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*J Immunol* 2008; 180:4476-4486; doi: 10.4049/jimmunol.180.7.4476
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Glucose Uptake Is Limiting in T Cell Activation and Requires CD28-Mediated Akt-Dependent and Independent Pathways

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T cell activation potently stimulates cellular metabolism to support the elevated energetic and biosynthetic demands of growth, proliferation, and effector function. We show that glucose uptake is limiting in T cell activation and that CD28 costimulation is required to allow maximal glucose uptake following TCR stimulation by up-regulating expression and promoting the cell surface trafficking of the glucose transporter Glut1. Regulation of T cell glucose uptake and Glut1 was critical, as low glucose prevented appropriate T cell responses. Additionally, transgenic expression of Glut1 augmented T cell activation, and led to accumulation of readily activated memory-phenotype T cells with signs of autoimmunity in aged mice. To further examine the regulation of glucose uptake, we analyzed CD28 activation of Akt, which appeared necessary for maximal glucose uptake of stimulated cells and which we have shown can promote Glut1 cell surface trafficking. Consistent with a role for Akt in Glut1 trafficking, transgenic expression of constitutively active myristoylated Akt increased glucose uptake of resting T cells, but did not alter Glut1 protein levels. Therefore, CD28 appeared to promote Akt-independent up-regulation of Glut1 and Akt-dependent Glut1 cell surface trafficking. In support of this model, coexpression of Glut1 and myristoylated Akt transgenes resulted in a synergistic increase in glucose uptake and accumulation of activated T cells in vivo that were largely independent of CD28. Induction of Glut1 protein and Akt regulation of Glut1 trafficking are therefore separable functions of CD28 costimulation that cooperate to promote glucose metabolism for T cell activation and proliferation. The Journal of Immunology, 2008, 180: 4476-4486.

The functional immune response requires rapid and extensive cell growth, proliferation, and production of effector proteins, such as cytokines. To perform these functions, the metabolic and biosynthetic demands of lymphocytes become dramatically increased after activation (1, 2). When resting, T lymphocytes meet basal energy demands primarily through mixed usage of glucose and glutamine (3). After activation, glucose metabolism increases as a source of energy and cellular biosynthesis (2-5). Although some regulation of cellular metabolism is anaplerotic and is met by enhanced flux due to relief of feedback inhibition in pathways whose end products are rapidly used, it has become increasingly apparent that regulatory mechanisms also exist to promote specific metabolic pathways and phenotypes (2, 6, 7). In lymphocytes, control of nutrient uptake by cell extrinsic signals has been proposed as a key point of regulation for cellular metabolism (8). If appropriate signals are not received, nutrient uptake decreases to a level below that capable of supporting cellular demands, leading to inhibition of cell proliferation and ultimately, apoptosis (8–10). In particular, signals and signaling pathways that control lymphocyte glucose uptake may be critical to cell function, viability, and immune response. Insufficient glucose can lead to deficient responses of activated T cells (11) and induction and activation of proapoptotic Bcl-2 family proteins such as Noxa and Bax (12, 13). However, the importance of elevated glucose uptake in T cell function and the signaling pathways that are required to promote increased glucose uptake in T cell activation are uncertain.

T cell activation and costimulation may regulate glucose transport in lymphocytes via control of glucose transporter expression, localization, and function. The primary glucose transporter in hematopoietic cells, Glut1, is expressed at low levels in resting T cells and is up-regulated upon T cell activation (2, 5, 14). Increased Glut1 levels and glucose uptake correlate with increased cellular growth and proliferation, such as in thymocytes, where increased Glut1 expression is highest in proliferating cells (15, 16). Control of Glut1 trafficking and activity are also key elements regulating glucose uptake. Similar to the insulin-responsive glucose transporter, Glut4, Glut1 cell surface localization is controlled by extrinsic signals (17). Among signaling pathways initiated in T cell activation, the PI3K/Akt pathway has been shown to promote both Glut1 cell surface trafficking and activity (17–20). In the absence of these signals, Glut1 remains intracellular and may be degraded in lysosomes to restrict glucose uptake (17, 19).

In resting T cells, glucose metabolism is maintained by cytokines such as IL-7 (9), while T cell activation increases glucose metabolism and supports T cell proliferation through the ligation of the TCR/CD3 and a second costimulatory signal (2). In particular, CD28 and its ligands B7.1 and B7.2 are sufficient to augment CD3 signals and promote glucose metabolism (5). Mechanistically, costimulation may promote glucose metabolism by enhancing Ag receptor initiated signaling pathways, such as NFAT, NF-κB, MAPK, and Ras activation. Costimulation may also preferentially activate specific signaling pathways, such as CD28 activation of the PI3K/Akt pathway, to promote glucose uptake.
and metabolism. Activation of PI3K may be a particularly important regulator of glucose uptake, as PI3K inhibition prevented increased glucose metabolism after lymphocyte activation or cytokine stimulation, and decreased glucose uptake of leukemic cells (5, 21–23). Conversely, in addition to its ability to cause autophagy and lymphoma, expression of constitutively active Akt promoted increased glucose uptake and consumption in cell lines (20, 23) and elevated glycolysis in primary T cells (24). The PI3K/Akt signaling pathway may, therefore, play an important role in regulating the immune response and lymphocyte homeostasis through the regulation of glucose metabolism.

