Interleukin-7 Inhibits Tumor-Induced CD27−CD28− Suppressor T Cells: Implications for Cancer Immunotherapy

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Abstract

**Purpose:** We have previously reported that many types of tumors can induce changes in human T cells that lead to the acquisition of suppressive function and phenotypic alterations resembling those found in senescent T cells. In the present study, we find a role for interleukin 7 (IL-7) in protecting T cells from these changes and further defined involved signaling pathways.

**Experimental Design:** We evaluated the ability of IL-7 treatment to prevent the gain of suppressive function and phenotypic alterations in human T cells after a short coculture with tumor cells in vitro. We then used inhibitors of components of the phosphoinositide 3-kinase (PI3K)/AKT pathway and short interfering RNA knockdown of Mcl-1 and Bim to evaluate the role of these signaling pathways in IL-7 protection.

**Results:** We found that IL-7 inhibits CD27/CD28 loss and maintains proliferative capacity, IL-2 production, and reduced suppressive function. The protective ability of IL-7 depended on activation of the PI3K/AKT pathway, which inhibited activation of glycogen synthase kinase 3β, which, in turn, prevented the phosphorylation and loss of Mcl-1. We further showed a key role for Mcl-1 in that its knockdown or inhibition abrogated the effects of IL-7. In addition, knockdown of the Mcl-1 binding partner and proapoptotic protein Bim protected T cells from these dysfunctional alterations.

**Conclusion:** These observations confirm the role for Bcl-2 family members in cytokine signaling and suggest that IL-7 treatment in combination with other immunotherapies could lead to new clinical strategies to maintain normal T-cell function and reduce tumor-induced generation of dysfunctional and suppressor T cells.

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Introduction

The immune response plays an important role in detecting and killing tumor cells, and the use of immunotherapies to take advantage of this process has been a major goal of medical research. Despite some successes, obstacles such as multiple processes of T-cell regulation and dysfunction remain unresolved. Recent studies have identified significantly increased populations of T cells in human patients that display many characteristics of senescent T cells normally associated with aging (1–3). Although the hallmark of these cells is the loss of CD27 and CD28 expression, they also possess other features of senescence, including shortened telomeres (4) and reduced proliferative capacity and cytokine production (5), as well as suppressor activity not associated with traditional senescence. These findings suggest that increased numbers of dysfunctional, senescent-like T cells directly support the local and systemic immunosuppression observed in cancer patients (3, 6). Indeed, increased numbers of these cells correlates with a poor prognosis and contributes to general immune hypo responsiveness (7–9), making these patients poor candidates for immunotherapy (2, 3, 10).

Working from the hypothesis that the tumors themselves were capable of inducing these dysfunctional changes in T cells, we have previously characterized an in vitro model in which normal human T cells were induced to become dysfunctional suppressors after a brief interaction with tumor cell lines of multiple origins (1). This model mimics a process in patients where normal T cells travel to the tumor microenvironment, are altered by tumor-generated factors, and then reenter the periphery where phenotypic alterations can be used to track them. We found that a brief coinoculation with tumor cells was sufficient to induce a significant loss of CD27 and CD28 expression in both CD4+ and CD8+ T cells as well as other senescence-related
Translational Relevance

In this study, we provide compelling data that IL-7, a cytoprotective cytokine, is also a potent inhibitor of tumor-induced suppressive activity and phenotypic alterations observed in human T cells. We further show that protective activities of IL-7 require involvement of the PI3K/AKT pathway and show the importance of Mcl-1 as a key regulator of these dysfunctional changes. Together these data provide new insights into the molecular action of IL-7. Perhaps of greater importance is the observation that T-cell alterations similar to those reported here are found in cancer patients and have been shown to be associated with poor prognosis and candidacy for immunotherapy. IL-7 may now have a broader appeal as an adjuvant to immune-based cancer treatment.

markers including telomere shortening, nuclear heterochromatization, and increased expression of p53, p21, and p16. Most significantly, we found that this process involved a soluble factor secreted by tumor cells (but not untransformed fibroblasts) and that both CD4+ and CD8+ populations gained a potent suppressive ability. These data suggest that this process of tumor-induced suppression could be a mechanism by which tumors could not only blunt T-cell responses against them directly but also enhance systemic hyporesponsiveness by redirecting effectors to become regulatory.

Several recent studies suggest that cytoprotective cytokines such as interleukin (IL)-7 and IL-12 may prevent the induction of altered T-cell function. For instance, IL-7 and IL-12 can induce telomerase activity, a key enzyme in maintaining telomere length (11–13). IL-12 has been found to restore CD28 expression in CD4+CD28null T cells sorted from human peripheral blood mononuclear cells (PBMC; ref. 13). Furthermore, in a recent clinical trial of cancer patients, exogenous IL-7 maintained CD27 expression in peripheral blood T cells (14). Therefore, we hypothesized that this cytokine could have the capacity to prevent the dysfunctional alterations that we observed in tumor-exposed T cells.

