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J Immunol 2008; 181:8363-8371; doi: 10.4049/jimmunol.181.12.8363
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Antigenic Experience Dictates Functional Role of Glycogen Synthase Kinase-3 in Human CD4+ T Cell Responses

Carlos A. Garcia,* Manjunatha R. Benakanakere,† Pascale Alard,* Michelle M. Kosiewicz,* Denis F. Kinane,† and Michael Martin2*†

Signals induced by the TCR and CD28 costimulatory pathway have been shown to lead to the inactivation of the constitutively active enzyme, glycogen synthase kinase-3 (GSK3), which has been implicated in the regulation of IL-2 and T cell proliferation. However, it is unknown whether GSK3 plays a similar role in naive and memory CD4+ T cell responses. Here we demonstrate a divergence in the dependency on the inactivation of GSK3 in the proliferative responses of human naive and memory CD4+ T cells. We find that although CD28 costimulation increases the frequency of phospho-GSK3 inactivation in TCR-stimulated naive and memory CD4+ T cells, memory cells are less reliant on GSK3 inactivation for their proliferative responses. Rather we find that GSK3β plays a previously unrecognized role in the selective regulation of the IL-10 recall response by human memory CD4+ T cells. Furthermore, GSK3β-inactivated memory CD4+ T cells acquired the capacity to suppress the bystander proliferation of CD4+ T cells in an IL-10-dependent, cell contact-independent manner. Our findings reveal a dichotomy present in the function of GSK3 in distinct human CD4+ T cell populations. The Journal of Immunology, 2008, 181: 8363–8371.

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ostimulation, in the form of either soluble mediators or cell ligand-to-receptor interactions has been shown to potently augment the responsiveness of TCR-stimulated CD4+ T cells (1). Initial work on costimulation led to the discovery of the B7/CD28 pathway, and ultimately to the confirmatory studies performed in CD28- and B7-deficient mice which altogether have underscored the importance of this pathway in adaptive immune responses (1). Subsequent work regarding the CD28 costimulatory pathway identified striking differences between naive and memory CD4+ T cells with respect to their dependency on CD28 costimulation for proliferative responses (2). In this regard, naive CD4+ T cell proliferation has been shown to be dependent on CD28 costimulation, whereas memory cell proliferation is less dependent on CD28 costimulation and can occur in its absence (2). Signaling differences between naive and memory CD4+ T cells have been reported (3, 4); however, the biochemical reasons for this differential requirement for CD28 remain ill defined. However, several laboratories have reported that the CD28 signaling pathway can still influence memory CD4+ T cell function (5, 6), thus raising the question of whether CD28 triggers distinct signaling pathways in memory CD4+ T cells and whether these signaling complexes play similar or distinct roles in naive and memory CD4+ T cells.

Although the downstream signaling pathways induced by CD28 are incompletely understood, CD28 costimulation has been shown to increase PI3K activity of which leads to the inactivation of the constitutively active serine/threonine kinase, glycogen synthase kinase-3 (GSK3) (7–12). Pharmacological inactivation of GSK3 in human T cells has been shown to mimic the CD28 costimulatory signal for T cell proliferation (9, 10). Although it is unclear exactly how GSK3 controls T cell proliferation, retrovirus-mediated expression of a constitutively active GSK3β mutant in murine TCR-transgenic CD8+ T cells also results in decreased proliferation with a concurrent suppression in IL-2 production upon TCR stimulation (13). In addition, both CD28 costimulation and pharmacological inhibition of GSK3 have been reported to promote the degradation of the cyclin-dependent kinase inhibitor p27kip1 in human CD4+ T cells, an event favoring the G1-S phase transition of TCR-stimulated CD4+ T cells (9). Altogether, these findings suggest GSK3 is a prominent target of CD28 signaling which influences p27kip1 stability, IL-2 production, and T cell proliferation.

Because naive and memory CD4+ T cells differ with regard to their dependence on CD28 costimulation for proliferation and because GSK3 inactivation can compensate for certain phenotypic properties associated with CD28 costimulation, we investigated the regulation and dependency on GSK3 inactivation in human naive and memory CD4+ T cell-proliferative and effector cytokine responses. We report that although CD28 signaling amplifies the cellular levels of inactivated GSK3 in both naive and memory CD4+ T cell populations, memory cells are less reliant on GSK3 inactivation for their proliferative responses. Rather, we find that GSK3 activity plays an additional role in memory CD4+ T cells by regulating their IL-10 recall response.