The role of glucose uptake in activation of naive T cells and the costimulatory signaling pathways necessary to promote it are not clear. In this study, we demonstrate that while not limiting in resting T cells or homeostasis, glucose uptake is limiting in T cell activation and elevated Glut1 expression enhanced T cell activation and led to increased serum Ig levels and Ig deposition in kidneys of aged mice. CD28 cosignaling is required to maximally increase glucose uptake through distinct signaling pathways. With low TCR signal strength, CD28 increased Glut1 expression, possibly by augmenting TCR-induced signals. In contrast, CD28 signaling through the Akt pathway was insufficient to up-regulate Glut1 proteins in naive T cells, possibly via enhanced cell surface Glut1 trafficking. Supporting dual mechanisms for CD28-mediated regulation of Glut1 protein levels and cell surface trafficking, transgenic expression of Glut1 together with constitutively active Akt synergistically increased glucose uptake. This diminished the requirement for CD28 cosignaling, and led to rapid accumulation of activated T cells in vivo. These data show that expression of Glut1 and regulation of glucose uptake are controlled by CD28 through separable Akt-dependent and Akt-independent pathways and are limiting in T cell activation.

**Materials and Methods**

**Mice**

Glut1 and myristoylated Akt (mAkt)3 transgenic mice have been previously described (24, 25) and were bred and maintained on the C57BL/6J background (The Jackson Laboratory). Double transgenic mice were produced at Duke University through selective breeding. Akt1+/− mice were a generous gift of Morris Birnbaum (University of Pennsylvania, PA). Mice were housed and cared for at Duke University and appropriate institutional boards approved all animal procedures. Unless otherwise indicated, experiments were performed on mice between 6 and 8 wk of age.

**T cell purification and culture**

T cells were purified via negative selection from spleen and mesenteric lymph nodes unless otherwise specified (StemSep) and cultured in RPMI 1640 (Mediatech) supplemented with 10% FBS (Gemini Bio-Products). Glucose-free RPMI 1640 (Invitrogen Life Technologies) with 10% dialyzed FBS (Gemini Bio-Products) was used for culture of cells in limited glucose. Glucose (Sigma-Aldrich) was filtered sterilized before being added to cultures to indicated concentrations. Following purification, T cell stimulation was achieved by culture of T cells on plates coated with anti-CD3ε (clone 145-2C11) at various doses and anti-CD28 (clone 37.51) (both from BD Pharmingen) at 5 μg/ml in PBS. The drug L1294002 (Calbiochem) was added to cultures at 10 μM to inhibit PI3K signaling where indicated. Cells were counted and cell size was determined on a Coulter Z2 particle counter (Beckman Coulter).

**Macrophage cocultures**

Macrophages were derived from murine bone marrow cultured in RPMI 1640 with addition of 3 ng/ml GM-CSF (PeproTech) for 7 days. Bone marrow cultures were washed and macrophages were trypsinized, replated, and treated with LPS from *E. coli* O111:B4 (Sigma-Aldrich) at 100 ng/ml with or without CTLA4-Ig (BD Pharmingen) at 1 μg/ml or anti-ICAM-1 (BD Pharmingen) at 2 μg/ml for 1 h before addition of T cells. T cell macrophage cocultures were cultured with or without addition of anti-CD3 (BD Pharmingen) at various doses for 1 day. To measure glucose uptake, nonadherent cells were removed and replated for 1 h. Cells that remained nonadherent were >90% T cells and were subject to glucose uptake analysis.

**Proliferation, survival, and flow cytometry**

Proliferation was determined by staining T cells with CFSE (Molecular Probes) before culture and analyzed flow cytometrically. Survival assays were performed through propidium iodide (Molecular Probes) exclusion and flow cytometry. T cells were stained with fluorescein-conjugated Abs against murine CD4, CD8, CD25, CD44, and CD69 (BD Pharmingen). Surface Myc-Glut1 was detected with anti-Myc (4A6, Upstate Biotechnology) followed by PE anti-mouse (BD Pharmingen). All flow cytometry was performed on a FACScan (BD Biosciences) and analyzed with FlowJo software (Tree Star).

**ELISA**

ELISAs were performed on supernatant from T cell cultures using rat Abs against murine IL-2 and IFN-γ (BD Pharmingen) as previously described (24). In some cases IL-2 ELISA was performed using Endogen Mouse IL-2 ELISA kit (Pierce Biotechnology) as per the manufacturer’s instructions. Blood Ig isotype levels were determined through use of Mouse Ig Isotyping ELISA kit (BD Pharmingen) per the manufacturer’s instructions.

