CD8⁺ Tumor-Infiltrating T Cells Are Deficient in Perforin-Mediated Cytolytic Activity Due to Defective Microtubule-Organizing Center Mobilization and Lytic Granule Exocytosis

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CD8⁺ Tumor-Infiltrating T Cells Are Deficient in Perforin-Mediated Cytolytic Activity Due to Defective Microtubule-Organizing Center Mobilization and Lytic Granule Exocytosis

Sasa Radoja,* Masanao Saio,* David Schaeer,† Mythili Koneru,* Stanislav Vukmanovic,† and Alan B. Frey†,*

Tumor-infiltrating lymphocytes (TIL) are well known to be functionally impaired typified by the inability to lyse cognate tumor cells in vitro. We have investigated the basis for defective TIL lytic function in transplantable murine tumor models. CD8⁺ TIL are nonlytically immediately on isolation even though they express surface activation markers, contain effector phase cytokine mRNAs, and contain perforin and granzyme B proteins which are packaged into lytic granules. Ag-specific lytic capability is rapidly recovered if purified TIL are briefly cultured in vitro and tumor lysis is perforin-, but not Fas ligand mediated. In response to TCR ligation of nonlytic TIL in vitro, proximal and distal signaling events are normal; calcium flux is rapid; mitogen-activated protein/extracellular signal-related kinase kinase, extracellular regulatory kinase 2, phosphoinositide-3 kinase, and protein kinase C are activated; and IL-2 and IFN-γ is secreted. However, on conjugate formation between nonlytic TIL and cognate tumor cells in vitro, the microtubule-organizing center (MTOC) does not localize to the immunological synapse, thereby precluding exocytosis of preformed lytic granules and accounting for defective TIL lytic function. Recovery of TCR-mediated, activation-dependent MTOC mobilization and lytic activity requires proteasome function, implying the existence of an inhibitor of MTOC mobilization. Our findings show that the regulated release of TIL lytic granules is defective despite functional TCR-mediated signal transduction. The Journal of Immunology, 2001, 167: 5042–5051.

Growth of cancer in the authochthonous host elicits anti-tumor immune response and is detected by both T and B cell antitumor immune responses (1). For example, immune cells infiltrating human tumors are seen in many different tumor types and, although the prognostic significance of infiltrates varies among tumor type, tumor-infiltrating lymphocytes (TIL) contain tumor-specific T cells. Patients often also have tumor-reactive T cells in lymph nodes draining tumor sites and in the circulation. These sources of T cells have been used to study the Ag specificity and frequency of T cell antitumor immune response and to prepare T cell lines used both in experimental adoptive immunotherapy (2) and as the basis for tumor Ag identification by expression gene cloning (3). Humoral antitumor immune response has also been noted in many human cancers (4), and patient sera have similarly been useful in identification of tumor Ags, most recently by the SEREX method (serological analysis of recombiant tumor cDNA expression libraries; Ref. 5). Patient humoral and T cell responses prove that human cancers are able to prime tumor-specific immune responses. Similar studies using experimental models have proven the immunogenicity of rodent tumors.

Human antitumor immune responses do not usually correlate with tumor remission but has motivated considerable research in cancer immunotherapy. Although a variety of protocols have proved successful in immunization of rodents to resist primary tumor challenges, only rarely have large preexisting tumors been eradicated (6). The failure to cure established rodent tumors and the modest success of human experimental immunotherapies, together with the realization that antigenic human cancers elicit immune response but are usually not eliminated, has sparked interest in understanding the basis for inadequate antitumor T cell immune responses (7).

The presence of TIL in human cancers demonstrates that tumor Ags are expressed in situ that are capable of eliciting antitumor T cell immune response, which nonetheless fails to eliminate tumors (8). The lytic function of CD8⁺ TIL has been investigated in various human (9) and murine (10, 11) tumors and found to be defective. Deficient lytic function is transient because, on purification from tumor cells and culture in vitro, tumor-specific killing can be detected (12).