Using our previously described in vitro model of tumor-induced T-cell dysfunction, we now show that IL-7 prevents the development of both phenotypic alterations and suppressive function in tumor-exposed T cells. We further show that this protection relies on the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway. Once activated, AKT inhibits the more downstream molecule glycogen synthase kinase 3β (GSK3β), preventing it from phosphorylating the cell survival molecule Mcl-1. Finally, we show that Mcl-1 plays a nonapoptotic role as a key downstream effector of IL-7 signaling by preventing tumor-induced dysfunction of T cells through well-known prosurvival pathways. These findings suggest that IL-7 is a potent adjuvant for preventing the development of tumor-induced suppressor T cells and may assist in cancer treatment, either alone or in combination with other immunotherapies.

Materials and Methods

Cell culture

Peripheral blood T cells from healthy donors were isolated as described previously (1). CD45RA+ T cells were enriched from purified donor T cells by positive magnetic bead selection (Miltenyi). Human solid tumor cell lines MCF7, 624mel, and Tu167 were cultured for 24 hours up to a 90% confluence. To induce suppressor T cells, purified, naïve, human T cells were added to wells containing solid tumor cells at a ratio of 1:1 for 6 hours. T cells were then collected, washed, and cultured for 7 days in a complete medium with or without each of recombimant human IL-2 (rIL-2; 100 U/mL; NCI BRB Preclinical Repository), IL-7 (rIL-7; 20 ng/mL; BD Pharmingen), IL-12 (rIL-12; 20 ng/mL; R&D Systems), and IL-15 (rIL-15; 10 ng/mL; eBioscience).

The following inhibitors at the indicated concentrations were used: 20 μmol/L LY294002 (Sigma); 0.5 μmol/L AKT inhibitor IV (Cal Biochem); 20 and 2 μmol/L GSK3β inhibitor X, and 5 μmol/L rapamycin (Calbiochem); 20 nmol/L TW37 was a gift from Shaomeng Wang (University of Michigan, Ann Arbor, MI), and 10 μmol/L ABT737 was provided by Abbott Laboratories.

Flow cytometry

Dysfunctional phenotypic markers were evaluated by CD27/CD28 staining and telomere length as described previously (1). CD3+CD27+CD28null subsets were collected by fluorescence-activated cell-sorting (FACS) sorting. For the intracellular Mcl-1 staining, cells were incubated with Mcl-1 antibody or IgG control (Sigma) followed by fluorescein isothiocyanate (FITC)-labeled secondary antibody (Jackson ImmunoResearch). For IL-2 staining, tumor-exposed cells with or without IL-7 were washed and stimulated with 1 μg/mL anti-CD3 monoclonal antibody (mAb) overnight; BD Golgi Stop (0.7 μL/mL) was added for 4 hours followed by intracellular staining with APC-anti-IL-2 and APC-anti-rabbit IgG (BD Pharmingen). For CFSE staining, T cells were labeled with 2 μmol/L carboxyfluorescein succinimidyl ester (CFSE; Molecular Probes) for 8 minutes at 37°C and then washed 3 times with 10% RPMI 1640 at room temperature. A total of 2 × 10^5 CFSE-labeled fresh responder cells were plated in 96-well plates with coated anti-CD3 mAb (1 μg/mL; BD Pharmingen) and cocultured with tumor-exposed cells with or without IL-7 for 48 hours (T cell ± IL-7-responder = 1:3). Apoptosis in responder cells was assessed by staining FITC-Annexin V and 7-aminoactinomysyn D (7-AAD; BD Pharmingen) per the manufacturer’s instructions. All samples were analyzed on a BD LSRII flow cytometer with FACS Diva Software (BD Bioscience). PBMCs from head and neck cancer patients were collected and frozen under an Institutional Review Board approved protocol at the University of Maryland (Baltimore, MD). Informed consent was obtained from all subjects. Expression of T-cell surface markers was evaluated by staining with PerCP-anti-CD3 (BD Bioscience), PE/cy7-anti-CD4 (BD Bioscience), APC-eFluor...
780-anti-CD8 (eBioscience), PE-anti-CD127 (BD Bioscience), APC-anti-CD28 (BD Bioscience), and FITC-anti-CD27 (BD Bioscience).