**Immunoblotting**

Cells were lysed for Western blotting for 1 h on ice in 1% Triton X-100 and 0.1% SDS containing protease inhibitors (BD Pharmingen) as previously described (17). In some cases, lysates were treated with PNgase F’ (New England Biolabs) as per the manufacturer’s instructions with the modification of boiling substituted with a 30-min incubation at room temperature to remove glycosylation. Equivalent protein concentrations were loaded on SDS-PAGE gels (Bio-Rad) and probed with primary Abs, rabbit anti-Glut1 (Abcam), mouse anti-actin (Sigma-Aldrich), and rabbit anti-phospho-ser473-Akt (Cell Signaling Technology). Secondary Abs anti-mouse HRP (BD Pharmingen) and anti-rabbit HRP (Cell Signaling Technology) were followed by ECL-Plus (Amersham Biosciences) for visualization.

**Microscopy**

Kidneys were flash frozen in liquid nitrogen embedded in Tissue-Tek OCT Compound (Sakura Finetek) and 12-μm sections were cut, washed in PBS, fixed in 1% parafomaldehyde, blocked with 2% FBS in PBS, and stained with FITC anti-Mouse IgG (BD Pharmingen). Sections were then washed and mounted with Fluoromount-G (Southern Biotechnology Associates) and examined microscopically at ×200 total magnification with a Zeiss Axio Imager upright wide field fluorescence microscope and Metamorph software (Universal Imaging Corporation).

**Glucose uptake**

Glucose uptake was performed as previously described with minor modification (17, 20). In brief, cells were suspended in Kreb’s Ringer HEPES and 2-deoxy-[H-3]glucose (2-Deoxy-2-[H]glucose) was added for 10 min at 37°C. The reactions were quenched with ice-cold 200 μM chloroform (Calbiochem), washed to separate cells from remaining radioactivity, and cells were solubilized with 1 M NaOH before measurement of radioactivity transported into cells with a scintillation counter.

**Bone marrow reconstitution**

C57BL/6J mice were injected with 5 mg fluorouracil (American Pharmaceutical Partners) 4 days before isolation of whole bone marrow. Bone marrow was cultured in DMEM (Invitrogen Life Technologies) with Pen Strep, glutamine, 15% FBS, 5% Wehi-3B conditioned media, 10 ng/ml IL3 (eBioscience), 50 ng/ml Stem Cell Factor (PeproTech), and 10 ng/ml IL6 (PeproTech). Cells were cultured for 1 day before infection with MSCV-Myc-Glut1 (Myc-Glut1 was a gift from Robert Farese, Université du Québec à Trois-Rivières, Canada). Cells were washed and injected via tail vein into C57BL/6J Rag2−/− (The Jackson Laboratory) mice that had been lethally irradiated the previous day. Cells were allowed to reconstitute mice for 2 mo before analysis.

**Results**

**Costimulation via CD28 is necessary for increased glucose uptake upon T cell activation**

In TCR-stimulated human peripheral T cells, an increase in glucose uptake has been previously demonstrated upon induction of...
the CD28 costimulatory signal when provided by Ab-coated beads (5). Consistent with this finding, glucose uptake in murine T cells increased dramatically at multiple doses of anti-CD3 Ab on coated plates in the presence of anti-CD28-mediated costimulation (Fig. 1A). In contrast, T cells stimulated with only anti-CD3 increased glucose uptake modestly compared with CD28-costimulated cells. These data demonstrate that CD28 signaling can potently increase glucose uptake of TCR-stimulated T cells, but do not demonstrate whether CD28 signaling is required among the myriad signals T cells may receive from APCs to maximally increase glucose uptake. To determine the requirement for CD28 signals to induce glucose uptake, primary T cells were cultured with bone marrow-derived macrophages. T cells were stimulated with anti-CD3 Abs with and without CTLA4-Ig (B) or anti-ICAM Ab (C). After 1 day of coculture, T cells were separated from macrophages and glucose uptake was analyzed (*, p < 0.005 and **, p < 0.01).

FIGURE 1. CD28 is required for maximal glucose uptake in T cell activation. A, Purified T cells were stimulated on plates coated with or without anti-CD28 and with indicated doses of anti-CD3. Glucose uptake was measured after 1 day. B and C, T cells were cocultured with LPS-stimulated bone marrow-derived macrophages. T cells were stimulated with anti-CD3 Abs with and without CTLA4-Ig (B) or anti-ICAM Ab (C). After 1 day of coculture, T cells were separated from macrophages and glucose uptake was analyzed (*, p < 0.005 and **, p < 0.01).

Glucose uptake in lymphocytes is mediated largely by the glucose transporter, Glut1 (8, 14). Changes in glucose uptake mediated by CD28 signaling may therefore be controlled through changes in expression or surface levels of Glut1. Low levels of Glut1 were expressed in unstimulated T cells and T cells stimulated with low concentrations of anti-CD3 (Fig. 2A). CD28 costimulation, however, elevated Glut1 protein levels in weakly stimulated T cells. With stronger anti-CD3 stimulation, Glut1 protein was induced in the absence of CD28 costimulation. CD28 costimulation may have additionally influenced Glut1 modification as Glut1 induced in the presence of CD28 demonstrated a lower mobility by SDS-PAGE at both anti-CD3 doses. This altered mobility may suggest enhanced Glut1 glycosylation due to altered protein trafficking (26). To determine whether this modification was due to elevated glycosylation, cell lysates were treated with the glycosylase, PNGase F. This treatment increased Glut1 mobility (Fig. 2B) indicating that costimulation elevated both levels and glycosylation of Glut1 protein. Enhanced glycosylation may suggest altered Glut1 intracellular trafficking and has been shown to increase glucose uptake activity (27, 28). Consistent with this regulation of Glut1 expression, blockade of CD28 signals on T cells stimulated by activated macrophages and anti-CD3 also showed that CD28 was necessary to maximally induce Glut1 in T cells receiving a weak TCR signal, but played no role in Glut1 up-regulation when a strong TCR signal was produced (Fig. 2C).