We have investigated the basis for defective TIL lytic function in transplantable murine tumor models. Freshly isolated TIL, although nonlytic in vitro, contain mRNAs encoding effector phase cytokines, serine esterases, and perforin characteristic of cytolytic effector T cells. Furthermore, perforin and granzyme B proteins are synthesized and packaged into lytic granules showing that TIL are mature CD8⁺ T cells. TIL recover perforin-mediated, Ag-specific lysis if purified and cultured briefly in vitro. After recovery of lytic function, on conjugate formation with cognate tumor cells in vitro, TIL mobilize cytotoxic granules to the immunological synapse, whereas granules in freshly isolated, nonlytic TIL do not migrate. In addition, the microtubule-organizing center (MTOC) does not...
localize to the immunological synapse in nonlytic TIL. This defect precludes movement of lytic granules to the immunological synapse and accounts for defective TIL lysis. Biochemical analyses demonstrated that TCR kinase-mediated signaling is functional in freshly isolated, nonlytic TIL. Our findings show that after arrival to the tumor microenvironment, antitumor T cells are unable to release cytolytic granules on contact with tumor which may permit tumor escape from T cell lysis.

Materials and Methods

Mice

Male C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed four per cage in a barrier facility and maintained on a 12-h light/dark cycle (7 a.m. to 7 p.m.) with ad libitum access to food and water. A sentinel program revealed that the mice were murine hepatitis virus negative and the tumor cell lines are murine hepatitis virus negative. Experiments involving animals were conducted with the approval of the New York University School of Medicine Committee on Animal Research.

Tumors

MCA-38 adenocarcinoma (13), P-815 cells (14), and 6-1 fibrosarcoma (15) have been described recently (12). Then 1–2 × 10^6 cells were injected s.c in 0.1 ml HBSS for tumor induction.

Tissue culture

Sources and use of all tissue culture reagents have been described recently (12).

Isolation of TIL

Isolation of TIL from tumors by immunomagnetic separation was recently described (12). Potentially inhibitory effects of the immunobead isolation protocol on TIL lytic function are unlikely because we have reisolated TIL with anti-CD8 magnetic beads after recovery in vitro, and those cells are lytic. As a further control, we have prepared CTL by primary MLR in vitro followed by isolation with anti-CD94 immunobeads. These cells are fully lytic, again showing that the isolation protocol is without effect on lytic activity.

Flow cytometric intracellular staining of T cells

Single-cell suspensions were prepared from tumors, primary MLR, or spleens of control mice by enzyme digestion and magnetic immunobeads as recently described (12). For granzyme B analysis of splenocytes, T cells were prepared by negative selection using anti-CD4 and anti-CD8-conjugated beads.

Flow cytometric analyses were performed as recently described (12). Some immunoblotting experiments used whole cell detergent extracts prepared from freshly isolated TIL (or TIL that were recovered in vitro before extraction) which were not activated in vitro. TIL subcellular fractionation and PKC analysis was performed essentially as described (17). Equal cell equivalents of soluble and membrane fractions were subjected to SDS-PAGE followed by immunoblotting with isofrom-specific Abs and detection with ECL (Amersham-Pharmacia Biotech, Little Chalfont, Buckinghamshire, England).

Immunoblot analysis

Activation of purified T cells in vitro and immunoblot analyses was performed as described (12). Some immunoblotting experiments used whole cell detergent extracts prepared from freshly isolated TIL (or TIL that were recovered in vitro before extraction) which were not activated in vitro.

Intracellular calcium mobilization studies

CD8^+ TIL were isolated and resuspended at 10^6 cells/ml in serum-free RPMI 1640. Cells were incubated with 1 μM Indo-1 (Sigma Chemical, St. Louis, MO) for 1 h, washed three times, and stimulated (10 μM ionomycin), and the OD 398 and 480 was measured over time. Excitation was at 340 nM in a Fluoromax 2 spectrofluorometer calibrated as described (18).

