previously described (15). Glycolysis was measured following the incubation of B cells (10^6 cells per 0.5 ml) with [5-^{3}H]glucose (Amersham Biosciences) for 90 min. B cells (100 μl) were removed and placed in 1.5-ml microcentrifuge tubes containing 100 μl of 0.2 N HCl. The production of ^{3}HOH resulting from enolase-mediated dehydration of [5-^{3}H]glucose was quantitated by evaporation diffusion (25°C) as previously described (4, 15).

One-dimensional heteronuclear multiple quantum coherence (1D-HMQC)

B cells (10^7) were cultured in RPMI 1640 containing 10 mM [1-^{13}C]glucose or [2-^{13}C]glucose (Cambridge Isotope Laboratories). At the indicated times, B cells were extracted with 70% (v/v) ethanol, the extract was subsequently frozen in liquid nitrogen and lyophilized. The dry material was resuspended in 0.5 ml of D_2O and the 1D-HMQC spectra (detecting only those protons coupled to ^{13}C) were acquired using an INOVA 500 spectrometer (Varian) as described (15).

TAT-Stat6 construct

Stat6 constructs were cloned into the pTAT-HA bacterial expression vector as described by Hirayama et al. (17). To generate an active form of Stat6 in which amino acids at Val^{547}/Thr^{548} were substituted by alanine residues, the following primers were used: Stat6-VT_F (5′-GGCTTTATTAGTAAGCAATAT-GCCGCTAGCCTTCTCCT CAATGAGC-3′; forward) and Stat6-VT_R (5′-GCTCATTGAGGAGAAGGCTAGCGGCATAT TGCTTACTAATA-AAGCC-3′; reverse). TAT-Stat6VT and TAT-Stat6 wild type (TAT-Stat6WT) fusion proteins were produced in BL21 *Escherichia coli* and purified as previously described (17).

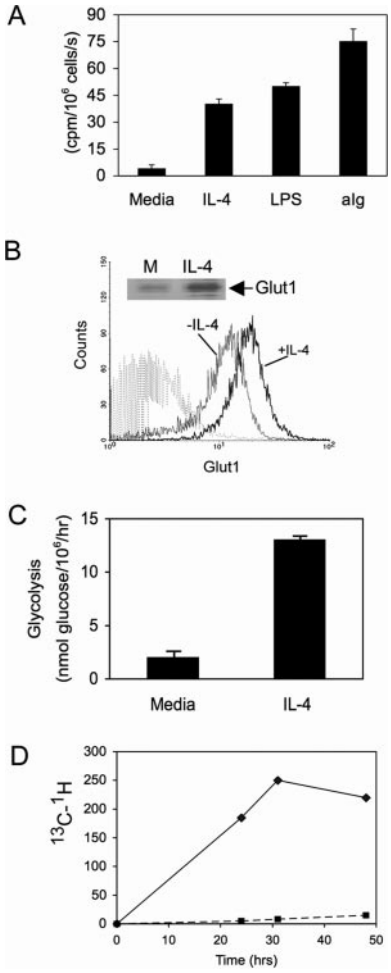

FIGURE 1. IL-4 increases glucose utilization in B lymphocytes. *A*, B cells were cultured in the absence (Media) or presence of 4 ng/ml IL-4, 10 μg/ml anti-Ig (aIg), or 25 μg/ml LPS for 15 h. Uptake of ^{3}H-labeled 2DG was measured for 60 s as previously described (15). *B*, B cells were cultured in medium alone (−IL-4) or with 4 ng/ml IL-4 (+IL-4) for 15 h. Glut1 staining was determined by flow cytometry as described in *Materials and Methods*. Cells were also stained with an isotype control Ab (gray dashed line). The *inset* shows a Western blot of Glut1 in extracts prepared from B cells cultured in the absence (M) or presence of IL-4 (15 h). *C*, B cells were cultured in the absence (Media) or presence of 4 ng/ml IL-4 (15 h); glycolysis was measured as described in *Materials and Methods*. Error bars in *A* and *C* reflect SD from the mean of triplicate measurements and the data are representative of three independent experiments. *D*, B cells were cultured in medium alone (0 h) or 4 ng/ml IL-4 containing 10 mM [1-^{13}C]glucose (♦) or [2-^{13}C]glucose (■). At the indicated times (0, 24, 31, and 48 h), cells were collected and analyzed by 1D-HMQC as described in *Materials and Methods*. The data are depicted as the integrated intensity of protons coupled to ^{13}C selected in the 1D-HMQC experiment. The data are representative of two independent experiments.