**Western blot analysis and coimmunoprecipitation**

Western blot analysis was carried out as previously described (1) by using the following antibodies: anti-AKT1, anti-phospho-AKT Ser473, and Thr308, anti-Mcl-1, anti-phospho-Mcl-1 (Ser159/Thr163), anti-GSK3β, anti-phospho-GSK3β (Ser9), anti-Bim, anti-STAT5, and anti-phospho-STAT5 (Cell Signaling Biotechnology). For coimmunoprecipitation, cell lysates were preclreated with protein G beads and incubated overnight at 4°C with 1 μg of anti-Mcl-1 (Santa Cruz Biotechnology) or anti-Bim (Alexis Biochemicals). Immunoprecipitates were collected by adding 30 μL protein G agarose beads (Sigma Aldrich) and incubated for 1 hour at 4°C.

**Proliferation suppression assay**

Autologous T cells (responder cells) were plated at a density of 2 × 10^5 cells per well in a flat-bottomed 96-well plate coated with 1 μg/mL of anti-CD3 mAb (BD Pharmingen). The next day, tumor-exposed T cells with or without IL-7 were washed and added to the cultures of responder cells for an additional 72 hours, 1 μCi (0.037 MBq) per well [3H]thymidine was pulsed overnight, and incorporation was measured with a scintillation counter.

**RNAi transfection**

The short interfering RNAs (siRNA) for Mcl-1, Bim, and nontargeting were purchased from Dharmacon and Santa Cruz Biotechnology. Purified primary T cells were transfected using the Amaxa Nucleofector Kit (Amaxa Biosystem) and analyzed 36 hours after transfection.

**Statistical analysis**

Student’s t test was used to evaluate the significance of differences between sample means. Statistical significance was defined at P < 0.05. All experiments were repeated at least 3 times, with error bars representing the SD.

**Results**

**IL-7 protects human T lymphocytes from tumor-induced T-cell suppression**

We have previously reported that tumor cells can directly induce suppressor T cells in vitro, which could be a mechanism that contributes to the ability of a tumor to evade immune recognition and killing (1). The cytokines IL-2, IL-7, or IL-12, which are known for their cytoprotective effects on T cells and are currently in clinical trials, were used to determine whether they can prevent these phenotypic and functional alterations. Initially, we treated human peripheral blood T cells with IL-2, IL-7, or IL-12 before and concomitantly with different tumor cell lines, which were chosen on the basis of our previous study (1). T cells were then recovered from coculture, washed, and plated in complete media in the presence or absence of cytokines for 7 days. Dysfunctional suppressor T cells were identified by the loss of CD27 and CD28 or telomere shortening. Prior research had found that tumor-exposed T cells undergo a 40% to 60% greater decrease in CD27 and CD28 expression than control T cells not exposed to tumor (1). The new findings show that IL-7 treatment (and to a lesser degree IL-2 but not IL-12) consistently protects tumor-exposed T cells from CD27/CD28 loss (Fig. 1A

![Image](373x454)

**Figure 1.** IL-7 prevents tumor-induced T-cell CD27/28 loss and telomere shortening induced by both hematopoietic and solid tumors in vitro. Purified T cells were incubated with various tumor cell lines (Tu167, MCF7, and 624mel) at a ratio of 1:1 for 6 hours and then washed and cultured for another 7 days with or without cytokines (100 U/mL IL-2, 20 ng/mL IL-7, and IL-12). On day 7, T-cell controls and tumor-exposed cells were evaluated for the loss of CD27 and CD28. A, representative dot plots depicting CD27 CD28 T cells induced by Tu167 are shown. B, tumor-exposed T cells incubated with different tumor cells (from at least 3 independent experiments) in the presence or absence of different cytokines are shown; error bars represent SD. C, analysis of telomere length with freshly isolated T cells, T cells on day 7, and tumor-exposed T cells on day 7 (with or without IL-2, IL-7, or IL-12). The bar chart shows the SEM relative telomere length in cells from 3 independent experiments. *, P = 0.027 versus control tumor-exposed cells. NS, no statistical significance between IL-7-treated, tumor-exposed cells, and untreated T cells alone.
and B). However, only IL-7–treated, tumor-exposed T cells maintained a telomere length comparable with unexposed controls (Fig. 1C). We then established that the best time for IL-7 treatment is immediately after coincubation with tumor (Supplementary Fig. S1), and for this reason, all subsequent experiments used this time point. We also determined that the combination of IL-7 with other common receptor γ-chain cytokines IL-2 and IL-15 did not result in any additive effect over IL-7 alone (Supplementary Fig. S2). To test whether IL-7 treatment could equally benefit naive versus memory T cells, naive CD45RA⁺ T cells were enriched with purified T cells by positive magnetic bead selection before both CD45RA-enriched and -depleted T cells were incubated with tumor and cultured in IL-7 (Supplementary Fig. S3). We found that the degree of CD27/CD28 loss was greater in naive CD45RA⁺ T cells than in CD45RO⁺ memory T cells but that IL-7 treatment was protective in both. These results suggest that naive T-cell populations may be more susceptible to tumor-induced alterations.