Results

TIL do not exhibit ex vivo cytotoxicity but recover perforin-mediated, tumor-specific cytolytic function after short term in vitro culture (12). For these analyses we studied two tumors, a fibrosarcoma made by expression of cDNAs encoding dominant-negative p53 plus activated Ha-ras genes in primary embryonic fibroblasts termed 6-1 (15), and separately a chemically induced adenocarcinoma, MCA-38 (13). For all experiments data achieved with either tumor were highly similar if not identical and representative data is shown. Furthermore, all experiments were performed with TIL isolated from individual mice (i.e., not mixed from different tumor preparations) and were repeated more than five times with equivalent results. CD8^+ TIL were purified by magnetic immunobeads from tumor single cell suspensions and tested for lytic ability (Fig. 1A). Freshly purified TIL are not lytic in standard four hr assays, however, if TIL are cultured in vitro in the presence of IL-2, substantial lytic activity is detected. The level of lysis is comparable to that demonstrated by primary MLR CTL at equivalent E:T ratios (Fig. 6).
Because of concern that the inflammatory milieu of the tumor microenvironment may attract $T$ cells which does not represent specific recruitment of antitumor $T$ cells, TIL were tested for Ag-specific lysis. Syngeneic MC57G tumor cells, which do not express cross-reactive tumor Ags), are not appreciably lysed by anti-MCA-38 TIL (Fig. 1B) (TIL were also tested for lytic function by redirected lysis assay (in which Ag specificity is irrelevant) and found to be nonlytic showing that the lytic defect is not caused by an unknown inhibitory MCA-38 tumor influence in vitro (data not shown)). TIL isolated from a variety of other tumors were similarly tested for Ag-specific lysis after recovery of lytic function in vitro and in all cases TIL demonstrate tumor-specific lysis (fibrosarcoma 6-1 (15), EL4 thymoma (19), EG.7 (EL4 expressing an MHC class I-restricted OVA peptide) (20), MC57G sarcoma (21), and mastocytoma P815 (14); data not shown).

The mechanism of cytolysis in recovered TIL was tested by inclusion of either EGTA (which inhibits requisite functional polymerization of perforin, MTOC mobilization, and lytic granule exocytosis by chelating free calcium (22)), or blocking Ab to Fas ligand (Fig. 1C). Anti-FasL Ab had no effect, however, EGTA completely abrogated lysis showing that target cell killing was perforin-mediated. In addition, the rapid kinetics of in vitro cytolysis by recovered TIL is characteristic of perforin- mediated, but not FasL-mediated lysis (23).

Collectively these experiments show that freshly isolated TIL are defective in tumor cell lysis in vitro but lytic function is restored after purification from tumor and brief culture in vitro. In addition, TIL lysis of cognate tumor cells is Ag-specific and perforin-mediated.

**Recovery of TIL lytic function**

The recovery of TIL lytic function was examined. In preliminary experiments TIL were incubated in various concentrations of rIL-2 for different periods of time and cytolysis determined. As little as 5 U/ml of rIL-2 for 12 h was sufficient to permit maximal recovery of lytic function (data not shown). Furthermore, TIL could recover significant lytic function if cultured in vitro for 6 h without additional medium supplements, typically 50–75% of maximal lysis (Fig. 2). Actinomycin D, cycloheximide, or brefeldin A had no effect on recovery of cytolytic function if included during the 6 h recovery period showing that recovery of lytic function does not require RNA, protein synthesis, or protein secretion. Recovery of maximal lytic activity required ~12 h in vitro culture, both RNA and protein synthesis (data not shown), and exogenous IL-2.

**TIL are mature CD8**$^+$** T cells**

One potential reason for the inability of TIL to lyse tumor target cells in vitro may be related to the state of TIL maturation which is reflected in the transcriptional activity of effector phase genes (24). The observation that protein or RNA synthesis is not required for recovery of significant TIL lytic function suggested that TIL are mature effector $T$ cells. We characterized TIL further by analyzing expression of genes characteristic of the effector phase. CD8$^+$ TIL were purified from tumors and used to prepare cDNA...
for programming of RT-PCR analysis of cytokine gene transcripts. Freshly isolated CD8\(^{+}\) TIL contain mRNAs encoding IL-2, IFN-\(\gamma\), perforin, granzyme A, and granzyme B (12).

In addition, expression of granzyme B and perforin proteins in TIL was examined (Fig. 3A). As anticipated, primary MLR CD8\(^{+}\) T cells demonstrating high levels of lysis in vitro (compare Fig. 6) contain both granzyme B and perforin. Freshly isolated, nonlytic TIL contain equivalent levels of perforin and slightly higher levels of granzyme B compared with MLR T cells. The level of perforin protein in TIL does not change when TIL are cultured in vitro for 12 h (data not shown) suggesting that recovery of lytic function is not due to increased expression of perforin.

The demonstration of perforin and granzyme B proteins in freshly isolated, nonlytic TIL proves that TIL lytic deficiency in vitro is not due to the absence of these enzymes, which would indicate that TIL are not fully mature, but does not address the possibility that cytolytic granules are not formed or loaded with lytic enzymes. To address this concern, we performed immunocytochemical localization of perforin and granzyme B, both in frozen thin sections of tumor (Fig. 3B) and in purified TIL (data not shown), and found that perforin and granzyme B (Fig. 5A) proteins are localized in cytoplasmic granules.