In addition to expression, Glut1 trafficking to the cell surface must be controlled (17) and regulation of glycosylation suggested CD28 may mediate this signal. To investigate costimulation-induced regulation of Glut1 surface levels, hematopoietic stem cells were retrovirovically transduced to express a Myc-tagged Glut1, and used to reconstitute the immune systems of lymphopenic mice. This approach allowed for fixed Glut1 expression from an exogenous promoter and detection of cell surface Glut1 via the Myc tag in cells derived from infected HSCs and for determination of the role of CD28 in the regulation of Glut1 cell surface trafficking. T cells purified from reconstituted mice and stimulated with a low or a high dose of anti-CD3 with or without CD28 costimulation produced equivalent amounts of Myc-Glut1 protein (Fig. 2D). However, addition of the CD28 costimulatory signal increased surface levels of Myc-Glut1 (Fig. 2E). Together, these data indicate that costimulatory signals are necessary to augment Glut1 expression in weakly TCR-stimulated cells and are necessary to promote maximal Glut1 cell surface levels regardless of TCR signal strength.

Glucose is required for maximal immune response

T cells can use both glucose and glutamine as carbon sources in cellular metabolism (3, 4). Therefore, it was possible that elevated glucose metabolism may not be required for T cell growth and metabolism upon activation and other carbon sources may be sufficient to meet cellular demands. To determine the relative importance of glucose uptake in T cell survival and function during stimulation for increased glucose uptake (Fig. 1B). If anti-CD3 was present in the culture, T cell glucose uptake was markedly increased after 1 day. Addition of CTLA-4 Ig to TCR-stimulated T cell/macrophage cocultures even at high doses of anti-CD3, resulted in a significant decrease in T cell glucose uptake (p < 0.01) relative to cultures that did not receive CTLA4-Ig. Although CD28 is necessary for maximal increases in glucose uptake, it was not uniquely required, as blockade of ICAM signaling also reduced TCR-stimulated glucose uptake in macrophage cocultures (Fig. 1C).

Costimulation increases Glut1 protein levels and trafficking to the cell surface

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activation, freshly purified resting T cells were unstimulated or stimulated with anti-CD3 Abs with or without anti-CD28 in media containing various concentrations of glucose at or below physiological levels that was supplemented with 2 mM glutamine. Cells that received no stimulation survived poorly after 2 days regardless of glucose availability (Fig. 3A). Stimulated T cells also failed to survive in the absence of glucose, but required only very low glucose concentrations to maintain viability. CD3-stimulated cells survived well at glucose levels as low as 0.05 mM and addition of the CD28 costimulatory signal further increased survival. Similarly to cell survival, T cells required glucose to produce IL-2, but a very low glucose concentration was sufficient to elicit maximal IL-2 secretion in T cells stimulated by anti-CD3 and anti-CD28 (Fig. 3B). No IL-2 was produced in unstimulated cells and very little IL-2 was secreted by CD3-stimulated T cells in the absence of CD28 costimulation at any glucose concentration. In contrast to cell survival and IL-2 production, T cell proliferation of stimulated cells was highly dependent on glucose availability (Fig. 3C). T cells stained with CFSE and stimulated as described above with two different doses of anti-CD28 proliferation was detected in cells stimulated with anti-CD3 with and without 5 μg/ml anti-CD28 for 1 day, and cells were immunoblotted for Myc-Glut1 levels (D) and stained with anti-Myc to detect surface levels of Glut1 by flow cytometry (E). Means and SDs of five samples are shown (*, p < 0.05).

**FIGURE 2.** Costimulation induced Glut1 protein expression and trafficking to the cell surface. A and B, Purified T cells were stimulated on plates coated with anti-CD3 and anti-CD28 Abs at the indicated concentrations for 1 day before lysis and (A) immunoblotting and (B) PNGase F treatment to deglycosylate followed by immunoblot. Numbers indicate the quantification of the deglycosylated Glut1 normalized to Actin control. C, T cells were cocultured with LPS-stimulated bone marrow-derived macrophages and stimulated with anti-CD3 Abs with and without CTLA4-Ig for 1 day before lysis and immunoblotting. D and E, T cells from mice reconstituted with hematopoietic stem cells infected with a Myc-tagged Glut1 were purified, stimulated on plates coated with the indicated dose of anti-CD3 with and without 5 μg/ml anti-CD28 for 1 day, and cells were immunoblotted for Myc-Glut1 levels (D) and stained with anti-Myc to detect surface levels of Glut1 by flow cytometry (E). Means and SDs of five samples are shown (*, p < 0.05).