Our data also show that IL-7 alone promoted T-cell survival (Supplementary Fig. S4A) and did not induce proliferation of tumor-exposed T cells (Fig. 2A) or T cells alone (data not shown). These findings suggest that IL-7 protects against T-cell dysfunction by inhibiting its development rather than causing the proliferation of nondysfunctional T cells. To address whether IL-7 treatment can reverse the dysfunctional phenotype, we evaluated tumor-exposed T cells (untreated) 7 days after coincubation and added IL-7. CD27/CD28 expression was checked at several time points after the introduction of IL-7, but only minor increases in expression of both markers was observed (data not shown). To determine whether loss of the IL-7 receptor (IL-7R) could account for this finding, we examined the expression of this receptor (CD127) on tumor-exposed T cells. We observed that the loss of CD27 and CD28 on target cells could be reversed by IL-7.

Figure 2. IL-7 enhances the proliferation of CD3-activated, tumor-exposed T cells and restores proliferation of responder cells in coculture with tumor-exposed cells. A, T cells were stained with 2 μmol/L CFSE and then cocultured with Tu167 at a ratio of 1:1 for 6 hours, washed, and cultured in anti-CD3 mAb–coated plates with or without IL-7 (20 ng/mL). Cell proliferation was examined by flow cytometry on days 1, 4, and 7. Tumor-exposed T cells cultured without anti-CD3 in medium alone (untreated) with IL-7 served as negative controls. B, tumor-exposed cells cultured with or without IL-7 for 7 days were washed and stimulated with anti-CD3 mAb for 24 hours and then IL-2 expression was detected by intracellular cytokine staining and FACS. C, tumor-exposed T cells were incubated with or without IL-7 for 7 days and then washed and placed into the cultures of normal donor responder cells prestimulated for 24 hours with anti-CD3 antibody at tumor-exposed cell:responder cell ratio of 1:3. Responder T-cell proliferation was evaluated by [3H]thymidine incorporation after 72 hours and represented as the mean ± SD of cpm from 3 independent experiments (left). The percentage of proliferation inhibition of responder T cells by tumor-exposed T cells and IL-7–treated, tumor-exposed T cells was calculated from 3 independent experiments (*, P = 0.027). Each bar represents mean ± SD of triplicate values. D, tumor-exposed T cells cultured 7 days with or without IL-7 were added to CFSE-labeled responder cells at a ratio of 1:3 and stimulated with anti-CD3 mAb for 48 hours. Apoptosis of the CFSE-labeled responder cells was evaluated by Annexin V and 7-AAD staining. The bar graph shows the percentage of double-positive staining cells from 3 independent experiments. Error bars represent the SD. R, responder cells.
correlated with significantly lower CD127 expression (Supplementary Fig. S5). To test whether the observed decrease in CD127 expression after tumor exposure has clinical relevance, we obtained peripheral blood from human head and neck cancer patients collected under an Institutional Review Board–approved protocol before chemotherapy treatment and examined CD127 expression on CD27+/CD28− T cells among the total CD8+ population as compared with healthy, age-matched donor controls. We chose to focus on the CD8+ T-cell population, as these cells display greater levels of CD27/CD28 loss in vivo than CD4+ cells (9). Similar to our in vitro observations, the CD27/CD28 double-negative CD8+ T-cell population in these patients had very low CD127 expression (Supplementary Fig. S6). These results suggest that the benefit of IL-7 therapy would be the protection of CD27+/CD28+ T cells from tumor-induced dysfunction rather than the reversal of already dysfunctional cells due to their lack of CD127 expression. Overall, our results show that IL-7 protects against tumor-induced phenotypic alterations, but that once T cells lose CD27 and CD28 expression, further IL-7 exposure is unlikely to have any effect.

**IL-7 prevents the functional changes associated with tumor-induced dysfunction**

In addition to the loss of CD27 and CD28 expression, we have also found that tumor-exposed T cells are hypoproliferative when stimulated with anti-CD3. To evaluate whether IL-7 treatment could affect this aspect of tumor-induced dysfunction, we evaluated the proliferative capacity of tumor-exposed T cells after 7 days in vitro culture with or without IL-7. Using CFSE-labeling and plate-bound anti-CD3 antibody, we observed an increase in the proliferation of tumor-exposed, IL-7–treated cells, but not in tumor-exposed T cells stimulated with anti-CD3 Ab or IL-7 alone (Fig. 2A).