Collectively, these experiments demonstrate that TIL contain both cytokine mRNAs (12) and cytolytic enzymes localized in cytotoxic granules characteristic of mature CD8\(^{+}\) effector T cells.

**TIL cytokine secretion**

The RNA analysis showed that TIL contain cytokine mRNAs (12) but did not reveal whether these cytokines are secreted. To address this, purified nonlytic TIL were stimulated in vitro and secretion of IL-2 and IFN-\(\gamma\) assessed by ELISA (Fig. 4). Secretion of IFN-\(\gamma\) was rapid: after 2 h of in vitro activation ~300 U/10\(^6\) cells were produced. After 4 h of activation, high levels of IFN-\(\gamma\) were produced, ~3000 U/10\(^6\) cells.

**TIL cytolytic granules do not localize to the immunological synapse**

For effector T cells to demonstrate lytic function in response to TCR-mediated signaling after target cell recognition, preformed cytotoxic granules must accumulate under the plasma membrane closely apposed to target cells, fuse to the membrane, and then release granule contents. Because TIL contain perforin and serine esterases but are nonlytic, we considered the possibility that granule exocytosis was defective. We tested whether localization of lytic granules to the immunological synapse occurred on TCR-mediated recognition of target cells by redirected assay in vitro (Fig. 5A). TIL, either freshly isolated or after in vitro recovery, were mixed with target cells for 5–10 min, and then the intracellular localization of lytic granules was determined by immunofluorescence microscopy using Ab to granzyme B. In freshly isolated, nonlytic TIL, only ~5% of T cells forming conjugates orient lytic granules to the area of tumor cell contact. However, in ~60–70% of conjugates formed between tumor cells and recovered TIL, granzyme B\(^{+}\) granules were reoriented to the immunological synapse. Representative pictures of granzyme B staining are shown in Fig. 5A, where in recovered but not freshly isolated TIL, lytic granules are easily visualized in close apposition with tumor cell targets.

**FIGURE 4.** Cytokine secretion from purified TIL. TIL were isolated and plated in triplicate (at 2 x 10\(^5\) cells/well) in 96-well dishes precoated with either anti-TCR (+) or control Ab (–). At the indicated times, culture supernatants were collected and assayed for IFN-\(\gamma\) or IL-2 by ELISA. Standard curves established using recombinant cytokines showed that the ELISA sensitivity was 19 pg/ml for IL-2 and 195 pg/ml for IFN-\(\gamma\). One nanogram IL-2 equals 37.5 U, and 1 ng IFN-\(\gamma\) equals 5.2 U.

**FIGURE 3.** TIL are mature CD8\(^{+}\) T cells. A, TIL contain perforin and granzyme B proteins. Single-cell suspensions were prepared, and CD8\(^{+}\) T cells were purified by magnetic immunobead (positive selection from tumors and negative selection from spleens). T cells were labeled for either perforin or granzyme B and CD8. Flow cytometric analyses were gated on CD8\(^{+}\) T cells. TIL (solid tracings in right panel) or primary MLR T cells (dashed tracings in right panel) were isolated and analyzed by flow cytometry for perforin and granzyme B proteins. Binding of primary Ab was detected using fluorochrome-conjugated secondary Abs. Species- and isotype-matched control Abs were used to establish the levels of nonspecific Ab labeling (shaded histograms). B, TIL perforin is localized in granules. Immunocytochemical detection of perforin protein (arrows) was made in frozen thin sections prepared from tumor tissue. There was no staining of cells using isotype-matched control Ab (data not shown). Magnification, ×100.
**FIGURE 5.** Mobilization of cytotoxic granules is defective in TIL. A, TIL do not localize cytotoxic granules to the immunological synapse. TIL were isolated, used in redirected assays with P-815 cells as targets, and analyzed by confocal microscopy using Ab to granzyme B either before recovery in culture or after as indicated. Nonimmune goat IgG was used as control. Arrows, granzyme B* granules. Identical results were obtained using cognate tumor cells as targets. B, TIL MTOCs do not reorient on contact with target cells. TIL were isolated and used in reorientation assays with cognate MCA-38 cells either immediately on isolation or after overnight recovery. Cells were stained using anti-β-tubulin and processed for immunofluorescence microscopy. Arrows, MTOC. Nonimmune murine Ig was used as control. At least 10 microscopic fields per sample were viewed, each with 25–75 T cells per field, and this experiment was repeated eight times. In fresh, nonlytic TIL, of those TIL forming conjugates, only 5–15% of cells have reoriented MTOC (toward target cells). In recovered, lytic TIL, the percentage is 45–70%. Magnification, ×150.