Materials and Methods

Media

Cells were cultured in RPMI 1640 supplemented with 10% FBS, 50 μM 2-ME, 1 mM sodium pyruvate, 2 mM L-glutamine, 20 mM HEPES, 50 μM penicillin, and 50 μg/ml streptomycin.

Received for publication July 28, 2008. Accepted for publication October 15, 2008.

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1 This research was supported by National Institute of Dental Research Grant RO1DE017680.

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Reagents

Anti-CD3/CD28-coupled and CFSE were purchased from Invitrogen. Anti-IL-10 (JES-5D7), anti-CD4, anti-CD45RA, anti-CD45RO, rat IgG1, rat IgG2a, anti-CD3 (OKT3), anti-CD28 (CD28.2), 7-aminocoumarin-conjugated 7-AAD, and IL-10 ELISPOT kits and IL-4, IL-5, IL-17A, IFN-γ, IL-10, and TGF-β ELISA kits were obtained from eBioscience. Anti-IL-10R1 was purchased from BD Pharmingen. SB216763, LiCl, and wortmannin were purchased from Tocris, Calbiochem, and LC Laboratories, respectively. Nontargeting pools of small interfering RNA (siRNA) and a mixture of four prevalidated siRNA duplexes specific for GSK3α and GSK3β (ON TARGET-plus) were purchased from Dharmacon. TaqMan probes were purchased from Applied Biosystems. Western blot Abs and flow cytometric Abs against phospho-GSK3 (T21) or (S21) were obtained from Cell Signaling Technologies.

Isolation of PBMCs and T cells

PBMCs were obtained from the venous blood of normal adult donors after isolation of the leukocyte layer and elimination of RBCs using a Ficoll density gradient (University of Louisville (Louisville, KY), Institutional Review Board, Human Subjects Protection Program, study 503.05). Naive, memory, and unfrac- tionated CD4⁺ T cells were purified from PBMCs by negative selection using kits from Miltenyi Biotech (>97.5% purity).

T cell stimulations

T cells (1 × 10⁶ cells) were pretreated for 2 h with media containing DMSO (0.1%), wortmannin, NaCl, LiCl, or SB216763 at the indicated final concentration and transferred to plates preincubated with PBS, anti-CD3, or anti-CD3 and anti-CD28 (10 µg/ml). Precoated anti-CD3/CD28 plates were prepared by incubating the tissue culture plate overnight at 4°C with PBS containing the indicated concentration of anti-CD3/CD28 Abs.

Flow cytometric analysis of phospho-GSK3

Purified CD4⁺ T cells were surface stained with anti-human CD4 and anti-human CD45RA for 10 min at room temperature and washed twice with medium. Cells were then counted and plated at 2.5 × 10⁶ cells/well in a 96-well flat bottom plate and pretreated with DMSO (0.1%) or wortmannin (100 nM) for 30 min and then transferred to plates precoated with DMSO, anti-CD3, or anti-CD3 and anti-CD28 (10 µg/ml). At the indicated time point, cells were transferred to 5-ml polystyrene round-bottom tubes and fixed by adding formaldehyde to a final concentration of 4% directly into the medium. The reaction was stopped by adding FCS to a final concentration of 10%. Cells were washed once in PBS, resuspended in 90% methanol, and incubated on ice for 10 min. Cells were washed in PBS containing 2% FCS, then resuspended in PBS containing 2% FCS and anti-phospho-GSK3 (S9/21) Ab, and incubated at room temperature for 30 min. Cells were washed twice in PBS containing 2% FCS and analyzed immediately by flow cytometry.

Cellular expansion and survival assays

Memory CD4⁺ T cells were pretreated with 6 µM SB216763 or DMSO (0.01%) before stimulation for 2 h. Cells were then transferred to 96-well flat-bottom plates coated with PBS or anti-CD3 (10 µg/ml). On the indicated day, each group containing triplicate samples was counted individually using a hemocytometer. Cells were then treated with 7-AAD and resuspended in PBS. Cells were electronically gated for a minimum of 10⁵ events, and 7-AAD was added with the capture Ab. ELISPOTs were developed on day 4 postculture and analyzed by Cellular Technology.

