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The CD28 Signaling Pathway Regulates Glucose Metabolism

Kenneth A. Frauwirth,[1,2,5] James L. Riley,[1,3,5] Marian H. Harris,[1,2] Richard V. Parry,[1,3] Jeffrey C. Rathmell,[1,2] David R. Plas,[1,2] Rebecca L. Elstrom,[1] Carl H. June,[1,3] and Craig B. Thompson[1,2,4]

[1]Abramson Family Cancer Research Institute
[2]Department of Cancer Biology
[3]Department of Pathology and Laboratory Medicine
University of Pennsylvania
Philadelphia, Pennsylvania 19104

Summary

Lymphocyte activation initiates a program of cell growth, proliferation, and differentiation that increases metabolic demand. Although T cells increase glucose uptake and glycolysis during an immune response, the signaling pathways that regulate these increases remain largely unknown. Here we show that CD28 costimulation, acting through phosphatidylinositol 3′-kinase (PI3K) and Akt, is required for T cells to increase their glycolytic rate in response to activation. Furthermore, CD28 controls a primary response pathway, inducing a level of glucose uptake and glycolysis in excess of that needed to maintain cellular ATP/ADP levels or macromolecular synthesis. These data suggest that CD28 costimulation functions to increase glycolytic flux, allowing T cells to anticipate energetic and biosynthetic needs associated with a sustained response.

Introduction

During the activation of a resting lymphocyte, large metabolic demands are placed on the cell as it initiates proliferation and cytokine production (Krauss et al., 2001). The cell grows to approximately double its resting size and then enters a program of rapid proliferation while also differentiating from a quiescent cell to one that is highly secretory. Reflecting these increased energy demands, activation of T cells by mitogenic lectins such as concanavalin A, phytohaemagglutinin, and pokeweed mitogen is associated with increases in glycolysis (Cooper et al., 1963; Culvenor and Weidemann, 1976; Hedeskov, 1968; Roos and Loos, 1970; Sagone et al., 1974). Unlike hepatocytes and myocytes, two cell types in which metabolic pathways have been well studied, resting lymphocytes do not have large internal glycogen stores, making them highly dependent on the import of extracellular glucose to meet increased metabolic needs.

Many studies suggest that glucose uptake and glycolytic regulation is homeostatically controlled by cellular ATP and metabolite levels. In this model, a primary signaling pathway is not required to allow the modulation of metabolism in response to changes in cellular conditions. However, it has recently been shown that signals from cell surface receptors are required to control the ability of resting cells to take up and utilize nutrients at levels sufficient to maintain viability (Rathmell et al., 2000). Further, in fat and muscle cells insulin induces glucose uptake in excess of that required to maintain cellular energy charge, allowing the synthesis of triglycerides and glycogen for storage. In T cells, the costimulatory receptor CD28 participates in an intracellular signaling pathway, some of whose proximal components are similar to those in the insulin receptor pathway. The binding of insulin to the insulin receptor results in the activation of intrinsic tyrosine kinase activity and leads to recruitment and phosphorylation of insulin receptor substrate-1 (IRS-1). Phosphorylated IRS-1, in turn, recruits and activates PI3K (Lee and Pilch, 1994). Similarly, upon ligation of the T cell receptor (TCR), activation of Lck or Fyn in the TCR/CD3 complex leads to the tyrosine phosphorylation of the cytoplasmic tail of CD28, which then acts as an adaptor for the recruitment and activation of PI3K (August and Dupont, 1994; Chuang et al., 1999; Pages et al., 1994; Prasad et al., 1994; Truitt et al., 1994).

The similarity in signaling pathways downstream of the insulin receptor and CD28 suggest that CD28 may act to regulate glucose utilization, coordinating the control of T cell activation and metabolism in a manner similar to that of the insulin receptor. Here we show that CD28 costimulation of human peripheral blood T cells enhances expression of glucose transporters, glucose uptake, and glycolysis, and that this enhancement is dependent on PI3K activity. Further, the majority of glucose processed by CD28-costimulated T cells is converted to lactate and secreted, rather than used for biosynthesis or oxidized for maximal energy extraction. These data indicate that the increased metabolism is not a homeostatic response to increased demands but is a result of a primary signal transduction pathway from CD28.

Results

The primary downstream mediator of the metabolic effects of insulin is the Ser/Thr kinase Akt (Summers et al., 1999). CD28 costimulation of human peripheral blood T cells induces a prolonged activation of Akt that is PI3K dependent. Stimulation of resting human peripheral blood T cells with magnetic beads coated with anti-CD3/anti-CD28 antibodies but not with anti-CD3 alone led to Akt activation within 5 min (Figure 1A). This activation peaked within 30 min but was still detectable 3 hr after stimulation (see Figure 3A) and was blocked by the PI3K inhibitor LY294002 (Figure 1A). Although some activation of Akt by anti-CD3 alone was detected, costimulation by CD28 was required for sustained activation.

These data support a model in which the TCR and CD28 synergize to activate Akt (Figure 1B). In the insulin signaling pathway, Akt activation stimulates the synthe-

[4]Correspondence: drt@mail.med.upenn.edu
[5]These authors contributed equally to this work.