Exocytosis of TIL lytic granules is blocked due to failure of the MTOC to localize to the T cell immunological synapse

After TCR-mediated recognition of cognate Ag, migration of lytic granules to the immunological synapse requires several sequential biochemical events including orientation of the MTOC to the T cell immunological synapse. The MTOC is a multicomponent structure that is the site for aggregation of cytoplasmic microtubules on which lytic granules migrate to the immunological synapse, where they fuse to the plasma membrane to release lytic enzymes. Because TIL were determined to contain mature lytic granules and because we showed that mobilization of granules to the T cell immunological synapse was defective, we asked whether TIL MTOC localized to the area of cell contact on formation of conjugates with cognate tumor target cells. As was found by analysis of lytic granule migration, TIL MTOC did not localize to the immunological synapse (Fig. 5B). In TIL, the lytic function is restored by purification and short term in vitro culture, the MTOC localizes to the immunological synapse within 5–10 min of contact with cognate tumor in vitro. Granule migration in recovered TIL does not occur if noncognate tumor is used as target cells (data not shown).

This finding shows that nonlytic, freshly isolated TIL are defective in an obligate early step in the process of TCR-mediated release of preformed cytotoxic granules, that of MTOC mobilization. Furthermore, recovery of lytic function is correlated with MTOC reorientation to the immunological synapse.

Conjugate formation by redirected assay does not restore lytic function or lytic granule mobilization

Freshly isolated, nonlytic TIL were observed to form fewer conjugates with cognate target cells in vitro compared with TIL after recovery (Table I). One possible interpretation of this finding is that target cell contact by TIL may be unstable, which might result in insufficient activation of T cell lytic function. We consider this interpretation unlikely: when conjugates are formed between nonlytic TIL and target cells by bridging TIL and target cells with anti-CD3 Ab (redirected assay), an equal number of conjugates are formed by nonlytic TIL as for lytic TIL, but cytolysis still does not occur (Table I). This finding shows that target cell conjugate formation is necessary but insufficient for cytolysis. Recovered TIL in the redirected conjugate formation assay are both lytic, and their lytic granules mobilize to the immunological synapse (Fig. 5A), but freshly isolated TIL are nonlytic and do not mobilize their lytic granules. Collectively, these observations show that even when nonlytic TIL form conjugates at the same frequency as lytic TIL, lytic granules do not localize to the immunological synapse.

**Role of different protein tyrosine kinases in TIL lytic function**

Release of granules from cytolytic cells involves a Ras-dependent and a Ras-independent pathway of signal transduction. NK cells activated to release granules, either on recognition of MHC class I-negative target cells or via FcyRIII ligation, use signal transduction involving MAPK-ERK, Vav, Pyk-2, PI3 kinase, and Syk 70

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* TIL were isolated and either tested for conjugate formation with P-815 cells or cognate tumor cells as targets immediately or after recovery in vitro. Aliquots were tested for lytic function, and conjugates were enumerated by light microscopy by counting the number of conjugates per microscopic field and the total number of T cells in the same field. The average percentage of TIL forming conjugates was obtained from viewing 5–10 microscopic fields each with 50–100 cells per field. Conjugate formation by redirected assay was dependent upon anti-CD3e Ab.
kinases (25). Granule release in T cells is less well understood: one report shows that MAPK-ERK activation is required, though to be activated primarily through the TCR pathway (26); whereas other reports implicate PI3 kinase (27) or PKC in lytic function (28). After recovery of TIL lytic function, the role of three major T cell protein tyrosine kinases in TIL lysis was investigated by inclusion of various inhibitors during lysis assays. For these experiments, the redirected assay was used, so that direct comparison of TIL function with primary MLR CTL T cells (used as positive controls) could be made under identical conditions. Because the results of lysis assays were consistent when conducted at different E:T ratios, for comparison of the effects of different inhibitors, only data from a single E:T ratio (10:1) is shown. Over a range of concentrations typically used in inhibition assays (27) inhibition of either PI3 kinase (wortmannin), ERK (PD98059), or PKC (bisindolmaleamide) caused dose-dependent reduction of lytic function (Fig. 6). MLR CTL lytic function was also inhibited in a manner similar to that for TIL, showing that the requirement for PI3 kinase, ERK, and PKC in target cell lysis is not restricted to TIL.