Transfections and immunoblots

Memory CD4⁺ T cells were transfected using the Amaxa Nucleofection apparatus and the Amaxa Nucleofector kit for unstimulated T cells according to the manufacturer’s instructions. Briefly, siRNA-treated memory CD4⁺ T cells (5 × 10⁶/100 µl) were mixed with 1 µg of the indicated siRNA. Samples were then transferred to cuvetts and transected using program V-24. Cells were rested in medium in the absence of any stimulus; on day 3, cells were washed, counted, and resuspended in fresh medium and stimulated with medium or plate-bound anti-CD3. Viability among the transfected groups was monitored by flow cytometry using 7-AAD and annexin V. GSK3α and GSK3β total protein levels were assessed on day 3 cells by Western blot. Whole-cell lysates were prepared as previously described (14). Twenty micrograms of total cellular protein from each group was suspended in lithium dodecyl sulfate buffer, heated for 10 min at 70°C, resolved by polyvinylidene difluoride membranes using the Novex system (Invitrogen). Probing and visualization of immunoreactive bands were performed using the ECL Plus kit (Amersham Pharmacia) following the manufacturer’s protocol. Images were acquired using the Kodak Image Station 4000MM system (Eastman Kodak).

Real-time PCR

RNA extraction and first-strand cDNA synthesis was performed using the Prime Script® PerfectRealtime PCRs (GIBCO). Real-time PCR was performed using an ABI 7500 system. GAPDH mRNA levels were determined for each time point and were used as the endogenous control. Fold increase was calculated according to the 2^(-ΔΔCt) method (15).

Suppression assays

Day 4 cell-free supernatants from nonstimulated and stimulated memory CD4⁺ T cells (10 µg/ml immobilized anti-CD3 and DMSO (0.1%) or 6 nM SB216763) were transferred to freshly isolated CD4⁺ T cells and stimulated with anti-CD3/CD28 microbeads (bead-to-cell ratio, 1:5). Proliferation was assessed on day 4 by BrdU incorporation during the last 20 h of culture using the colorimetric BrdU assay (Calbiochem). OD values represent the difference of the mean of the experimental groups and the mean of BrdU-labeled nonsuppressed cells.

Statistical analysis

Data are expressed as mean ± SEM. Statistical significance between groups was evaluated by ANOVA and the Tukey multiple comparison test using the Instat program. Differences between groups were considered significant at p < 0.05.

Results

CD28 costimulation increases the frequency of phosphorylation-dependent GSK3 inactivation in naive and memory CD4⁺ T cells

CD28 costimulation has been shown to increase the cellular levels of inactivated GSK3 in TCR-activated human CD4⁺ T cells (9, 10). This process is dependent on the activation of the PI3K pathway by CD28 which leads to the phosphorylation of GSK3α (αβ) on S21 or S9 (respectively), and repression of GSK3 activity (10, 16). However, previous studies investigating GSK3 function in human T cells utilized unfractionated T cells or unfractionated CD4⁺ T cells (9, 10). Thus, we initially investigated whether human naive and memory CD4⁺ T cells display differences with regard to the ability of CD28 to promote the phosphorylation of GSK3 (S9/21) in the presence of TCR stimulation.

Although no perfect marker(s) exists to absolutely identify human naive and memory CD4⁺ T cell subsets, we nonetheless utilized CD45RA which is routinely used for discriminating naive and memory T cells (17–19). Multiparameter flow cytometric analysis of autologous naive (CD45RA⁺) and memory (CD45RA⁻) CD4⁺ T cells revealed that TCR stimulation increases the frequency of cells containing phospho-GSK3 in both subsets (Fig. 1). The presence of CD28 costimulation further augmented the frequency of naive and memory CD4⁺ T cells containing phospho-GSK3. Pretreatment of CD4⁺ T cells with the PI3K inhibitor, wortmannin, abrogated the ability of anti-CD3/CD28 stimulation to induce the phosphorylation of GSK3, indicating that both naive
and memory CD4+ T cells utilize a similar pathway for the inactivation of GSK3 under these stimulation conditions (Fig. 1).

These findings are consistent with previous observations demonstrating that PI3K activity is required for the phosphorylation of GSK3 on S9/21 in CD45RA+ and CD45RA- populations. Results are representative of at least three individual experiments.