**FIGURE 3.** T cells require glucose for cell survival, IL-2 production, proliferation, and IFN-γ production. Purified T cells were stained with CFSE, stimulated with 5 μg/ml anti-CD3 with or without 1 μg/ml or 5 μg/ml anti-CD28 in glucose-free media supplemented with glucose to the indicated concentrations, and cultured for 2 days before analysis. A, Cell survival was determined by propidium iodide exclusion as analyzed by flow cytometry. B, IL-2 production was assayed through ELISA on T cell supernatants. C, Reduction in CFSE staining was used to determine cell division by flow cytometry. D, IFN-γ production was assayed through ELISA on T cell supernatants.

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proliferating more than those that received a lower dose of co-stimulation and despite continued IL-2 production, failed to proliferate at lower glucose concentrations. Glucose availability also affected the ability of stimulated T cells to produce the inflammatory and Th1-promoting cytokine IFN-γ. IFN-γ was undetectable in media from unstimulated cells and low in CD3-only stimulated cells regardless of glucose concentration (Fig. 3D). CD3-stimulated and CD28-costimulated cells produced IFN-γ with cells exposed to a low dose of anti-CD28 producing only slightly more IFN-γ than CD3 only stimulated cells, but unlike the nearly glucose-independent regulation of IL-2 secretion, IFN-γ levels decreased with reduced glucose concentration. These data from naïve T cells support the findings of Cham et al. (11), who demonstrated with activated T cell blasts that IFN-γ production is more sensitive to glucose availability than the production of IL-2. Therefore, regulation of glucose uptake is critical for proper immune function in both initial T cell activation and in effector T cell populations.

Glut1 over-expression does not alter T cell development

Diminished glucose availability led to impaired T cell proliferation and IFN-γ production following activation. To determine whether glucose uptake is rate-limiting for proper T cell development, homeostasis, or activation, the role of increased glucose uptake capacity was analyzed in transgenic animals that overexpress Glut1 specifically in T cells (25). Glut1 protein expression was elevated in both thymocytes and peripheral T cells from Glut1 transgenic animals compared with nontransgenic littermates (Fig. 4A). Transgenic expression of Glut1 increased glucose uptake capacity compared with nontransgenic cells in both thymocytes and mature T cells, with a larger effect in peripheral T cells (Fig. 4B). This increase in glucose uptake did not appear to alter T cell development, as percentages of thymic subpopulations and CD4 to CD8 ratios were normal in young mice (Fig. 4C). Total thymocyte cell numbers and the number of splenic CD4 and CD8 T cells and B cells were also not significantly altered by Glut1 overexpression (data not show, and Fig. 4D). Further, Glut1 expression and its associated increase in glucose transport did not affect the activation phenotype of resting peripheral T cells. Flow cytometric analysis of CD25 and CD44 levels on CD4 and CD8 T cells showed little difference between Glut1 transgenic and nontransgenic littermates (Fig. 4E). These data suggest that glucose uptake was not limiting in developing or resting T cells.
matched IFN-γ production of Glut1 transgenic T cells. These data show that increased expression of Glut1 is sufficient to augment both T cell growth and cytokine secretion upon stimulation, demonstrating that glucose uptake is a limiting factor for T cell growth and stimulation.

**Chronic exposure to Glut1 over-expression results in increased T cell activation**

Despite the generally normal phenotype of young Glut1-transgenic mice, the enhanced in vitro activation of Glut1-transgenic T cells suggested that T cells may accumulate a previously activated phenotype in vivo with age. To investigate this possibility, 1-year-old Glut1 and nontransgenic littersmates were analyzed. Transgenic mice showed no overt signs of illness, yet flow cytometric analysis demonstrated an increased percentage of CD4+ T cells with elevated levels of the activation markers CD25, CD44, and CD69 in old Glut1 transgenic mice compared with age-matched nontransgenic mice (Fig. 6A). An increase in CD44 staining was also observed in Glut1 transgenic CD8+ T cells. Consistent with a previously activated or memory phenotype in vivo, in vitro stimulation of aged Glut1 T cells resulted in greatly increased production of both IL-2 and IFN-γ (Fig. 6, B and C; ND, not detectable). T cells from Glut1 transgenic mice produced more of both cytokines than nontransgenic cells following a 1-day stimulation with CD3 alone, as well as with CD28 costimulation.

To further determine the impact of Glut1 over-expression on immune homeostasis, levels of immunoglobulin isotypes were determined in the serum of 1-year-old Glut1 transgenic mice and nontransgenic littersmates. All Ig isotypes were higher in the Glut1 transgenic mice than nontransgenic littersmates (Fig. 6D). In addition, Glut1 transgenic mice had increased Ig deposits in the kidneys (Fig. 6E). Together, these data demonstrate that glucose uptake is a limiting aspect of T cell activation and that increased Glut1 expression is capable of promoting accumulation of memory-phenotype T cells and possible immune pathology in aged animals.