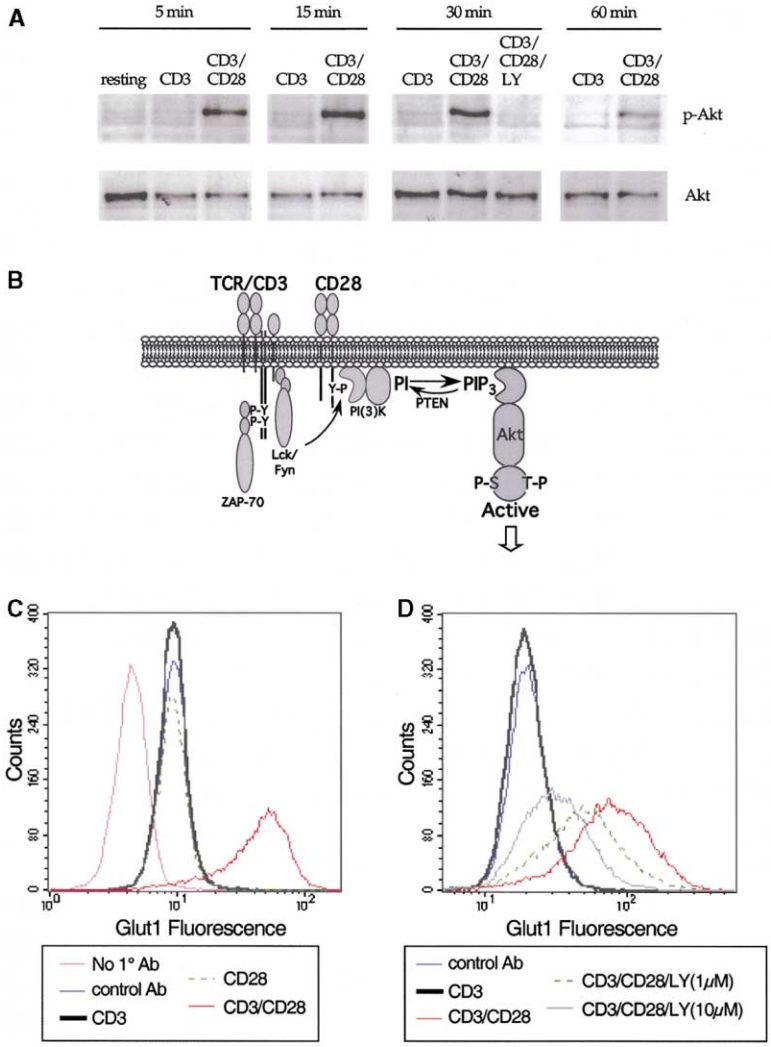

Figure 1. CD28 Costimulation Induces Sustained PI3K-Dependent Akt Activation and Increased Expression of Glut1

(A) CD28 costimulation activates Akt in human peripheral blood T cells in a PI3K-dependent fashion. Purified T cells were cultured in the absence of antibodies (resting) or stimulated with anti-CD3 (CD3) or anti-CD3/anti-CD28 (CD3/CD28) antibodies for the indicated times. Akt activation was detected by Western blot for Ser473-phosphorylated Akt (top panels, p-Akt). The blot was then stripped and reprobed for total Akt (bottom panels, Akt). PI3K dependence was determined by stimulation in the presence of 10 μM LY294002 (CD3/CD28/LY).

(B) Pathway for Akt activation in T cells.

(C) CD28 costimulation induces Glut1. T cells were stimulated for 20 hr with the indicated antibodies, and total cellular Glut1 was measured flow cytometrically. Anti-MHC antibody was used as a nonstimulatory control (control Ab). Cells were also stained in the absence of primary anti-Glut1 antibody (No 1° Ab) as a specificity control.

(D) Glut1 induction is PI3K dependent. T cells were stimulated with control, anti-CD3, or anti-CD3/anti-CD28 Abs in the absence or presence of 1 μM or 10 μM LY294002, and Glut1 levels were measured as in (C). Higher doses of LY294002 could induce further suppression of Glut1 induction but also appear to be associated with nonspecific toxicity (not shown). Data are representative of three independent experiments.

sis and surface expression of glucose transporters and regulates glucose utilization (Barthel et al., 1999; Cong et al., 1997; Kohn et al., 1996; Taha et al., 1999; Ueki et al., 1998; Wang et al., 1999). We therefore tested the ability of CD28 to regulate glucose metabolism during human T cell activation. The first point of regulation of glucose utilization by a cell is the control of uptake via surface glucose transporters. Resting T cells express low levels of Glut1, the primary glucose transporter of hematopoietic cells (Rathmell et al., 2000). Neither activation by crosslinking the TCR/CD3 complex nor ligation of CD28 alone resulted in a significant change in Glut1 expression. In contrast, stimulation with anti-CD3/anti-CD28 led to a synergistic induction of Glut1 expression (Figure 1C) that was sensitive to inhibition by LY294002 in a dose-dependent fashion (Figure 1D). Thus, CD28 costimulation is required to increase glucose transporter expression during T cell activation, and this is dependent on the ability of CD28 to induce PI3K activation.

Increased synthesis and secretion of interleukin-2 (IL-2) and other cytokines are also hallmarks of T cell activation in the presence of costimulation, and binding of IL-2 to the IL-2 receptor on T cells can activate PI3K (Williamson et al., 1993). Furthermore, activation of Akt by CD28 has been suggested to function primarily to upregulate IL-2 expression. (Kane et al., 2001; Parry et al., 1997). To determine if the Glut1 induction was secondary to IL-2 signaling, we tested the effects of supplementing anti-CD3 cultures with recombinant IL-2. At levels comparable to those seen in anti-CD3/anti-CD28 cultures, IL-2 was unable to mimic the effects of CD28 costimulation on Glut1 levels (Figure 2A). To further test the role of IL-2, we also cultured T cells in the presence or absence of neutralizing anti-IL-2 antibody during stimulation with anti-CD3/anti-CD28-coated beads. Addition of the blocking anti-IL-2 antibody reduced levels of functional IL-2 in anti-CD3/anti-CD28 culture supernatants by >90% (data not shown) but did not result in a decrease in Glut1 expression (Figure 2A).