Human naive and memory CD4+ T cell proliferative responses vastly differ on their reliance for GSK3 inactivation

Previous work performed with unfractionated human CD4+ T cells and murine transgenic CD8+ T cells implicated GSK3 as a negative regulator of proliferation as the inactivation of GSK3 augmented the proliferative responses of TCR-stimulated cells (9, 10, 13). Because CD28 costimulation enhanced the S9/21-mediated inactivation of GSK3 in both naive and memory CD4+ T cell subsets, we next investigated whether human naive and memory CD4+ T cell proliferation was similarly dependent on GSK3 inactivation. CD28 costimulation increased the proliferative response of naive CD4+ T cells as compared with cells stimulated solely with anti-CD3. Inhibition of GSK3 using the GSK3-specific inhibitor, SB216763 (20) exhibited effects similar to those of the CD28 costimulatory signal, given that naive CD4+ T cells pretreated with SB216763 also exhibited enhanced proliferative responses in response to stimulation with immobilized anti-CD3 (Fig. 2A). In contrast, the ability of GSK3 inactivation and CD28 costimulation to augment memory CD4+ T cell proliferation was dependent on the strength of the TCR signal (Fig. 2B). At low TCR signal strengths (0.1 μg/ml), memory CD4+ T proliferation was augmented upon GSK3 inactivation or CD28 costimulation, however, these increases were consistently less than that observed with naive cells (Fig. 2B). However, under stronger TCR signals (10 μg/ml; anti-CD3), memory CD4+ T cell proliferation was not significantly different upon GSK3 inactivation or CD28 costimulation.
despite the ability of CD28 costimulation to enhance the levels of phospho-GSK3 at this concentration of anti-CD3 (Fig. 1). The observed differences in the sensitivity of TCR-induced proliferation required for naive and memory CD4+ T cells are in line with the previous published work demonstrating memory CD4+ T cells respond to lower levels of TCR stimulation (21).

Because we observed vast differences regarding the reliance on GSK3 repression for naive and memory CD4+ T cell-proliferative responses and because GSK3 has been previously shown to regulate the production of IL-2 by murine T cells (13), we next examined whether these differences were due to a differential ability of GSK3 to regulate IL-2 production in human naive and memory CD4+ T cells. No detectable level of IL-2 was observed in naive CD4+ T cells upon anti-CD3 stimulation at any concentration tested (Fig. 2C). However, upon CD28 costimulation or inhibition of GSK3, naive CD4+ T cell-derived IL-2 was detected (Fig. 2C). In contrast, IL-2 production by memory CD4+ T cells activated with TCR signals alone was detected and was significantly enhanced by either CD28 costimulation or GSK3 inhibition (at 1 and 10 μg/ml anti-CD3; Fig. 2D). Memory CD4+ T cell proliferation under weak TCR signals (0.1 μg/ml anti-CD3) occurred in the absence of detectable levels of IL-2, suggesting that memory CD4+ T cell proliferation is likely less dependent on IL-2 than their naive CD4+ T cell counterparts in which the detection of IL-2 correlated with proliferative responses (Fig. 2, B and D). Similar results were obtained using LiCl, which also inhibits GSK3 albeit by a different mechanism as compared with SB216763 (data not shown). Thus, despite the decreased dependence on GSK3 inactivation for memory CD4+ T cell proliferation, the production of IL-2 by memory CD4+ T cells remains strongly dependent on GSK3 inactivation.

We further examined naive and memory CD4+ T cell proliferative requirements by CFSE dilution analysis which allows for a more detailed examination of proliferation at the single-cell level. For these experiments, we used anti-CD3 at 10 μg/ml, because this concentration was used in Fig. 1 and induced detectable levels of naive CD4+ T cell proliferation (Fig. 2A). However, for these studies the GSK3 inhibitor SB216763 was replaced with the GSK3 inhibitor LiCl, because SB216763-treated cells exhibited fluorescent properties partially overlapping the fluorescent spectra emitted by CFSE-treated cells. Pretreatment of naive CD4+ T cells with LiCl significantly increased the frequency of cells undergoing at least one cellular division as compared with NaCl-pretreated cells (Fig. 3, A and B). Moreover, naive cells treated with LiCl also
displayed a higher mean cycle number for those cells displaying at least one cellular division than did NaCl-treated cells (Fig. 3, A–C). These differences in proliferation between naive and memory (data not shown) as compared with control cells (Fig. 3, D). Cell lysates were prepared from purified memory CD4+ T cells and the THP-1 cell line (positive control). Blots were initially probed for GSK3β, stripped, and reprobed for GSK3β or total p38 (loading control). E. Relative levels of GSK3β and GSK3β mRNA to GAPDH in resting human memory CD4+ T cells. F. Memory CD4+ T cells were mock transfected or transfected with GSK3β- or GSK3β-specific siRNA. Cellular lysates were prepared on day 3 and analyzed by Western blot. G. Memory CD4+ T cells were mock transfected or transfected with equal amounts of nontargeting siRNA control (CTRL), GSK3β-specific siRNA, or GSK3β-specific siRNA. On day 3, transfected cells were transferred to anti-CD3 coated plates (1 μg/ml) and supernatants were analyzed for IL-10, IL-5, IL-17A, and IFN-γ by ELISA four days later. Results are mean values ± SEM of triplicate cultures and representative of three individual experiments. *, p < 0.05 determined by ANOVA and post hoc Tukey test.