**Akt signaling plays a role in the regulation of glucose uptake but cannot substitute for CD28 signaling effects on glucose uptake**

These data show that the increase of glucose uptake that occurs upon CD28-mediated costimulation is vital for a proper immune response, and chronically increased uptake alone is capable of promoting T cell activation, particularly in aged mice. However, it remained unclear how glucose uptake was up-regulated by CD28 costimulation. Activation of the PI3K/Akt pathway has been implicated as a transducer of CD28 signals to increase glucose uptake following T cell stimulation (5). Therefore, we sought to determine the role of the Akt pathway in CD28-dependent up-regulation of Glut1 expression and glucose uptake. T cells were purified from Akt1+/− animals and stimulated with a low level of anti-CD3 with or without CD28 costimulation. Despite the continued presence of Akt2 and Akt3, which are expressed in T cells and can also regulate glucose uptake (29), glucose uptake appeared to be reduced in Akt1−/− costimulated T cells compared with Akt1+/− T cells (Fig. 7A), suggesting a role for the Akt isoforms in T cell glucose uptake. To determine the sufficiency of Akt signaling in CD28-mediated regulation of glucose uptake, T cells were isolated from transgenic mice that express constitutively active mAkt in T cells and from nontransgenic littersmates. Expression of the mAkt transgene has been shown to decrease T cell dependence on CD28 for cell growth, cytokine production, and proliferation (24). Akt activation due to transgenic expression was comparable or higher than that achieved in nontransgenic cells by CD3 stimulation and
CD28-costimulation as determined by phospho-Akt levels 1 h after stimulation (Fig. 7B). Therefore, transgenic mAkt expression provided a model to analyze the role of this pathway in CD28 signaling to stimulate glucose uptake.

Akt may promote glucose uptake through multiple mechanisms that include both regulation of protein expression as well as posttranslational control of Glut1 protein trafficking and activity (17, 19, 30, 31). Therefore, Glut1 protein levels were observed in resting and stimulated T cells from non- or mAkt-transgenic mice. Expression of mAkt was found to have no discernible effect on total Glut1 protein levels in unstimulated, stimulated, or costimulated T cells after one day (Fig. 7C). Although mAkt did not appear to affect Glut1 protein levels, Glut1 glycosylation was regulated by PI3K activity as treatment of cells with the PI3K inhibitor

FIGURE 6. Glut1 over-expression led to accumulation of memory-phenotype T cells in aged mice. A, Resting T cells purified from Glut1 transgenic and age-matched nontransgenic animals >1 year old were stained with fluorescently labeled Abs to detect the T cell activation markers CD25, CD44, and CD69 and analyzed by flow cytometry. B and C, Resting T cells purified from Glut1 transgenic and age-matched nontransgenic animals >1 year old were stimulated with 1 μg/ml anti-CD3, with or without 5 μg/ml anti-CD28, for 1 day and supernatants were collected and analyzed for cytokine production by ELISA (*, p < 0.05; **, p < 0.005; ND = Not Detected). D, Serum from representative aged-matched Glut1 transgenic and nontransgenic mice was analyzed by ELISA for Ig isotypes (*, p < 0.04; **, p < 0.01). E, Kidney sections from 8-wk-old and 1-year-old aged matched Glut1 transgenic and nontransgenic mice were stained for anti-Mouse Ig and examined microscopically.
LY294002 (LY) resulted in a decrease in the mobility shift normally seen in costimulated T cells at both the low and high dose of anti-CD3 (Fig. 7D). Despite the apparent inability of Akt activity to alter Glut1 protein levels, glucose uptake of unstimulated mAkt T cells was significantly higher than that of unstimulated nontransgenic T cells (p < 0.02) (Fig. 7E). These data suggest that Akt may not regulate Glut1 expression, but consistent with the role of Akt to regulate Glut1 trafficking in response to cytokine signaling (17, 21, 32), may increase Glut1 surface trafficking or glucose uptake activity.

The ability of Akt to stimulate Glut1 trafficking and activity may comprise a significant portion or all CD28-mediated glucose uptake. To determine whether Akt activation was sufficient to replace CD28-mediated glucose uptake in T cell activation, nontransgenic and mAkt transgenic T cells were stimulated for 1 day and glucose uptake was analyzed (Fig. 7F). In the absence of CD28 costimulation, a low dose of anti-CD3, which was incapable of inducing Glut1 protein (Fig. 2A), modestly increased glucose uptake in nontransgenic T cells. Glucose uptake of CD28-costimulated T cells increased compared with CD3-only stimulated cells. Importantly, mAkt expression was not capable of mimicking CD28 costimulation and failed to induce additional glucose uptake in T cells stimulated with a low dose of anti-CD3 alone. In contrast, at higher doses of anti-CD3 where Glut1 protein was induced in the absence of CD28 costimulation (Fig. 2A), Akt activation was sufficient to augment glucose uptake to an extent similar to that seen in nontransgenic costimulated cells. Therefore, while Akt signals may play an important role in CD28-mediated glucose uptake, Akt is not sufficient to replace CD28-mediated increases in glucose uptake in weakly TCR-stimulated cells. Other TCR or CD28-stimulated pathways, such as those that lead to Glut1 induction or modification, are required for Akt to promote glucose uptake.