Although IL-2 was not sufficient to replace CD28 signaling in the induction of Glut1, CD28 can augment the expression of other cytokines in addition to IL-2. To rule out other secreted cytokines as mediators of Glut1 upregulation, we also stimulated T cells with anti-CD3 in conditioned medium harvested from anti-CD3/anti-CD28-stimulated cells. Unlike direct stimulation with anti-CD3/anti-CD28, addition of anti-CD3/anti-CD28-

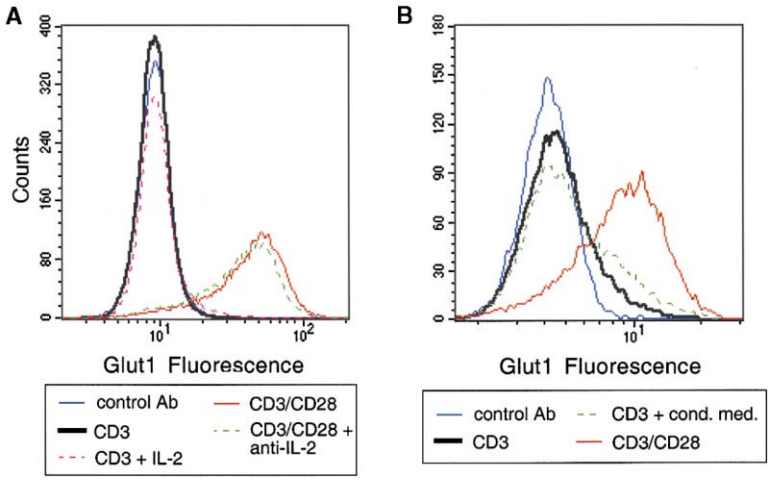

Figure 2. Increased Glut1 Expression after CD28 Costimulation Is Not Mediated by Secreted Cytokines

T cells were stimulated as in Figure 1. Anti-CD3-stimulated cells were cultured with anti-CD3 alone (CD3) ([A] and [B]), anti-CD3 with 300 U/ml IL-2 (CD3 + IL-2) (A), or anti-CD3 with conditioned medium from anti-CD3/anti-CD28-stimulated cultures (CD3 + cond. med.) (B). Anti-CD3/anti-CD28-stimulated cells were cultured without (CD3/CD28) ([A] and [B]) or with 1 μg/ml anti-IL-2 Ab (CD3/CD28 + anti-IL-2) (A). Glut1 levels were determined by flow cytometry as in Figure 1.

conditioned medium failed to enhance Glut1 expression during anti-CD3 stimulation (Figure 2B).

In order to examine the significance of the increased Glut1 expression in CD28-costimulated cells, we measured glucose uptake rates in cells stimulated as described above. Anti-CD3/anti-CD28 significantly increased glucose uptake, while either antibody alone had no effect on the uptake rate (Figure 3A). As with Glut1 expression, the increase in glucose uptake was not reduced by anti-IL-2 antibody, and exogenous IL-2 was unable to substitute for CD28 costimulation. Stimulation with anti-CD3/anti-CD28 for as little as 6 hr was sufficient to produce a significant increase in glucose uptake.

We next investigated whether the enhanced glucose uptake resulted in an increased glycolytic rate. Stimulation with anti-CD3 alone had a minimal effect on glycolytic rate, as measured at the dehydration reaction catalyzed by enolase, the penultimate step of glycolysis (Figure 3B). However, the anti-CD3 stimulation conditions were sufficient to initiate signal transduction and regulate the transcription of over 500 genes (Riley et al., 2002). In contrast, CD28 costimulation dramatically increased the ability of T cells to perform glycolysis (Figure 3B), an increase inhibitable by LY294002 (data not shown). Consistent with the Glut1 and glucose uptake data, the CD28 enhancement of the glycolytic rate was independent of IL-2 (Figure 3B) and not mimicked by addition of anti-CD3/anti-CD28-conditioned medium (Figure 3C).

CD28 costimulation enhances T cell proliferation and cytokine production. Therefore, the increases in glucose uptake and glycolysis we observed could be secondary to a primary effect of CD28 costimulation on transcription or translation. If glycolytic control during lymphocyte activation is regulated homeostatically by ATP levels and glycolytic intermediates, then the increase in glucose utilization we observed could be a result of the need to regenerate ATP and metabolites consumed during macromolecular synthesis. However, we found that the major product of the increased glucose metabolism stimulated by CD3/CD28 was lactate (Figure 3D). As much as 90% of the glucose consumed by CD3/CD28-stimulated T cells was converted to lactate and secreted. This represents a significant decrease in the efficiency of glucose utilization by CD28-costimulated T cells to support either ATP production or macromolecular synthesis.

One concern in considering the ability of CD28 to upregulate glucose metabolism is that this upregulation could be due to the established ability of CD28 costimulation to induce T cell proliferation at low levels of TCR engagement. To address this issue, T cells were stimulated with increasing amounts of anti-CD3. At mitogenic doses, anti-CD3 induced a small but consistent increase in glycolysis when compared to unstimulated cells. Nevertheless, at all doses of anti-CD3 tested, the addition of CD28 costimulation led to a >5-fold further increase in glucose metabolism without inducing a comparable increase in proliferation (Figure 4).