To further validate this observation, we repeated the experiment using [3H]thymidine incorporation as a read-out for proliferation. Initially, we did not observe proliferation in any of the treatment groups (data not shown). Because IL-7–treated T cells contain small numbers of CD27+/CD28 double-negative T cells, we hypothesized that the lack of proliferation could be due to suppression by these cells. To evaluate this possibility, CD27−/CD28− T cells were electronically sorted from IL-7–treated or untreated groups 7 days after tumor exposure and stimulated with plate-bound anti-CD3 antibody for 72 hours (Supplementary Fig. S2B). Interestingly, we observed that CD27−/CD28− T cells not receiving IL-7 treatment were hypoproliferative. This observation could be due to the fact that tumor-induced dysfunction is a process and that the loss of proliferative capacity precedes the loss of CD27 and CD28 expression. Tumor-exposed CD27+/CD28 double-positive cells treated with IL-7 displayed proliferation similar to control T lymphocytes not exposed to tumor. As expected, the CD27−/CD28− population from both untreated and IL-7–treated, tumor-exposed T cells did not proliferate (data not shown). These observations indicate that IL-7 prevents tumor-induced dysfunction in most, but not all, T cells and IL-7–treated CD27+/CD28+ T cells have normal proliferative capacity.

Another distinguishing characteristic of both senescent and dysfunctional T-cell populations in cancer patients and those generated by our tumor-exposure model is reduced production of IL-2 upon stimulation (15). We discovered IL-7 treatment can significantly increase IL-2 production in tumor-exposed T cells after anti-CD3 stimulation (Fig. 2B)

Perhaps the most significant functional alteration of tumor-exposed T cells is their ability to suppress the proliferation of normal T lymphocytes in response to anti-CD3 and mixed lymphocyte reaction stimulation in vitro (1). Thus, we examined whether IL-7 treatment could reduce or prevent this suppression. Fresh allogenic T lymphocytes (responder cells) were pretreated with plate-bound anti-CD3 Ab for 24 hours. Tumor-exposed T cells cultured with or without IL-7 were then added to the responder cells at a ratio of 1:3. We observed that IL-7–treated cells had less suppressive function than their untreated counterparts (Fig. 2C). We then evaluated whether tumor-exposed, T-cell–mediated suppression occurs through induction of apoptosis in responder T cells by labeling them with CFSE prior to coincubation. After 2 days, responder cell apoptosis was measured by 7-AAD and Annexin V staining. The 20% baseline level of apoptosis was expected because anti-CD3 stimulation induces proliferation and activation-induced cell death. In the presence of tumor-exposed T cells, however, apoptosis in responder T lymphocytes nearly doubles, whereas coincubation with IL-7–treated, tumor-exposed cells did not significantly increase responder apoptosis (Fig. 2D). Taken together, these results show that IL-7 inhibits the functional alterations seen in tumor-induced dysfunctional T cells.

**PI3K/AKT is required for IL-7 protection against tumor-induced T-cell dysfunction**

To better understand the effects IL-7 treatment has on protecting T cells from tumor-induced dysfunction, we next investigated the role of several pathways known to be involved in IL-7 signaling, namely, JNK/STAT5 and PI3K/AKT (16). We found that though inhibition of STAT5 (17) could block its phosphorylation during IL-7 treatment, the blockade had little effect on the protective ability of IL-7 (Supplementary Fig. S7). We next evaluated the PI3K/AKT pathway, which has been shown to be involved in mediating IL-7–driven T-cell survival (16, 18). AKT is a key downstream target of PI3K and is activated by dual phosphorylation at Thr308 and Ser473. As shown in Figure 3A and B, IL-7 cannot protect T cells from tumor-induced CD27/CD28 loss when cultured with PI3K (LY294002) or AKT inhibitors (AKT inhibitor IV). We then analyzed whether AKT is activated by IL-7 in tumor-exposed cells. As shown in Figure 3C, IL-7 can induce AKT phosphorylation at Ser473 and Thr308 within 30 minutes, with phosphorylation increasing for at least 12 hours. The PI3K and AKT inhibitors, as expected, blocked IL-7–induced AKT activation in tumor-exposed cells. Similar results occurred with another inhibitor of PI3K, wortmannin (data not shown).
IL-7 prevents tumor-induced T-cell dysfunction through inhibition of GSK3β