Glut1 and Akt additively increase T cell stimulation
As Akt did not lead to induction of Glut1 protein in CD3-stimulated cells, we sought to determine whether directly increased expression of Glut1 may alter the role of Akt in T cell costimulation. For this purpose, Glut1/mAkt double transgenic animals were generated and T cells from double transgenic mice were compared with Glut1 and mAkt single transgenic and nontransgenic littermates. Importantly, double transgenic cells demonstrated a synergistic increase in glucose uptake compared with either single transgenic (Fig. 8A). Resting T cells purified from young nontransgenic, Glut1, mAkt, and Glut1/mAkt transgenic mice also had genotype-dependent variations in cell size as determined by a particle size analyzer (Fig. 8B). Glut1 and mAkt transgenic cells were similar in size to each other yet larger than nontransgenic cells. The increased T cell size and glucose uptake observed in the presence of Akt activation or Glut1 over-expression suggested that these two pathways may synergize to promote cell growth. Consistent with this notion, double transgenic T cells were significantly larger than...
cells from either single transgenic ($p < 0.005$ and $p < 0.01$). In addition, T cells in Glut1/mAkt double transgenic mice had a more memory-like phenotype than either single transgenic. As shown above (Fig. 4E), expression of the Glut1 transgene alone did not alter expression of CD25 or CD44 on resting CD4 T cells. As previously reported, CD4 T cells expressing the mAkt transgene had increased expression of CD44 (Fig. 8C) (24). However, double transgenic CD4 T cells had higher expression of CD25, CD44, and CD69 activation markers than either single transgenic. These data indicate that increases in Glut1 protein levels and Akt signaling combined to further augment glucose uptake, T cell activation, and accumulation of previously activated T cells.

In addition to increased activation of T cells in vivo, transgenic expression of Glut1 and mAkt enhanced T cell activation after in vitro stimulation and further reduced T cell dependence on CD28 costimulation. To examine T cell activation, T cells from non, mAkt, Glut1, and mAkt/Glut1 transgenic mice were labeled with CFSE, stimulated with 5 μg/ml anti-CD3, with and without 5 μg/ml anti-CD28, for 3 days, and levels of CFSE depletion to indicate cell proliferation were determined by flow cytometry.

**Discussion**

In this study, we address the role of glucose uptake in T cell development, homeostasis, and activation as well as explore the mechanism by which the CD28 costimulatory signal regulates glucose uptake. Limiting glucose uptake resulted in decreased T cell proliferation and IFN-γ production, while increasing Glut1 protein levels through transgenic expression did not appear to affect T cell development or homeostasis, but did result in increased cell size and cytokine production upon activation. In addition, aged Glut1 transgenic mice showed increased Ig levels in the serum and deposition in the kidney. We implicate the involvement of the PI3K/Akt pathway (5, 24), and demonstrate that other CD28-signaling pathways are also required to augment TCR-mediated induction of Glut1. In particular, constitutive activation of Akt was sufficient to increase glucose uptake in resting T cells, but was insufficient to...
achieve levels of glucose uptake seen during costimulation unless Glut1 protein was up-regulated independently by strong TCR signals. This suggested that Akt activation downstream of CD28 signaling may regulate Glut1 surface localization or activity, but a separate pathway was necessary to up-regulate Glut1 protein. Together, these data indicate that regulation of glucose uptake is a critical aspect of T cell activation, and that Akt-independent regulation of Glut1 expression and Akt-dependent regulation of Glut1 trafficking and glucose uptake activity may account for the central role of CD28 in glucose metabolism of activated T cells.

This work delineates a vital role for glucose and glucose uptake in immunity. Despite availability of other nutrients, glucose was required for increased growth, IL-2 production, and proliferation upon T cell stimulation. Conversely, increased T cell activation that occurred when Glut1 was over-expressed suggested that glucose uptake is normally limiting in T cell stimulation. It should be noted, however, that in addition to decreased glucose metabolism, glucose limitation can lead to ER stress and activation of 5′AMP-activated protein kinase that may complicate these analyses (33). Nevertheless, increased activation of Glut1 transgenic T cells demonstrates that glucose uptake is normally insufficient to allow maximal immune response. The extensive increase (>10-fold) in glucose uptake that occurs after 1 day of stimulation (Fig. 1A) relative to the increase glucose uptake provided by the Glut1 transgene (3–4-fold; Fig. 4B) suggests that glucose may be particularly limiting in the early phases of T cell activation. Consistent with glucose limitation early in T cell activation, Tamás et al. (34) recently showed an important role for 5′AMP-activated protein kinase activation following TCR stimulation, which may promote more efficient ATP generation or use of alternative fuels while Glut1 protein is synthesized and glucose uptake increases to meet cellular demands. Although increases in energy availability are necessary for early T cell activation, maintaining the appropriate balance of nutrient uptake and energy production is also critical for T cell homeostasis as increased glucose uptake led to possible immune pathology with age.