The data above suggest that CD28 signal transduction can increase glucose consumption beyond what is strictly required to maintain the bioenergetics of a proliferating cell. The T cell appears to deal with this excess in glucose consumption by secreting it as lactate. Increased production of lactate by mitogen-activated T cells and leukemic T cell lines has been reported (Cooper et al., 1963; Culvenor and Weidemann, 1976; Hedeskov, 1968; Roos and Loos, 1970) and has been suggested to indicate that lymphocytes have a limited ability to carry out oxidative phosphorylation. To address this issue, T cells were cultured under conditions of limited extracellular glucose. T cells cultured in reduced glucose (0.4 mM) had lower glycolytic rates than those cultured under standard glucose conditions (11 mM) (Figure 5A). This represents a decrease in actual glycolytic capacity, as it was observed even when cells were provided with 10 mM glucose in the assay buffer (data not shown). When glucose became limiting, the oxygen:glucose consumption ratio for anti-CD3-stimulated cells rose from 2.5:1 in 11 mM glucose to over 6.0:1 in 0.4 mM glucose. These data indicate that there was a shift to essentially complete oxidation of glucose, resulting in maximal ATP generation from the limited glucose supply (Figure 5B). Thus, anti-CD3-stimulated cells in low glucose are unable to meet their bioenergetic needs through compensatory increases in glycolysis. The ability to regulate oxidative phosphorylation in re-

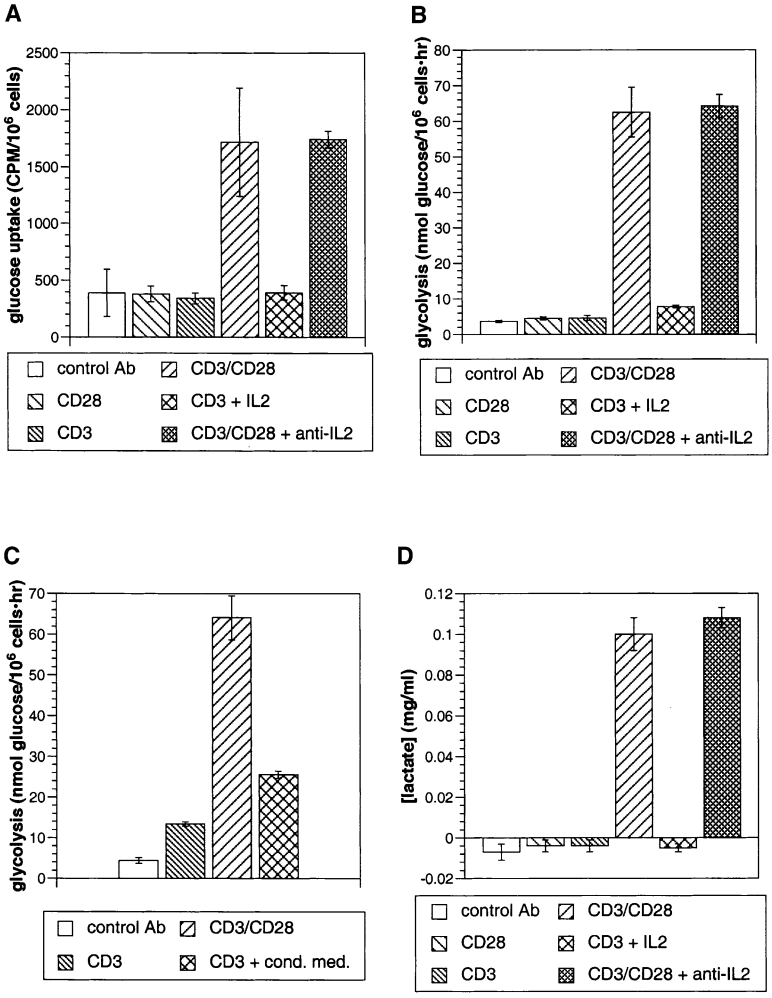

Figure 3. CD28 Costimulation Increases Glucose Uptake, Glycolysis, and Lactate Production

T cells were stimulated for 20 hr with the indicated antibodies and uptake of [^{3}H]-2-deoxyglucose (A), glycolysis of 5-[^{3}H]-glucose ([B] and [C]), and the increase in lactate in culture medium ([D]; calculated as total lactate concentration minus the lactate concentration in medium alone) were measured. Data are representative of three independent experiments. In (C), "CD3 + cond. med." represents stimulation with anti-CD3 in the presence of conditioned medium from anti-CD3/anti-CD28 cultures.

sponse to glucose deprivation was even more apparent in anti-CD3/anti-CD28-stimulated cells. When glucose levels were reduced in CD3/CD28-stimulated cultures, there was a 4-fold increase in the absolute rate of oxygen consumption, demonstrating that the cells had the capacity to increase their rate of oxidative metabolism under conditions of reduced extracellular glucose (Figure 5B). This compensatory regulation of bioenergetics is reflected in the ability of anti-CD3/anti-CD28-stimulated T cells to survive and proliferate when cultured in 0.4 mM glucose (data not shown). Thus, the production of lactate by costimulated T cells is not due to an inability to increase their rate of oxidative phosphorylation, but reflects that glycolytic flux is occurring at a rate sufficient to suppress ADP-coupled oxidative phosphorylation. Together, these data also suggest that activated T cells lack homeostatic mechanisms to produce compensatory increases in glycolysis in response to glucose deprivation.

Cytotoxic T lymphocyte antigen-4 (CTLA-4) is a negative regulator of CD28 costimulation (Krummel and Allison, 1995; Walunas et al., 1996). Although resting T cells express very little surface CTLA-4, expression is induced within 1 hr of stimulation (Lindsten et al., 1993), and inhibitory effects of CTLA-4 on IL-2 expression can be detected within 4 hr (Blair et al., 1998; Brunner et al., 1999). We therefore tested whether CTLA-4 can inhibit the increases in glucose metabolism induced by CD28. Crosslinking CTLA-4 with anti-CTLA-4 antibody blocked Akt activation by anti-CD3/anti-CD28 (Figure 6A), similar to the effects of LY294002 (Figure 1A). Consistent with its blockade of Akt activity, anti-CTLA-4 inhibited the induction of Glut1 expression (Figure 6B), glucose uptake (Figure 6C), and glycolysis (Figure 6D) by anti-CD3/anti-CD28. Thus, CTLA-4 blocks T cell activation in such a manner as to prevent the upregulation of glucose transport and glycolysis that results from CD28 costimulation.