We next evaluated downstream cell survival molecules related to the PI3K/AKT pathway by examining the effector molecular complex, mTOR (19, 20). Using the mTOR inhibitor rapamycin, we did not observe any reduction in the protective function of IL-7 (Supplementary Fig. S8), suggesting that mTOR is dispensable for IL-7 effects. We next studied GSK3β, one of the downstream substrates of PI3K/AKT, which is inactivated by direct phosphorylation on the Ser9 residue, promoting cytokine-driven cell survival (21). These previous reports suggested that GSK3β might effect IL-7–mediated protection through PI3K/AKT pathway activation. We observed that the development of tumor-induced CD27/CD28 loss was prevented by the GSK3β inhibitor X (20 μmol/L) even without IL-7 (Fig. 4A and B). We also observed that the GSK3β inhibitor at a lower dose (2 μmol/L) and observed less protection. With a lower GSK3β inhibitor dose, IL-7 could prevent CD27/CD28 loss (data not shown). We also observed that IL-7 causes a time-dependent increase in phosphorylation of GSK3β, which suggests that this process is part of its protective signaling (Fig. 4C).

IL-7 upregulates Mcl-1 expression by dephosphorylation in tumor-exposed T cells

Studies indicate that Mcl-1 levels are highly regulated by GSK3β phosphorylation at Ser159 in cytokine-deprived cells, which causes Mcl-1 degradation (22). We observed via Western blot analysis that Mcl-1 phosphorylation occurs in tumor-exposed cells (tumor 0 hour; Fig. 5A). IL-7 addition reduced Mcl-1 phosphorylation and upregulated total Mcl-1 in a time-dependent manner. Both PI3K and AKT inhibitors can prevent IL-7–induced Mcl-1 dephosphorylation, whereas GSK3β inhibitor with IL-7 prevented almost completely Mcl-1 phosphorylation (Fig. 5A). To further evaluate Mcl-1 expression, we used FACS analysis of tumor-exposed T cells in a 3-period time course over 1 week. Our data show that fluorescence intensity of Mcl-1 increased and was maintained throughout this course after a single IL-7 administration. As control, we investigated Mcl-1 levels in tumor-exposed cells treated with IL-2 and IL-12 (Fig. 5B), concluding that IL-7 had the greatest impact on Mcl-1 expression. These experiments show that IL-7 increases Mcl-1 levels by inhibiting its phosphorylation induced by GSK3β, further elucidating the role of IL-7 in protecting T cells from tumor-induced dysfunction.

Depletion of Mcl-1 abrogates IL-7 protection against tumor-induced dysfunction

To determine whether Mcl-1 plays a role in mediating the protective activity of IL-7, we reduced Mcl-1 levels through siRNA-mediated knockdown and examined the resulting influence on cell survival. T cells were transfected with Mcl-1 siRNA or a control siRNA. Transfection with Mcl-1–specific siRNA produced a profoundly decreased Mcl-1 expression that IL-7 did not upregulate. Importantly, protective activity of IL-7 was significantly abrogated in T cells
with reduced Mcl-1 levels. IL-7 still had a small effect in Mcl-1 knockdown of T cells; however, this may be due to the fact that the siRNA transfection is not absolute. To further verify the lack of off-target effects, we used a distinct Mcl-1 siRNA from another company and obtained similar expression and functional results (Supplementary Fig. S9). Notably, downregulation of Mcl-1 in T cells can induce moderate losses of CD27/CD28 compared with controls (Fig. 5C). To confirm the specific role of Mcl-1 in IL-7–mediated protection, we introduced 2 pharmacologic drugs, TW37 (a nonpeptide pan small-molecule inhibitor of Bcl-2, Mcl-1, and Bcl-xL) and ABT737 (a small-molecule BH3 mimetic that binds to and antagonizes Bcl-2/Bcl-xL). Figure 5D illustrates that IL-7 can reduce CD27/CD28 loss with ABT737 but not with TW37 treatment. Collectively, these results indicate the involvement of Mcl-1 in IL-7–mediated protection from tumor-induced T-cell dysfunction.

**The importance of the Mcl-1 binding partner Bim in IL-7 protection**

After examining the role of Mcl-1 in IL-7–mediated protection from tumor-induced T-cell dysfunction, we explored its well-known binding partner Bim, whose inhibition is essential in IL-7–mediated lymphocyte survival (23–25). To assess the role of Bim in IL-7–mediated signaling, we acutely knocked down its expression by using siRNA (from 2 different sources) in tumor-exposed cells with and without IL-7 (Fig. 6A and Supplementary Fig. S9B). Bim loss abrogated tumor-induced CD27/CD28 loss, and IL-7 addition could not provide further protection (Fig. 6A). Finally, to assess whether the function of IL-7 was dependent on binding of Mcl-1 with Bim, we carried out immunoprecipitation (IP) experiments for these proteins. Interestingly, when we carried out IP for Mcl-1, almost all Bim was pulled down in tumor-exposed T cells whether or not IL-7 was administered. In contrast, after the IP for Bim, the level of free Mcl-1 was significantly increased by IL-7 in tumor-exposed cells (Fig. 6B and C). These data suggest that IL-7–driven Mcl-1 expression prevents tumor-induced T-cell dysfunction and that the level of free (unbound to Bim) Mcl-1 is key to this process.