Blockade of costimulation with CTLA4-Ig or anti-ICAM on CD3-stimulated T cells cultured on LPS-stimulated macrophages prevented the maximal increase of glucose uptake in T cells. Despite the wide variety of other cell-cell interactions and soluble factors that LPS-activated macrophages may provide, these costimulation pathways provide a necessary combination of signals for the regulation of T cell glucose uptake upon activation. Common features of costimulation include enhancement of TCR signals and activation of PI3K/Akt. The combination of these signaling pathways may be critical for up-regulation of glucose uptake. Conversely, receptors that decrease these signaling pathways, such as CTLA4, may decrease glucose uptake. Although signals downstream of CD28 that regulate glucose metabolism are not entirely clear, the PI3K/Akt pathway appears to play a critical role. The Akt pathway is well known to control trafficking of the insulin-sensitive glucose transporter Glut4 (35) and we have directly shown Akt regulation of Glut1 cell surface trafficking in a lymphoid cell line in response to cytokine signaling (17, 32). Akt has also been shown to regulate cell surface trafficking of Glut1 in the mammary gland and was required to meet the increased metabolic demand associated with lactation (36). In addition to regulation of cell surface levels, Akt may also control Glut1 transporter activity through activation of the mammalian target of rapamycin (17). Glut1 glycosylation also appeared to be PI3K-dependent, although Akt activation was not sufficient to substantially alter this Glut1 modification. Therefore, Akt may play a critical role in response to costimulation to promote trafficking of Glut1 protein to the cell surface and to stimulate Glut1 activity.

The capacity of Akt signaling to control Glut1 localization or activity may combine with pathways that up-regulate or modify Glut1 to jointly mediate CD28-induced glucose uptake. In addition to activation of the PI3K/Akt pathway, CD28 also augments CD3 signals that may play a critical and underappreciated role to up-regulate and modify Glut1 protein. Other likely signaling pathways that may regulate Glut1 protein levels downstream of CD28 include activation of the TEC kinases, TEC and ITK, which can play a role in positive regulation of PLC-γ (37). Another signaling pathway that may link CD28 to increased Glut1 protein levels may be the small GTPase GEF, Vav1 (38). Vav1 associates with the membrane in a PI3K-independent mechanism (39) and is known to potently induce activation of NFAT, NF-κB, and AP1 (40). Each of these transcription factors could be responsible for induction of Glut1 protein expression downstream of CD28 and explain how CD3 signals alone, when strong enough, are capable of inducing increased Glut1 expression.

Increased glucose uptake may benefit T cell activation through a number of mechanisms. T cell stimulation increases the intracellular need for ATP and other high energy molecules (41). This need is met primarily by an increase in glycolysis, resulting in substantial lactate production (3). However, glycolysis is not the only cellular metabolic pathway that may benefit from increased intracellular glucose levels. The pentose phosphate pathway, which produces pentose sugars required for nucleic acid synthesis and NADPH for reducing power and lipid synthesis, is also up-regulated in response to T cell activation (42). In addition, increased glucose metabolism may alter signaling pathways to enhance activation and survival. In both hematopoietic cell lines and Glut1 transgenic T cells, we have shown elevated levels of glucose metabolism to lead to increased levels of phospho-GSK3α/β (25). This inhibitory phosphorylation of GSK3 may augment cellular activation by reducing nuclear export of transcription factors such as NFAT (43) as well as protease against apoptosis by stabilizing the anti-apoptotic Bcl-2 family member Mcl-1 (25, 44). Together, these pathways establish signaling pathway coordination between nutrient sensing, proliferation, and cell survival.

The evidence presented in this study implicates regulation of glucose uptake and Glut1 as critical features of immune function. In diabetes research, it has become clear that pathways that regulate glucose transporter translocation are a central aspect of insulin-dependent promotion of metabolism and growth of insulin-responsive tissues. T cells do not typically respond directly to insulin, yet comparable to insulin regulation of Glut4, CD28 provides a signal to T cell metabolism and growth that is mediated by induction of Glut1 and regulation of Glut1 trafficking. Similar to T cell activation, cancer cells also often show elevated Glut1 expression and glycolytic metabolism (45, 46). Understanding the regulatory pathways underlying this metabolic phenotype may therefore provide useful insight into immunological and neoplastic diseases, as well as illustrate the critical role that nutrition may have on immune function.

Acknowledgments
We thank Dr. Kenneth Frauwirth, Emily Ferguson, and Nicholas Holifield for critical evaluation of this manuscript and helpful comments, and Brian Emmenegger and Dr. Robert Wechsler-Reya for help with tissue sectioning. We would also like to thank Department of Pharmacology and Cancer Biology shared microscopy facility and the Duke University Flow Cytometry Shared Resource.

Disclosures
The authors have no financial conflict of interest.
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References

12. 2000. IL-7 promotes Glut1 trafficking and glucose uptake via STAT5-mediated activation of Akt to support T cell survival. Blood 111: 2101–2111.