Proximal TCR-mediated signal transduction is normal in nonlytic TIL

Using either NK cell or T cell lines, others have shown that pharmacological inhibition of ERK (29), PKC (28), or PI3 kinase (30) blocks lytic function. We analyzed TIL for the presence and activation status of those kinases suggested to play a role in granule release. First, we examined TIL for the pattern of total tyrosine-phosphorylated proteins by immunoblot analysis of lysates prepared from freshly isolated TIL before and after activation in vitro with anti-TCR Ab. As a control, spleen CD8\(^+\) T cells prepared by identical procedures were used for comparison. Similar to spleen cells, TIL have a very low basal level of tyrosine-phosphorylated proteins and, on activation in vitro, several prominent protein species are rapidly phosphorylated (Fig. 7A) including ZAP 70 and mitogen-activated protein/extracellular signal-related kinase kinase (data not shown). This observation shows that TIL proteins are not hyperphosphorylated due to overstimulation in the tumor microenvironment, which might inhibit subsequent activation events, and also that TCR signal transduction is intact at least at the level of the proximal components of the pathway.

Because ERK has been implicated in granule release from both T cells and NK cells we next examined the levels and activation status of ERK protein. Again, like spleen CD8\(^+\) T cells, freshly isolated TIL contain ERK1/2 protein (Fig. 7A). Stripping the immunoblot membrane and reprobing with Ab specific for activated (phosphorylated) ERK revealed enhanced phosphorylation of the p42 form of ERK (ERK2) in TIL, but not ERK1, in contrast to activated spleen T cells which contain both ERK1 and ERK2.

On discovery that TIL do not contain p44 phospho-ERK, we considered that the failure to activate both forms of ERK may be the biochemical basis for defective MTOC orientation and lytic function. This possibility was further investigated by assessment of
the levels of ERK proteins in TIL whose lytic function was restored. Lysates were prepared from either freshly isolated or recovered TIL and, as positive control, purified CD8+ T cells from primary MLR cultures, and analyzed by immunoblotting. Lysates were prepared directly after purification or following activation of TIL in vitro with anti-TCR Ab. Aliquots of both recovered TIL and MLR cells were determined to be lytic (~40% lysis at E:T of 10:1: data not shown). Nonlytic and lytic TIL and lytic MLR T cells have phospho-ERK2 but do not contain activated p44 ERK demonstrating, we believe for the first time, that ERK1 is not required for perforin-mediated lytic function in T cells and eliminating the hypothesis that the failure to activate p44 ERK in TIL is the basis for lytic dysfunction.

Because activity of PI3 kinase has been shown to be involved in cytolytic function of NK cells (30) and T cells (Fig. 6), we asked whether PI3 kinase was activated in TIL. For this determination we analyzed the level and activation status of the immediate downstream target of PI3 kinase, Akt. Primary MLR CD8+ T cells, again used as positive controls, and freshly isolated TIL were prepared and either activated or not before lysate preparation followed by immunoblot analysis. TIL contain nonphosphorylated Akt protein which is rapidly phosphorylated on activation showing that TCR-mediated signal transduction through PI3 kinase is intact in nonlytic TIL.

Because the inhibitor experiments implicated a role for PKC in CTL function and also because of a previous report suggesting the same requirement (28), activation of PKC was also tested. Cell extracts were prepared either from freshly isolated or from TIL recovered by in vitro culture. The subcellular localization of several PKC isoforms was determined by fractionation of extracts into membrane and cytosolic fractions that were subjected to SDS-PAGE and immunoblotting with isoform-specific Abs (Fig. 7B). TIL contain similar levels of membrane-associated PKC compared with control T cells, except PKC-α which has higher membrane-associated levels than control spleen cells. (Because both nonlytic or recovered TIL have higher PKC-α levels, this observation appears unrelated to lytic status.) On activation in vitro, additional PKC protein becomes membrane associated. Compared with spleen cells, the kinetics of activation-induced PKC membrane association in TIL is slightly delayed: by 10 min of stimulation, a portion of PKC in spleen T cells becomes membrane associated; whereas a shift in localization does not occur in TIL until after 10 min of activation in vitro. Because the kinetics of membrane association of PKC is identical for lytic and nonlytic TIL, the delay in activation of PKC relative to primary spleen CD8+ T cells cannot be directly related to defective TIL lysis. This analysis shows that there is no dramatic difference between nonlytic and lytic TIL in activation of (several isoforms of) the third protein tyrosine kinase the function of which is suggested to be required for lytic activity, PKC.