The above data suggest that CD28 costimulation acts to regulate nutrient uptake in lymphocytes through its ability to act as an adaptor molecule in the activation of PI3K and, thus, Akt. To confirm that sustained activation of Akt is sufficient to account for the observed metabolic effects of CD28 costimulation, we used the lymphoid cell line FL5.12 and transfected it with either constitutively active Akt1 or empty vector (Figure 7A). In the presence of IL-3, Akt-transfected FL5.12 cells proliferated at a rate similar to control-transfected cells (Figure 7B), indicating that net macromolecular synthesis of control and Akt transfectants was comparable. However, Akt transfectants had a much higher rate of glycolysis than control cells (Figure 7C). Consistent with the increased glycolysis, Akt-transfected cells consumed glucose from the culture medium more rapidly

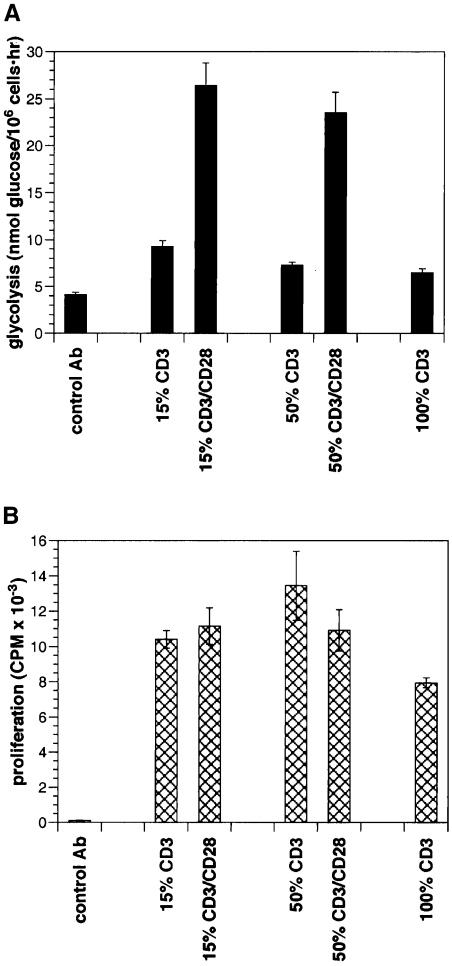

Figure 4. Mitogenic Doses of CD3 Do Not Augment Glycolysis to CD3/CD28 Levels

T cells were stimulated with control Ab, anti-CD3, or anti-CD3/anti-CD28, and glycolysis (A) and incorporation of [^{3}H]thymidine (B) were measured after 20 hr and 3 days, respectively. Error bars denote standard deviation from the mean of triplicate measurements. Total amount of antibody bound to beads was kept constant; percentages indicate the fraction of total antibody that is anti-CD3. In CD3 samples, the remainder is control antibody; in CD3/CD28 samples, the remainder is anti-CD28 Ab. For comparison, stimulations for Figures 1–3 used 5% CD3.

than vector transfectants (Figure 7D) and produced higher levels of lactate (Figure 7E). In fact, Akt transfectants depleted their glucose to such an extent within 3 days of culture that the cells began consuming lactate (Figures 7D and 7E), which can be converted back to pyruvate by lactate dehydrogenase. Therefore, as with costimulated primary T cells the increase in lactate production is unlikely to be due to an inability to carry out oxidative phosphorylation, but rather is due to a glycolytic flux that is in excess of energy demand. Thus, increased Akt activity enhances glucose consumption to a level above that needed to meet energetic and biosynthetic demands.

Discussion

The regulation of glycolysis is generally portrayed as a homeostatic response to the immediate metabolic demands of the cell. As the levels of intracellular ATP and/or glycolytic intermediates drop, allosteric inhibition of key glycolytic enzymes is relieved, resulting in an increase in the rate of glycolysis. Conversely, negative feedback prevents a cell from consuming nutrients beyond its short-term needs. We report that T cells receiving CD28 costimulation rapidly induce glucose uptake and glycolysis to a level that is sufficient to suppress oxidative phosphorylation. Furthermore, the rate at which CD3/CD28-stimulated T cells process glucose is in excess of that needed to sustain macromolecular synthesis. The majority of the glucose taken up by the cells is not incorporated into cellular macromolecules, but instead is disposed of as lactate. The ability of CD28 to drive glucose metabolism beyond the level needed for the immediate metabolic demands indicates that cell surface signaling can result in a primary regulation of glucose utilization. Such regulation would allow T cells to anticipate the demands of activation, inducing synthesis and activity of metabolic machinery before it becomes a bottleneck for T cell proliferation and function, thus ensuring the availability of glycolytic intermediates for biosynthetic and energetic reactions. This likely contributes to the ability of CD28 costimulation to prolong T cell viability and prevent the apoptotic death that characteristically follows T cell stimulation via the TCR/CD3 complex alone.

Our results also bring into question whether T cells have a homeostatic ability to increase either glucose uptake or glycolysis in response to a decrease in the ability to produce ATP. When glucose levels were lowered in the culture medium, anti-CD3-treated cells switched to virtually complete oxidation of glucose through oxidative phosphorylation but failed to maintain glycolytic rate. However, the same concentration of glucose was able support a 10-fold higher glycolysis rate in anti-CD3/anti-CD28-stimulated cells. Thus, glucose levels were not limiting per se, but rather anti-CD3-stimulated cells were unable to compensate by increasing Glut1 expression and glucose transport capacity to maintain homeostasis in the face of reduced extracellular glucose concentration.