FIGURE 4. GSK3β is the predominant isofrom of GSK3 in human memory CD4+ T cells and suppresses their production of IL-10. A, Memory CD4+ T cells were pretreated for 2 h with DMSO (0.1%) or with the indicated concentration of SB216763 (SB) and then transferred to plates precoated with anti-CD3 (10 μg/ml), and supernatants were analyzed by ELISA on day 4. B, Autologous naive or memory CD4+ T cells were pretreated for 2 h with DMSO (0.1%) alone or with the indicated concentration of SB216763 and then transferred to plates precoated with anti-CD3 (10 μg/ml) and analyzed for IL-10 levels by ELISA on day 4. C, Memory CD4+ T cells were pretreated with DMSO (0.1%), wortmannin (100 nM), or wortmannin and SB216763 (6 μM) and then stimulated for 4 days with plate-bound anti-CD3 (10 μg/ml) alone or in conjunction with anti-CD28 (10 μg/ml). On day 4, cell-free supernatants were analyzed for IL-10 by ELISA. D, Cell lysates were prepared from purified memory CD4+ T cells and the THP-1 cell line (positive control). Blots were initially probed for GSK3α, stripped, and reprobed for GSK3β or total p38 (loading control). E. Relative levels of GSK3β and GSK3β mRNA to GAPDH in resting human memory CD4+ T cells. F, Memory CD4+ T cells were mock transfected or transfected with GSK3β- or GSK3β-specific siRNA. Cellular lysates were prepared on day 3 and analyzed by Western blot. G. Memory CD4+ T cells were mock transfected or transfected with equal amounts of nontargeting siRNA control (CTRL), GSK3β-specific siRNA, or GSK3β-specific siRNA. On day 3, transfected cells were transferred to anti-CD3 coated plates (1 μg/ml) and supernatants were analyzed for IL-5, IL-10, IL-17A, and IFN-γ levels by ELISA four days later. Results are mean values ± SEM of triplicate cultures and representative of three individual experiments. *, p < 0.05 determined by ANOVA and post hoc Tukey test.

Because CD28 costimulation further repressed GSK3 activity in a PI3K-dependent manner (Fig. 1) coupled with the finding that the direct inactivation of GSK3 in the absence of CD28 costimulation augmented IL-10 production upon TCR cross-linking (Fig. 4B), we next investigated whether CD28 costimulation of memory CD4+ T cells could also promote IL-10 production and whether this attribute required PI3K activity. CD28 costimulation of TCR-stimulated memory CD4+ T cells significantly increased IL-10 levels as compared with anti-CD3 stimulation alone (Fig. 4C). Inhibition of the PI3K pathway led to significant reductions in the levels of IL-10 upon CD3/CD28 stimulation, demonstrating that PI3K activity was required for the induction of IL-10 upon CD3/CD28 stimulation (Fig. 4C). The inactivation of GSK3 using the GSK3-specific inhibitor SB216763 in CD3/CD28-stimulated memory CD4+ T cells partially rescued the deficiencies in IL-10 production observed upon PI3K inhibition (Fig. 4C). Thus, these findings suggest that GSK3 is responsible for the ability of the PI3K pathway to regulate IL-10 production by CD3/CD28-stimulated human memory CD4+ T cells.