Results and Discussion

IL-4 modulates glucose metabolism in B cells

We initially determined whether IL-4 modulates the acquisition of extracellular glucose in B cells. IL-4 treatment of small dense B cells resulted in an approximate 7-fold increase in glucose uptake (Fig. 1*A*). By comparison, glucose uptake in anti-Ig- or LPS-stimulated B cells was increased ~12- and 8-fold, respectively. The increase in glucose uptake by IL-4 was accompanied by increased Glut1 expression (Fig. 1*B*). We also evaluated the metabolic fate of glucose to determine whether IL-4 modulates glucose catabolism. To monitor glycolysis, the production of ^{3}HOH resulting from the dehydration of [5-^{3}H]glucose catalyzed by enolase was measured (3, 15). B cells exhibited a measurable baseline rate of glycolysis, which was increased ~5-fold by IL-4 (Fig. 1*C*). Nuclear magnetic resonance spectroscopy was also used to monitor ^{13}C fixation from [1-^{13}C]glucose into glycolytic metabolites (15). A 1D-HMQC sequence to select only those ^{1}H coupled to ^{13}C nuclei revealed enhanced glycolysis during an initial 31-h period of IL-4 stimulation as evidenced by the increased ^{13}C incorporation into the methyl group of lactate from [1-^{13}C]glucose (Fig. 1*D*). If [2-^{13}C]glucose is used instead of [1-^{13}C]glucose, there will be no ^{13}C incorporation into the lactate methyl group by the glycolytic pathway; however, if the pentose phosphate pathway is operational ^{13}C will be incorporated into the methyl group of lactate (15). B cells cultured in [2-^{13}C]glucose revealed no substantial glucose flux through the pentose phosphate pathway following IL-4 treatment (Fig. 1*D*). The increase in glycolysis was accompanied by a 2-fold increase in cellular ATP concentration in B cells treated with IL-4 (ATP levels in naive B cells corresponded to 8.6 nM; ATP levels in IL-4-stimulated B cells

for 18 and 22 h corresponded to 12.0 nM, and 17.7 nM, respectively). Taken together, these results indicate that glucose uptake and metabolism via the glycolytic pathway is up-regulated by IL-4.

Glycolysis contributes to IL-4-mediated survival of B cells

To determine whether increased glycolytic flux by IL-4 contributed to the rescue of naive B cells from apoptosis by IL-4, we used the glycolysis inhibitor 2-DG, which can be phosphorylated by hexokinase but not further metabolized by glycolytic enzymes (18). 2-DG was added to B cells at the start of culture with IL-4, and at various times B cells were harvested and viability was determined by propidium iodide exclusion. B cells cultured in the absence of IL-4 exhibited decreased viability over time, whereas the addition of IL-4 was sufficient to provide substantial protection from death (Fig. 2*A*). By contrast, 2-DG reduced the percentage of viable B cells in cultures containing IL-4 (Fig. 2*A*). The decreased cell viability was representative of apoptosis, as demonstrated in parallel 2-DG-treated, IL-4-stimulated B cells by a TUNEL assay (Fig. 2*B*).

IL-4-induced glucose utilization does not require IRS-2 or PI3K activity

To evaluate the contribution of PI3K activity to IL-4-induced glycolysis, B cells were cultured with the PI3K inhibitor wortmannin, and the effect on glycolysis following the addition of IL-4 was measured. Wortmannin did not significantly decrease IL-4-induced glycolysis in comparison to IL-4-stimulated B cells cultured without wortmannin (Fig. 3*A*). The efficacy of wortmannin as an inhibitor of endogenous PI3K activity was