The identification of Akt as a potential signaling link between CD28, CTLA-4, and T cell metabolism reveals an interesting parallel with the insulin signaling pathway. Ligation of the insulin receptor causes the phosphorylation of IRS-1, which can then recruit and activate PI3K (Lee and Pilch, 1994). Membrane recruitment of Akt by PI3K-generated phospholipids allows Akt phosphorylation and activation, which has been shown to be centrally involved in insulin-mediated increases in glucose transport and synthesis of glucose transporters (Barthel et al., 1999; Cong et al., 1997; Kohn et al., 1996; Taha et al., 1999; Ueki et al., 1998; Wang et al., 1999). Phosphorylation of CD28 by TCR/CD3-associated Lck or Fyn initiates a similar set of events (August and Dupont, 1994; Chuang et al., 1999; Prasad et al., 1994; Truitt et al., 1994), resulting in increased glucose metabolism. However, while IRS-1 is directly recruited to the insulin receptor upon binding of insulin, recruitment of CD28 to the TCR/CD3 complex is dependent on binding of CD28 to its ligands B7-1 or B7-2, adding a further level of control to the regulation of T cell metabolism.

Insulin signaling is negatively regulated by phosphatases, such as PTEN, which opposes the activity of PI3K

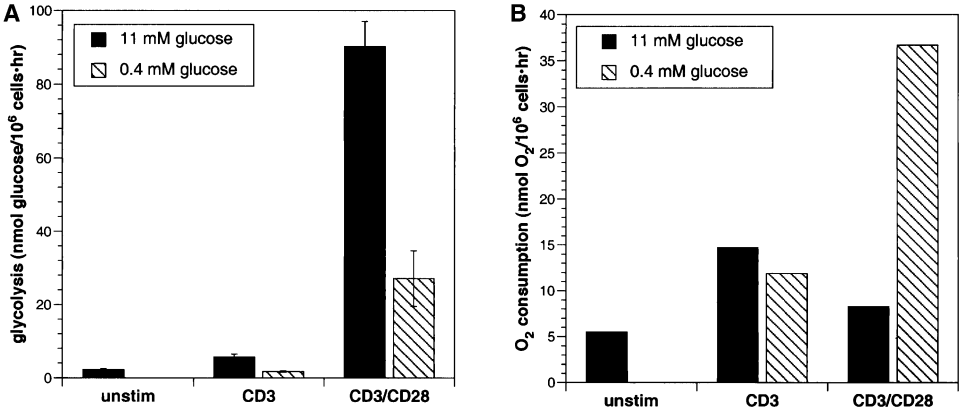

Figure 5. CD28-Costimulated T Cells Can Increase Oxidative Phosphorylation When Glycolysis Is Restricted by Glucose Limitation

T cells were left unstimulated (unstim) or stimulated with anti-CD3 mAb (CD3) or anti-CD3/anti-CD28 mAb (CD3/CD28) in either standard (11 mM) or reduced (0.4 mM) glucose for 3 days, and rates of glycolysis (A) and oxygen consumption (B) were measured. Error bars denote standard deviation from the mean of triplicate measurements. Data are representative of three independent experiments.

(Simpson and Parsons, 2001), and PP2A, which deactivates Akt (Millward et al., 1999). The parallels between the insulin receptor and CD28 pathways raise the possibility that CD28 signaling is similarly regulated. The inhibitory receptor CTLA-4 has been shown to associate with the phosphatases SHP-2 and PP2A (Chuang et al., 2000; Lee et al., 1998; Marengere et al., 1996), and we have found that CTLA-4 crosslinking inhibits the activation of Akt and the metabolic effects of CD28 costimulation. While these studies do not identify how CTLA-4 prevents sustained Akt activity and Glut-1 expression, the ability to prevent an upregulation of T cell metabolism may be an important component of the inhibitory activity of CTLA-4.

A rapid and sustained increase in lymphocyte metabolism is required to support the proliferation, differentiation, and effector function resulting from an immune response. Our results implicate CD28 costimulation acting through the PI3K/Akt pathway as an important component in the regulation of glucose metabolism. TCR