Because GSK3 can exist as two major isoforms encoded by separate genes (16), GSK3α and GSK3β, we next investigated which isoform was suppressing IL-10 production. GSK3β, but not GSK3α, was detected in whole-cell lysates derived from memory CD4+ T cells by Western blot (Fig. 4D). Moreover, only phospho-GSK3β (S9) was observed in memory cells by Western blot analysis (data not shown). mRNA analysis of resting memory CD4+ T cells confirmed the predominance of the GSK3β isoform (Fig. 4E). We next used siRNA-mediated gene silencing to confirm the functional role of the GSK3β isoform in selectively suppressing IL-10 production from memory CD4+ T cells. The specificity of GSK3β
siRNA pools was confirmed by Western blot given that cells transfected with GSK3β, but not GSK3α-specific siRNAs, exhibited reduced levels of GSK3β protein as compared with mock-transfected cells (Fig. 4F). Knockdown of GSK3β protein using pools of siRNA significantly increased IL-10 production, but not of the other cytokines examined by human memory CD4+ T cells upon TCR activation, as compared with cells transfected with nontargeting siRNA (Fig. 4G). In contrast, cells transfected with pools of GSK3α siRNA did not produce significantly different amounts of IL-10 as compared with cells transfected with nontargeting siRNA (Fig. 4G). These data demonstrate that the GSK3β isoform selectively suppresses IL-10 production by human memory CD4+ T cells.

GSK3 regulates the magnitude of IL-10 production by memory CD4+ T cells

We next assessed whether GSK3 activity influences the steady-state levels of IL-10 mRNA in memory CD4+ T cells. No differences were found in the levels of IL-10 mRNA between GSK3-inactivated and control cells at either 4 or 24 h postactivation (Fig. 5A). However, by 48 h, significantly higher levels of IL-10 mRNA were observed in GSK3-inactivated cells than in cells stimulated in the presence of DMSO. By 72 h, GSK3-inactivated cells contained 23-fold higher levels of IL-10 mRNA than did cells stimulated in the presence of DMSO (0.1%) at 72 h (Fig. 5A).

We next examined the kinetics of IL-10 production by memory CD4+ T cells upon GSK3 inactivation. Significantly higher levels of IL-10 were present in supernatants derived from GSK3-inactivated memory CD4+ T cells than in cells stimulated in the presence of 0.1% DMSO or medium alone on day 3 (Fig. 5B). No difference in the kinetics of IL-10 production between GSK3-inactivated or DMSO (0.1%)-treated memory CD4+ T cells was observed (Fig. 5B). GSK3 inhibition increased IL-10 levels at all concentrations of anti-CD3 tested and the magnitude of IL-10 produced upon GSK3 inhibition was dependent on the strength of the TCR signal (Fig. 5C). Thus, rather than regulate the kinetics of IL-10 production, GSK3 regulates the magnitude of the IL-10 response upon TCR stimulation by memory CD4+ T cells.

Repression of GSK3 activity enhances the productivity and frequency of IL-10-producing memory CD4+ T cells

We next assessed the regulation of IL-10 production by GSK3 at the single-cell level by ELISPOT which measures the secretion of IL-10 throughout the entire duration of the assay. Upon GSK3 inhibition, the frequency of IL-10-producing cells significantly increased by ~300%, as compared with anti-CD3-treated cells (Fig. 6A). GSK3 inhibition also significantly increased the net amount of IL-10 produced per cell (Fig. 6B). Moreover, the absolute number of IL-10-producing memory CD4+ T cells induced upon GSK3 inhibition increased with stronger TCR signals (data not shown).
High IL-10 producing CD4+ T cells can suppress bystander T cell proliferation in an IL-10-dependent, cell contact-independent manner (22). To determine whether GSK3 inhibition promotes regulatory activity via IL-10, cell-free supernatants from activated memory CD4+ T cells stimulated in the presence of GSK3 inhibition were transferred to CD4+ T cells (Fig. 7). CD4+ T cells stimulated in the presence of supernatants derived from activated memory CD4+ T cells in which GSK3 was inhibited exhibited significantly decreased proliferation, as compared with cells cultured in supernatants derived from memory cells stimulated with anti-CD3 alone (Fig. 7). Blocking the IL-10 receptor on the target cells (Fig. 7A) or neutralizing IL-10 (Fig. 7B) significantly reversed the suppressive capacity of the supernatants derived from memory cells activated in the presence of GSK3 inhibition, demonstrating the suppression was dependent on IL-10. The IL-10-dependent suppressive capacity of GSK3-inactivated memory CD4+ T cells was observed at suppressor-to-responder cellular ratios of 5:1 and 1:1 but was insignificant at a ratio of 1:5 (Fig. 7A). In addition, supernatants derived from nonstimulated cells in which GSK3 was inhibited were unable to suppress CD4+ T cell proliferation (Fig. 7B). Thus, the inactivation of GSK3 upon TCR stimulation can promote IL-10-dependent regulatory activity by human memory CD4+ T cells.