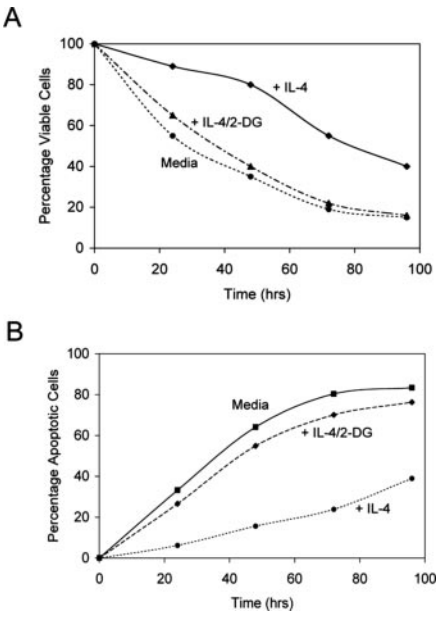

FIGURE 2. IL-4-induced glycolysis is necessary for the prevention of apoptosis in B cells. *A*, B cells were cultured in the absence (Media) or presence of 4 ng/ml IL-4 alone (+IL-4) or IL-4 plus 5 mM 2-DG (+IL-4/2-DG). At the indicated times, cells were collected and viability was determined by propidium iodide staining/flow cytometry. *B*, B cells were cultured in the absence (Media) or presence of 4 ng/ml IL-4 alone (+IL-4) or IL-4 plus 5 mM 2-DG (+IL-4/2-DG). B cells were monitored for apoptosis by TUNEL assay/flow cytometry as described in *Materials and Methods*. Note that 2-DG did not significantly affect viability or apoptosis in B cells cultured in medium alone. The results are representative of three independent experiments.

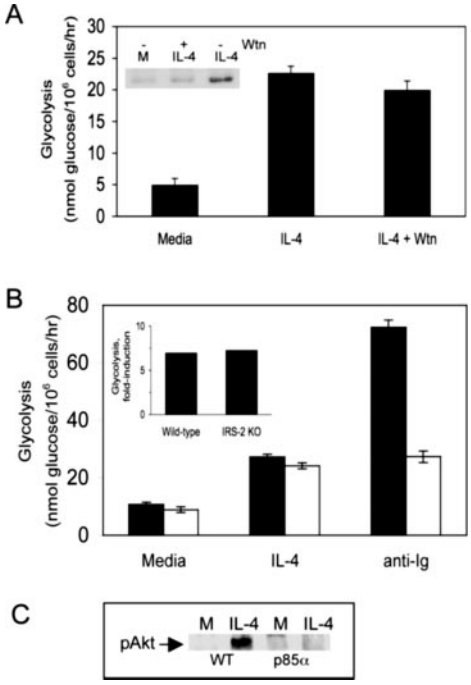

FIGURE 3. IL-4-mediated induction of glycolysis does not require IRS-2 and occurs independently of PI3K and MAPK activation. *A*, B cells were cultured in the absence (Media) or presence of 4 ng/ml IL-4 containing a DMSO vehicle for 12 h; parallel IL-4-stimulated B cells were cultured with 20 nM wortmannin (Wtn). The *inset* shows a Western blot of pAkt (Ser^{473}) in B cells cultured in the absence (M) or presence of 4 ng/ml IL-4 for 30 min; Wtn corresponds to B cells pretreated with 20 nM wortmannin for 30 min. *B*, B cells from WT (closed bars) and p85α-deficient (open bars) mice were cultured in the absence (Media) or presence of 4 ng/ml IL-4 or 10 μg/ml anti-Ig for 12 h. The *inset* shows parallel B cells from WT and IRS-2-deficient mice with the data expressed as the fold-induction (i.e., glycolytic rate of cells treated with IL-4/glycolytic rate of cells cultured in medium alone). *C*, Western blot of pAkt (Ser^{473}) in WT and p85α-deficient B cells cultured in the absence (M) or presence of 4 ng/ml IL-4 for 30 min. In *A* and *B* glycolysis was measured as described in *Materials and Methods*. Error bars reflect SD from the mean of triplicate measurements, and the data are representative of three independent experiments.

confirmed in that IL-4-stimulated Akt Ser^{473} phosphorylation was blocked (Fig. 3*A*, *inset*). The requirement for PI3K activity in IL-4-induced glycolysis was further evaluated in B cells deficient in the p85α-regulatory subunit of PI3K (19). Increased glycolytic flux was not significantly impaired in IL-4-treated, p85α-deficient B cells in comparison to WT cells (Fig. 3*B*). As a control, enhanced glycolysis following anti-Ig stimulation was reduced in p85α-deficient B cells (Fig. 3*B*), an event shown previously to be dependent on the p85α subunit (15). Of note, p85α-deficient B cells exhibited impaired IL-4-induced Akt phosphorylation (Fig. 3*C*). We also observed no measurable difference in IL-4-induced glycolysis between WT and IRS-2-deficient B cells (Fig. 3*B*, *inset*). Collectively, these results suggest that IRS-2/PI3K signaling is dispensable for IL-4-induced glycolysis in B cells.