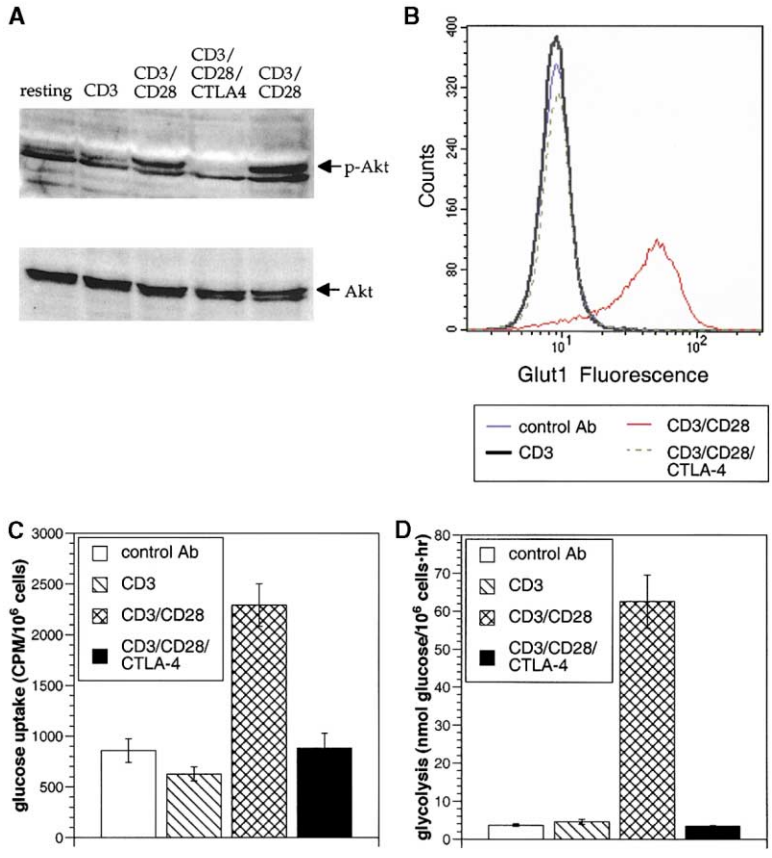

Figure 6. CTLA-4 Inhibits CD28 Effects on Metabolism

(A) CTLA-4 inhibits Akt activation. T cells were cultured without stimulation (resting) or stimulated with the indicated antibodies for 3 hr. Akt activation was detected by Western blot (top panel; p-Akt) as in Figure 1A, and the blot was stripped and reprobed for total Akt (bottom panel; Akt). In one sample, an optimal dose of anti-CD3/anti-CD28 mAb was used for maximal Akt activation (far right lane).

(B–D) CTLA-4 prevents CD28-mediated increases in glucose metabolism. T cells were stimulated with the indicated antibodies for 20 hr, and induction of Glut1 expression (B), glucose uptake (C), and glycolysis (D) were measured as above. Error bars denote standard deviation from the mean of triplicate measurements. Results are representative of three independent experiments.

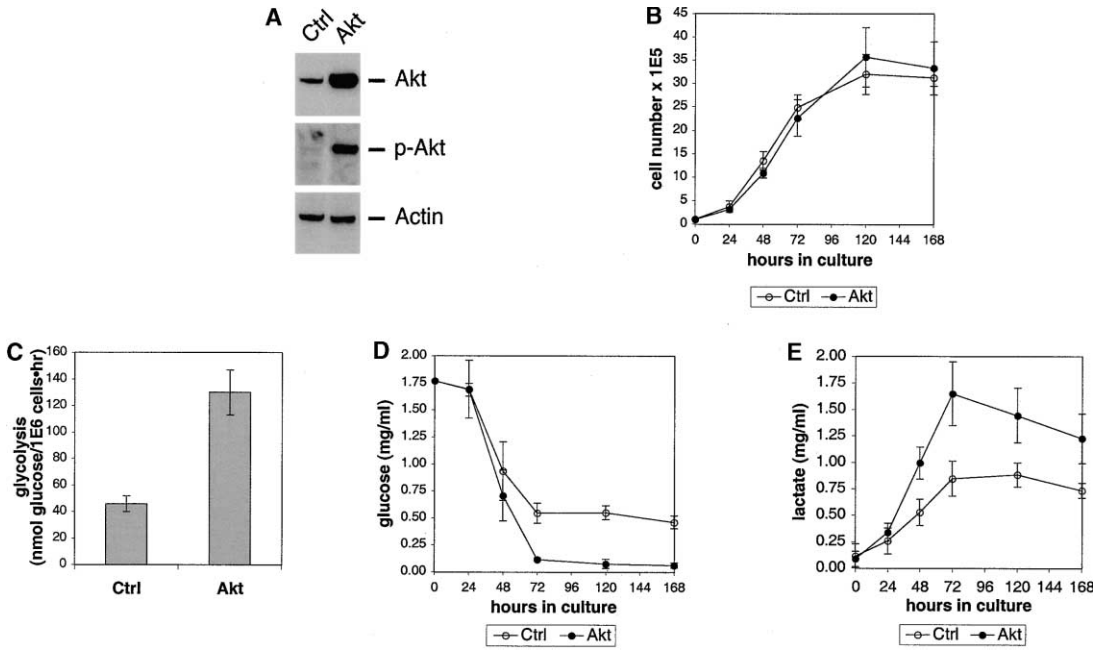

Figure 7. Constitutive Akt Activity Increases Glucose Metabolism

(A) FL5.12 cells stably transfected with either control (Ctrl) or Akt constructs were assayed for total Akt (top panel; Akt) by Western blot. Blots were subsequently stripped and reprobed for Akt activation-specific phosphorylation (middle panel; p-Akt) and actin (bottom panel).

(B) Ctrl-FL5.12 and Akt-FL5.12 cells were cultured for the indicated times in the presence of IL-3 and cells were counted. Error bars denote standard deviation from the mean of three independent experiments.

(C) Comparison of glycolysis rates of Ctrl-FL5.12 and Akt-FL5.12 cells. Error bars represent standard deviation from the mean of triplicate measurements. (D and E) Glucose (D) and lactate (E) levels in culture medium were analyzed over time for Ctrl-FL5.12 and Akt-FL5.12 cells. Error bars denote standard deviation from the mean of three independent experiments.

signal transduction alone has the capacity to activate Akt under some conditions (Jones et al., 2000; Lafont et al., 2000) and a limited ability to stimulate glycolysis (Figure 4). Enhancement of cellular bioenergetics has been reported in TCR-activated cells to result from stimulation of oxidative phosphorylation (Krauss et al., 2001), a property of TCR engagement confirmed by our studies (Figure 5). In contrast, CD28 costimulation leads to prolonged Akt activation and upregulation of glycolysis. Although cytokines also have the ability to induce Akt activation in antigen-primed T cells (Kelly et al., 2002; Lafont et al., 2000; Scheel-Toellner et al., 2002), they have not been reported to activate Akt in resting cells. Thus, while cytokines could be important in maintaining the metabolism of activated cells, they do not appear capable of replacing CD28 in the activation of glycolysis during the initial stages of T cell activation.