**Discussion**

We demonstrate a contrast between human naive and memory CD4+ T cells in that inactivation of GSK3β strongly favors proliferation in naive cells whereas in memory CD4+ T cells it favors IL-10 production. The ability of GSK3 to enhance IL-10 levels by memory CD4+ T cells resulted in their ability to suppress bystander T cell proliferation in an IL-10-dependent manner.

The finding that GSK3 regulates NF-AT was the first study to demonstrate GSK3 was a component of the cellular signaling machinery present in cells of the immune system (23). Subsequent work performed with human and mouse cells implicated GSK3 as a negative regulator of T cell proliferation and IL-2 production (10, 13). Moreover, GSK3 was later found to regulate p27kip1 levels and the phosphorylation state of the retinoblastoma tumor suppressor protein in the presence of TCR activation, thus adding mechanistic support for the role of GSK3 in T cell proliferation (9). However, because of the number of differences described in the intracellular signaling pathways present in naive and memory cells (3, 24), we hypothesized GSK3 may play discrete roles depending on the antigenic experience of the CD4+ T cell. In this regard, our findings identify the existence of a dichotomy present in the role of GSK3 in naive and memory human CD4+ T cells. In this respect, our findings demonstrate GSK3 was a component of the cellular signaling machinery present in cells of the immune system (23). Subsequent work performed with human and mouse cells implicated GSK3 as a negative regulator of T cell proliferation and IL-2 production (10, 13). Moreover, GSK3 was later found to regulate p27kip1 levels and the phosphorylation state of the retinoblastoma tumor suppressor protein in the presence of TCR activation, thus adding mechanistic support for the role of GSK3 in T cell proliferation (9). However, because of the number of differences described in the intracellular signaling pathways present in naive and memory cells (3, 24), we hypothesized GSK3 may play discrete roles depending on the antigenic experience of the CD4+ T cell. In this regard, our findings identify the existence of a dichotomy present in the role of GSK3 in naive and memory human CD4+ T cells. Because GSK3 acts primarily as a negative regulator of proliferation in naive cells, whereas GSK3 inhibition in memory CD4+ T cells leads to enhanced IL-10 production and IL-10-dependent suppressive activity. The current findings clarify the role of GSK3 by demonstrating that the ability of GSK3 to negatively regulate proliferation is largely dependent on the antigenic experience of the CD4+ T cell.

Inhibition of GSK3β in memory CD4+ T cells increased IL-10 productivity upon TCR stimulation. The mechanism responsible for control of IL-10 by GSK3β in memory CD4+ T cells is unknown at the present. Various possibilities exist from the direct molecular regulation of IL-10 transcriptional activity (25) to indirectly by regulating activation-induced cell death (26) or cellular proliferation (10, 13). We explored the latter possibility that GSK3 may suppress IL-10 production by indirectly influencing the expansion or survival of memory CD4+ T cells postactivation. We found no evidence supporting this hypothesis. Specifically, we found that despite the ability of GSK3 to suppress the frequency of IL-10 producing cells present upon TCR activation, inactivation of GSK3 did not significantly alter the growth dynamics of activated memory CD4+ T cells as no significant increase in the percentage of viable cells or change in the absolute number of cells was observed throughout the duration of the assay. In contrast, increases in the steady-state levels of IL-10 mRNA were observed upon the inactivation of GSK3 suggesting that GSK3 either is involved in regulation of IL-10 mRNA stability or negatively controls a transcriptional event. The latter has been reported to be the mechanism by which GSK3 differentially controls pro- and anti-inflammatory...
cysteine production by TLR-stimulated monocytes (25). Specifically, repression of GSK3 activity lead to a decrease in the transcriptional activity of NF-kB p65, an effect mediated by the ability of GSK3 to control nuclear CREB levels which compete with NF-kB p65 for the nuclear coactivator, cAMP-binding protein (25). Recently, the Epstein-Barr viral protein, LMP1, was shown to induce IL-10 production from B cells via a GSK3/CREB-dependent pathway (27). However, we did not observe any significant change in the transcriptional activity of CREB upon GSK3 inhibition in TCR-stimulated human peripheral blood T cells (C. A. Garcia, unpublished observation). Thus, although a comorbidity exists with TLR-stimulated innate immune cells, LMP1-expressing B cells, and TCR-stimulated memory CD4+ T cells with respect to the ability of GSK3 to suppress the production of IL-10 (25, 27–29), distinct differences are also apparent because GSK3 does not appear to regulate proinflammatory cytokine production by memory CD4+ T cells and the downstream mechanism for controlling IL-10 production appears to be unique in memory CD4+ T cells.