**Discussion**

The link between senescence and tumor-induced T-cell suppression has yet to be completely understood. Many studies have found elevated levels of T cells bearing the characteristics of replicative senescence normally associated with aged individuals in peripheral blood and tumor-infiltrating lymphocytes of cancer patients (3, 9). Other studies have focused on phenotypically similar
populations that exhibit suppressor function (3). Our previous data suggest that the answer may be more complex. Specifically, we observe in appropriate coincubation conditions that tumor cells can induce senescent-like changes in T cells, with strongly associated gain of suppressor function, in the absence of activation and proliferation (1). Interaction with tumors has been shown to induce a large array of changes in T cells in addition to the senescent-like dysfunctional changes reported here, including the induction of preapoptotic features such as the loss of \( \zeta \) chain and CCR7 expression (26). Our previous work has found that the T-cell alterations we observe are a process distinct from tumor-induced apoptosis, though we report here that the protection conferred by IL-7 treatment involves several signaling pathways known to be antiapoptotic. Thus, the signaling relationship between tumor-induced apoptosis and dysfunction remains to be completely resolved.

The presence of tumor-induced dysfunctional T-cell populations in cancer patients is associated with systemic...
immunosuppression and poor prognosis. Therefore, regulating their formation could improve clinical outcomes (8, 27). Although cytokines are an attractive option for immunomodulation, little is understood about their effects on natural and induced suppressor cell populations. For instance, some cytoprotective cytokines, such as IL-15, induce immunosuppressive cellular responses including increases in senescence-associated markers on T cells (28, 29). Our in vitro model, in which tumor cells can induce similar changes in normal donor T cells, provides a platform to identify cytokines that regulate formation of dysfunctional T cells and dissect the molecular pathways responsible for these effects. Using this model, we observed that IL-7 is a potent inhibitor of tumor-induced dysfunctional T-cell phenotypic and functional changes. Furthermore, we show that IL-7 exerted its protective function through common molecular pathways involved in preventing apoptosis.

We initially began with 3 cytokines currently used in clinical trials, namely, IL-2, IL-7, and IL-12. IL-2 and IL-7 share the same γ-chain receptor but seem to have disparate effects on the immune system (30). For instance, whereas both IL-2 and IL-7 promote T-cell survival, IL-2 also causes expansion of natural regulatory T cells. IL-12, a non-γ-chain cytokine, not only promotes antitumor immunity but has also been shown to increase CD28 expression in CD28null T cells (13, 31). We found a striking difference between these cytokines in that only IL-7 consistently protects against tumor-induced T-cell phenotypic and functional changes and that combination with other IL-2 family cytokines (IL-2 and IL-15) does not produce an additive effect over IL-7 alone. This observation is notable in that both IL-2 and IL-15 have been shown to enhance the survival of effector and memory T-cell populations similar to IL-7 (30). Several phase I clinical trials have been conducted with IL-7 in the context of cancer (14, 32, 33) and HIV infection (34, 35). In total, these trials show that IL-7 can be well tolerated by patients with minimal toxicities and it induces the expansion of naive and memory CD4+ and CD8+ T-cell populations. However, overall antitumor efficacy was nominal whether used alone or in combination with vaccine. Our data showing that induction of a suppressive, dysfunctional phenotype is accompanied by the loss of IL-7R suggests that the timing of

Figure 6. Free Mcl-1 is important for IL-7 protection and is upregulated by IL-7 treatment. A, T cells were transfected with either control (ctrl) siRNA or Bim siRNA for 36 hours, coincubated with Tu167 for 6 hours at a ratio of 1:1, and cultured in fresh medium with or without IL-7 for 5 days. The loss of CD27/CD28 was determined by FACS (top). Bim expression was examined by Western blotting (bottom). One representative of 3 experiments is shown. B and C, tumor-exposed T cells with and without IL-7 were lysed in IP buffer and immunoprecipitated with anti-Mcl-1 and Bim antibody. The immunoprecipitated proteins, total extracts, and depleted supernatant (sup) were analyzed by SDS-PAGE and immunoblotted with indicated antibodies.
administration would be important not only in the depletion of existing dysfunctional suppressors but also in protecting subsequent waves of effector T cells induced either endogenously by vaccine or injected via adoptive transfer. Indeed, preclinical mouse models have indicated that IL-7 can successfully be combined with a vaccine to significantly boost antitumor immunity (36).