The role of PI3K in CD28 signaling has been controversial. Although several groups have reported PI3K activity to be dispensable for CD28 costimulatory function (Crooks et al., 1995; Harada et al., 2001a; Okkenhaug et al., 2001; Truitt et al., 1995), others have found that the PI3K pathway is a significant component of CD28 signaling, particularly early in a T cell response. (Burr et al., 2001; Cai et al., 1995; Harada et al., 2001b; Pages et al., 1994). Part of the confusion may come from the use of PTEN-deficient cells, such as Jurkat (Shan et al., 2000), for studies of CD28 function. In the absence of PTEN, cell lines have constitutive activation of Akt and therefore increased rates of glycolysis, independent of CD28-mediated PI3K activation. The loss of PTEN from lymphoid cell lines may reflect the importance of the PI3K/Akt pathway in the regulation of lymphocyte growth. Enhancing access to nutrients through increased levels of glucose transporters and activity of the glycolytic pathway appears to be one important way in which costimulatory receptors can stimulate T cell growth.

Experimental Procedures

T Cell Purification

Human peripheral blood T cells were isolated by magnetic bead negative selection as previously described (June et al., 1987).

Cell Lines and Culture

FL5.12 cells (an IL-3-dependent cell line) were cultured as previously described (Vander Heiden et al., 1997). Tet-inducible mAkt-FL5.12 cells have been described (Plas et al., 2001). FL5.12 cells constitutively expressing mAkt1 were generated by retroviral transduction with pBABE expression vectors (Kennedy et al., 1997), provided by Dr. Nissim Hay (University of Illinois, Chicago, IL), and used for some experiments. Cells were counted on a Coulter Z2 particle counter (Beckman Coulter, Miami, FL).

Antibodies, Bead Preparation, and Cell Stimulation

Anti-CD3 (OKT3), anti-CD28 (9.3), anti-CTLA-4 (ER5.3D6), and anti-MHC class I (W6/32) mAbs were covalently attached to tosyl-activated Dynabeads (Dynal, Great Neck, NY) following the manufacturer's instructions, as previously described (Blair et al., 1998). In order to see effects of CTLA-4, a suboptimal dose of anti-CD3 antibody was used (Blair et al., 1998).

Beads were mixed with T cells at a 3:1 ratio of beads:cells. Where

indicated, 300 U/ml of IL-2 (Chiron Therapeutics, Emeryville, CA) or 1 μg/ml of polyclonal goat anti-human IL-2 Abs (R&D Systems, Minneapolis, MN) was added to the cultures. In some experiments, cultures were supplemented with the PI3K inhibitor LY294002 (Calbiochem, San Diego, CA) at 1–10 μM. IL-2 in the culture medium was measured by sandwich ELISA using reagents from R&D Systems.

Western Blots

T cells were left unstimulated or stimulated with bead-linked antibodies for times ranging from 5 min to 3 hr. Cells were lysed in buffer containing 1% NP-40, 150 mM NaCl, 50 mM Tris-Cl (pH 7.4), 5 mM EDTA, and 10 mM iodoacetamide and supplemented with the following protease and phosphatase inhibitors: NaF (10 mM), β-glycerophosphate (10 μg/ml), Na_3VO_4 (150 μM), PMSF (1 mM), leupeptin (1 μg/ml), chymostatin (1 μg/ml), pepstatin A (1 μg/ml), and antipain (1 μg/ml). Lysates were resolved by SDS-PAGE and transferred to nitrocellulose. Blots were probed with anti-phospho-Ser473-Akt Ab (Cell Signaling Technology, Beverly, MA), stripped, and reprobed with anti-total-Akt Ab (Cell Signaling Technology). Some blots were further probed with anti-actin Ab (Santa Cruz Biotechnology, Santa Cruz, CA). Blots were visualized using ECL+ (Amersham/Pharmacia, Piscataway, NJ) or Super Signal (Pierce, Rockford, IL) chemiluminescence reagents.

Glut1 Intracellular Staining

T cells were stimulated for 20 hr with bead-linked Abs and fixed overnight at 4°C with 1% paraformaldehyde in PBS. Fixed cells were permeablized with 0.3% saponin and stained with rabbit anti-Glut1 Ab (Research Diagnostics Inc., Flanders, NJ) followed by FITC-labeled goat anti-rabbit Ab (Pharmingen, San Diego, CA). Glut1 staining was measured by flow cytometry on a FACSCalibur cytometer (Becton Dickinson, Mountain View, CA), and data were analyzed with CellQuest software (Becton Dickinson).

Glucose Uptake, Glycolysis, and Oxygen Consumption

Glucose uptake was measured using a modified protocol of Whetton et al. (1984). Briefly, stimulated T cells were incubated for 15 min at 37°C in glucose uptake buffer (8.1 mM Na_2HPO_4, 1.4 mM KH_2PO4, 2.6 mM KCl, 136 mM NaCl, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$ [pH 7.4]) to deplete intracellular glucose stores. Triplicate samples of 1×10^6 cells were incubated with 1 μCi of [^{3}H]-2-deoxyglucose (NEN, Boston, MA) in glucose uptake buffer for 2 min at room temperature and immediately spun through a layer of bromododecane (Sigma, St. Louis, MO) into 20% perchloric acid/8% sucrose, stopping the reaction and separating the cells from unincorporated [^{3}H]-2-deoxyglucose. The perchloric acid/sucrose/T cell layer was removed and analyzed by liquid scintillation using a 1450 Microbeta scintillation counter (Wallac, Turku, Finland). Measurement of glycolysis and oxygen consumption were performed as described previously (Vander Heiden et al., 2001).

Measurement of Glucose and Lactate in Cultures

Glucose and lactate levels in culture supernatants were measured using enzymatic Diagnostic kits (Sigma) following the manufacturer's instructions.

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