Although T cell differentiation is critical for the outcome of the adaptive immune response, the events regulating memory CD4+ T cell recall responses are likely to also be important, particularly in humans whose resident T cell population contains a considerable fraction of memory cells (30). Recent evidence suggests that despite diminished requirements of the memory CD4+ T cell on costimulation for proliferative responses and their ability to produce effector cytokines upon TCR stimulation alone (2), memory CD4+ T cells retain considerable plasticity and control with regard to their effector responses. To this accord, Sallusto et al. (31–33) have reported that human and murine CD4+ T cells, despite polarization into a specific lineage (i.e., Th1), can produce effector cytokines of a distinct and separate lineage (i.e., Th2) under particular conditions. The memory recall response has also been shown to be regulated by extrinsic factors like OX40, ICOS, and CD28 costimulation (6, 34, 35). Recently, it was shown that blockade of B7/CD28 signaling using CTLA-4Ig can inhibit the IL-2 and IFN-γ recall response of influenza-specific memory CD4+ T cells in mice (5) and that TLR stimulation and CD28 costimulation can augment IFN-γ production by TCR-stimulated human memory CD4+ T cells (36). Our finding that the level of endogenous GSK3 activity is a critical parameter involved in the regulation of the IL-10 recall response by human memory CD4+ T cells, taken together with our findings and those of others (37) demonstrating that the inhibition of PI3K activity abrogates IL-10 production by human memory CD4+ T cells, suggests that the ability of the PI3K pathway to regulate IL-10 production by human memory CD4+ T cells is due to its ability to inactivate GSK3. Indeed, blockade of PI3K activity abrogated the ability of anti-CD3/CD28 stimulation to induce the phosphorylation-dependent inactivation of GSK3 and promote IL-10 production by memory CD4+ T cells. Because the PI3K pathway can be induced in memory CD4+ T cells via distinct extrinsic signals like CD28, GSK3 may function as a molecular rheostat that regulates the amplitude of IL-10 production. Indeed, we demonstrate that CD28 costimulation can augment the production of IL-10 from TCR-stimulated memory CD4+ T cells in a PI3K-dependent manner. GSK3 may serve as a biochemical link between the state of the immunological milieu and the memory CD4+ T cell for the regulation of IL-10. The importance of GSK3 activity in vivo IL-10 responses by memory CD4+ T cells responses should be explored in future studies.

Memory CD4+ T cells have been implicated in a vast number of autoimmune diseases including rheumatoid arthritis, psoriasis, multiple sclerosis, and type 1 diabetes mellitus (38). Yet despite their likely participation in diverse autoimmune pathologies, memory CD4+ T cells in many cases are resilient to therapeutic strategies shown to be successful with their naive counterparts (38). This resiliency has been attributed to qualities such as enhanced Ag sensitivity and lesser dependency on costimulation for their execution of effector responses. Although human memory CD4+ T cells can produce multiple effector cytokines upon TCR cross-linking alone, costimulatory molecules can still alter the memory CD4+ T cell recall response. However, strategies relying on costimulatory blockade may not always be effective when memory CD4+ T cells are a principal etiological agent because even in the absence of costimulation, memory CD4+ T cells are capable of producing Th1-, Th2-, and Th17-type cytokines. Our current work suggests that rather than inhibiting memory CD4+ T cell activation, an alternative approach for the modulation of memory CD4+ T cell responses may be feasible by targeting regulatory kinases such as PI3K and GSK3β that are intimately involved in the memory CD4+ T cell cytokine response. We demonstrate that although TCR-stimulated memory CD4+ T cells upon GSK3 inactivation can still produce proinflammatory cytokines, their propensity toward IL-10 production as a consequence of GSK3 inactivation, renders a state where the suppression of bystander CD4+ T cell proliferation is observed. Thus, GSK3 may provide a valuable target for modulating immune responses involving memory CD4+ T cells due to the unique ability of GSK3 to govern IL-10 production.

Acknowledgments

We thank Panagiotis Stathopoulos and Kobe Garcia for technical assistance.

Disclosures

The authors have no financial conflict of interest.

References