IL-7 is known to activate cell survival signaling pathways, including the JAK/STAT and the PI3K/AKT pathways (37). However, these pathways may work in concert or be mutually exclusive (38). In addition, consequences of IL-7 signaling can differ depending on the T-cell lineage. We hypothesized that the protective effects of IL-7 may be mediated through these known cytoprotective pathways and identified only the PI3K/AKT pathway as significant. Our data show that PI3K and AKT are required for IL-7–mediated protection from dysfunctional changes and, in doing so, causes phosphorylation of AKT at both possible sites, indicating full activation of this molecule. Finally, our findings show that telomere length is maintained with IL-7 administration, which is consistent with the reported ability of AKT to activate telomerase (39).

A number of recent studies have shown that in cancer, constitutive AKT expression promotes cellular senescence (40). Others have shown, however, that AKT can promote proliferation through inhibition of RAF (41). This discrepancy may be a cell-type–dependent phenomenon and, at least in mouse and human T cells, AKT seems to maintain normal function. Thus, our data implicating the PI3K/AKT pathway may represent changes in lymphocytes only, or may be related to its function in combination with other IL-7–induced pathways. Ultimately, these findings describe a novel IL-7–mediated T-cell–protective pathway dependent on PI3K and AKT.

The AKT molecule has many downstream targets. Two of the most studied are the mTOR complex and GSK3β, which are both involved in promoting T-cell proliferation (42, 43). We observe that inhibition of mTOR using rapamycin did not affect protective activities of IL-7. This observation is interesting because it has been recently reported that AKT induces cellular senescence through mTOR and may also explain why AKT in IL-7 signaling is antisenescent (44). As a result, we then tested the importance of GSK3β. Our data show that chemical inhibition of GSK3β was sufficient to almost completely inhibit tumor-induced phenotypic alterations in T cells even without IL-7. We further show that IL-7 causes the phosphorylation and inhibition of GSK3β. Overall, our data illustrate that during IL-7 signaling, AKT is activated, which then prevents GSK3β signaling and leads to reduced CD27/CD28 loss.

One of the main targets of phosphorylation by GSK3β is the antiapoptotic Bcl-2 family member Mcl-1 (22). Although there is significant data regarding the role of Mcl-1 in apoptosis, there are no data about its ability to modulate function and phenotypic changes in T cells. Other antiapoptotic Bcl-2 molecules, however, were shown to inhibit (Bcl-xL) or promote (Bcl-2) similar processes associated with senescence (45, 46). We observe that IL-7 consistently causes the phosphorylation of AKT (activation) and GSK3β (inhibition) and prevents the increase in phosphorylation of Mcl-1. These findings seem to occur as early as 30 to 60 minutes after administration of IL-7, indicating their temporal proximity. Although the increase in Mcl-1 expression could be due to lack of phosphorylation and degradation, others have shown that AKT can induce increases in Mcl-1 expression via a translational mechanism (47). Regardless, the importance of these findings was confirmed through knockdown and chemical inhibition of Mcl-1 in tumor-exposed T cells, showing its importance in preventing dysfunctional phenotypic changes and indicating a novel nonapoptotic role.

Given that Mcl-1 has important function in resisting the tumor-induced T-cell phenotypic changes in our model, we decided to investigate whether a related proapoptotic molecule, Bim, also played a role. Other and we have already shown that Bim binds to and is inhibited by Mcl-1 in apoptosis (24). Our data show that not only does IL-7 reduce the phosphorylation and increase the overall expression of Mcl-1 but also causes an increase in free Mcl-1 protein (not bound to Bim), resulting in a protective effect.

In summary, we present the novel conclusion that the cytokine IL-7 can prevent the formation of dysfunctional T cells that display suppressor function and senescent phenotype. We further show novel data indicating that a major IL-7–induced antiapoptotic pathway is also protective (see Supplementary Fig. S10 for a diagrammatic overview). Despite recent advances in immunotherapy, successful clinical outcomes remain limited (27, 48) and tumor-induced immunosuppression could be a major contributing factor (49). As a result, there is new enthusiasm for cytoprotective strategies that are designed to overcome these processes (50). Cytokines such as IL-7 reduce many aspects of cancer immunosuppression, including induction of dysfunctional phenotypic and functional immune changes, such as those elucidated in this study. Therefore, future approaches to treat cancer using immunotherapies will require the incorporation of cytoprotective strategies, such as IL-7, to bring them to clinical reality.

Disclosure of Potential Conflicts of Interest

S.E. Strome is a cofounder and major stockholder in Gliknik Inc., a biotechnology company. He also receives royalties through the Mayo Clinic College of Medicine for licensure of intellectual property related to B7-H1 (PD-L1) and 4-1BB (CD137) to third parties.

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