A recent report implicated p38 MAPK in IL-4 signaling of B cells (20). To investigate whether p38 MAPK activity was required for IL-4-induced glycolysis, B cells were pretreated with SB203580, an inhibitor of p38 MAPK, before stimulation with IL-4. We found that SB203580 had no significant effect on the increase in glycolysis by IL-4 (Fig. 4*A*). The possibility that ERK signaling may contribute to IL-4-induced glycolysis was

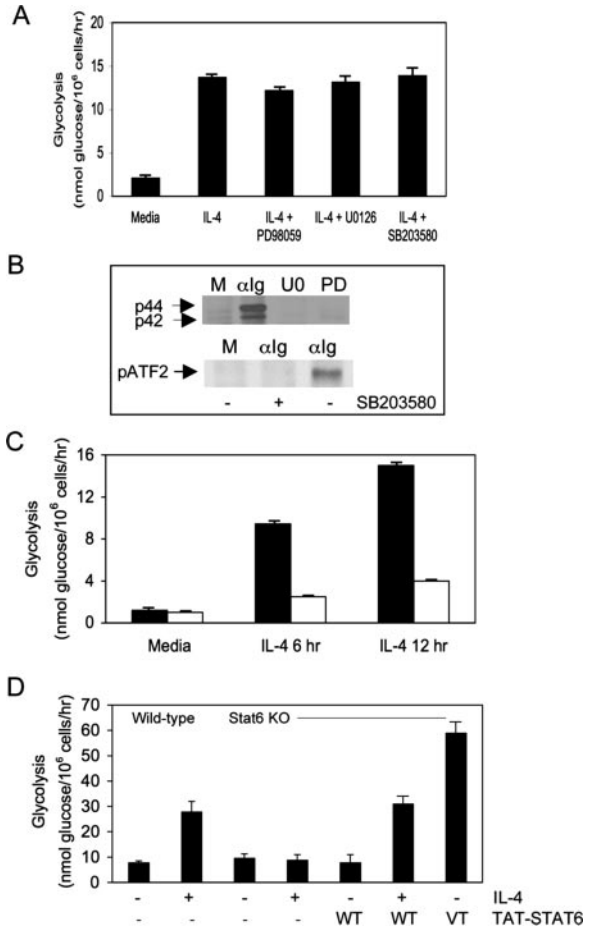

FIGURE 4. IL-4-induced glycolytic flux requires Stat6. *A*, B cells were pretreated with DMSO vehicle alone (*lanes* marked Media and IL-4) or with one of the following: 20 μM PD98059, 10 μM U0126, or 10 μM SB203580 for 30 min. Cells were then cultured with 4 ng/ml IL-4 for 12 h. *B*, Western blot of p44/42 phospho-MAPK (*upper panel*) in B cells cultured in the absence (M) or presence of 10 μg/ml anti-Ig (αIg) for 30 min; PD and U0 correspond to anti-Ig-stimulated B cells pretreated (30 min) with 20 μM PD98059 and 10 μM U012620, respectively. In the *lower panel* is a Western blot of pATF2 in B cells cultured in the absence (−) or presence (+) of 10 μM SB203580 (30 min) followed by culture in the absence (M) or presence of 10 μg/ml anti-Ig (αIg) for 20 min. *C*, B cells from WT (closed bars) and Stat6-deficient (opened bars) mice were cultured in the absence (Media) or presence of 4 ng/ml IL-4 for 6 and 12 h. *D*, WT B cells were cultured in the absence (−) or presence of 4 ng/ml IL-4 (+) for 48 h. Stat6-deficient (Stat6 KO) B cells were cultured in the absence (−) or presence of 4 ng/ml IL-4 (+) or cultured with cell-permeable forms (200 ng/ml) of WT TAT-Stat6 (WT −), WT TAT-Stat6 plus IL-4 (WT +), or constitutively active TAT-Stat6VT (VT) for 48 h. In *A*, *C*, and *D* glycolysis was measured as described in the *Materials and Methods*. Error bars reflect SD from the mean of triplicate measurements and the data are representative of three independent experiments.

also investigated in parallel B cells pretreated with U0126 or PD98059, inhibitors of ERK-kinase I (MEK-1). Inhibition of MEK-1 had little effect on IL-4-induced glycolysis (Fig. 4*A*); inhibition of the JNK pathway by pretreatment with SP600125 did not affect IL-4-induced glycolysis (data not shown). The efficacy of U0126 and PD98059 as inhibitors of MEK-1 was confirmed in that anti-Ig-stimulated p44/42 MAPK phosphorylation on Thr^{202}/Tyr^{204} was blocked, whereas SB203580 blocked anti-Ig-induced ATF2 phosphorylation on Thr^{71} (Fig. 4*B*).

Increased glycolysis by IL-4 requires signaling through Stat6

To determine whether IL-4 induces glucose utilization in a Stat6-dependent manner, glycolytic flux from WT and Stat6-deficient splenic B cells was evaluated. IL-4-induced glycolysis was significantly reduced in Stat6-deficient B cells in comparison to WT B cells (Fig. 4*C*). We note that 12 h following IL-4 stimulation there remained an approximate 2-fold increase in glycolysis (in comparison to control Stat6-deficient B cells), suggesting the presence of a Stat6-independent pathway. Currently, the identity of this pathway is unknown. IL-4-induced Akt phosphorylation on Ser^{473} was intact in Stat6-deficient B cells (data not shown). Notwithstanding, these findings suggest that Stat6 is indispensable for IL-4-induced glycolytic catabolism in B cells. To test this further, we determined whether the reconstitution of Stat6-deficient B cells with WT Stat6 was sufficient to rescue IL-4-induced glycolysis. A TAT-Stat6WT protein was generated that contains a short peptide sequence from the HIV TAT protein (17). The TAT sequence confers the ability to rapidly and efficiently (>90%) transduce proteins into mammalian cells (21). IL-4 was capable of inducing glycolysis in ex vivo Stat6-deficient splenic B cells reconstituted with TAT-Stat6WT (Fig. 4*D*). We also evaluated the effects on glycolysis with a constitutively active form of Stat6 (TAT-Stat6VT), which has been shown to bind DNA and to activate transcription in the absence of IL-4 stimulation (17). The addition of TAT-Stat6VT to Stat6-deficient B cells was sufficient to increase glycolytic flux in the absence of IL-4 stimulation (Fig. 4*D*). Interestingly, this level of glycolysis surpassed that of IL-4-stimulated Stat6-deficient B cells reconstituted with TAT-Stat6WT.

The findings reported herein represent the first demonstration that IL-4 signaling regulates glucose energy metabolism in primary B cells. The data also point to a critical role for glycolytic metabolism in maintaining primary B cell viability. Our findings raise the intriguing question as to why increased glucose uptake and glycolytic metabolism is required for the survival pathway driven by IL-4. One possibility is that, in the absence of IL-4, B cells cannot import and use glucose to levels sufficient to maintain normal housekeeping functions such as maintenance of ionic integrity and volume regulation (22). Indeed, restricting glycolytic metabolism results in a failure to maintain cellular homeostasis, eventually triggering apoptosis (2, 3, 5).

Interestingly, we find that IRS-2 and PI3K activity are dispensable for IL-4-induced glycolytic flux. Our results differ from reports demonstrating a role for PI3K activity in the survival of T cells and IL-3-dependent hemopoietic cell lines (2, 3, 14). This discrepancy may point to inherent differences in the prosurvival signaling pathways used by B cells and these other cell types. In keeping with this, we have identified a heretofore unknown function of Stat6-dependent signaling in regulating glucose energy metabolism. The molecular basis underlying Stat6-dependent up-regulation of glycolytic flux is currently unknown. It is plausible that Stat6 may control the transcriptional activation of genes encoding glycolytic enzymes in response to IL-4. In support of this, recent gene expression profiling studies have revealed increased levels of transcripts encoding several glycolytic enzymes following IL-4 treatment of B cells (23). It is also possible that members of the Pim family of serine/threonine kinases, which are downstream targets of

IL-4-mediated Stat6 signaling, may regulate glucose energy metabolism via the phosphorylation of rate-limiting glycolytic enzymes (5, 24). Additional studies will be required to delineate the regulatory functions of Stat6 in glycolytic homeostasis.

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Disclosures

The authors have no financial conflict of interest.

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