**TIL calcium flux is normal**

Because TCR activation-induced release of lytic granules has been shown to be dependent on calcium flux (31), we tested TIL calcium mobilization. Although freshly isolated TIL are nonlytic, calcium flux in response to activation was indistinguishable in comparison with lytic TIL (Fig. 8). Supporting this observation, treatment of nonlytic TIL with PMA plus ionomycin did not restore lytic function (data not shown), showing that although requirement for activation of the proximal signaling pathway was circumvented, TIL lytic function may require another activating event or may be blocked. Because rapid calcium flux requires TCR-dependent signal transduction, this finding further supports the notion that proximal TCR-mediated signaling is intact in TIL.

Collectively, these pharmacological and biochemical analyses demonstrate that the major signal transduction pathways in nonlytic TIL are intact and that the three protein tyrosine kinases suggested to be essential in cytolytic function, ERK, PKC, and PI3 kinase, as well as calcium mobilization, are functional in TIL.

**TIL localization of PYK-2 or Rab 27a**

Membrane translocation of the nonreceptor tyrosine kinase PYK-2 has been recently shown to be essential for NK cell cytolyis (32). We compared nonlytic and lytic TIL for levels of PYK-2 protein (by immunoblotting) and also for PYK-2 subcellular localization on conjugate formation in vitro (by confocal microscopy). We found both lytic and nonlytic TIL have equivalent levels of PYK-2 protein, suggesting that down-regulation of PYK-2 expression does not contribute to the TIL lytic defect. Furthermore, and in contrast to PYK-2 function in NK cells, PYK-2 does not localize to the immunological synapse in fully lytic T cells, thereby excluding a potential PYK-2 defect in nonlytic TIL (data not shown).

Additionally, the intracellular movement of vesicles to the plasma membrane during activation-induced lysis has been recently shown to be dependent on the function of a small GTPase, Rab 27a (33, 34). Therefore, we examined nonlytic and lytic TIL for levels (Fig. 9A) and subcellular localization of Rab 27a (Fig. 9B). The level of Rab 27a protein is identical in lytic and nonlytic TIL (Fig. 9A), eliminating the possibility that nonlytic TIL do not produce sufficient Rab 27a (or degrade it abnormally), thereby inhibiting lytic function. Also, Rab 27a localization was investigated by confocal microscopy (Fig. 9B). Lytic and nonlytic TIL were labeled with both anti-Rab 27a (green) and anti-granzyme B (red) to visualize colocalization of Rab 27a with lytic granules. Rab 27a protein is distributed widely in T cells, but granzyme B+ lytic granules colocalize with anti-Rab 27a (yellow in Fig. 9B), showing that nonlytic TIL contain Rab 27a protein which colocalizes with lytic granules albeit, as shown previously, not at the immunological synapse.

Acquisition of the lytic dysfunction in TIL involves induction of a repressor

Two types of data suggest that acquisition of the lytic dysfunction in TIL may involve induction of a repressor: signal transduction is functional in nonlytic TIL (and the levels and localization of other...
components of the lytic machinery are normal (PYK-2 and Rab 27a)) and recovery of lytic function does not require protein synthesis. According to this notion, a putative repressor is degraded during TIL recovery in vitro. This was directly tested by performing recovery of TIL function in the presence or absence of two different inhibitors of proteasome function, lactacystin and MG132 (Fig. 10). If proteasome inhibitors are included during the 6-h incubation in vitro, there is a dose-dependent inhibition of recovery of lytic function. However, if TIL are cultured in vitro in the absence of inhibitor treatment, full lytic activity is recovered, and addition of proteasome inhibitors after recovery is without effect on lytic function.

These observations suggest that freshly isolated, nonlytic TIL contain an inhibitor of lytic function the proteasome-dependent degradation of which is required to regain lytic activity.

FIGURE 9. Lytic and nonlytic TIL contain equivalent amounts of Rab 27a which colocalizes with granzyme B+ lytic granules. A, Immunoblot analysis of Rab 27a. Whole cell detergent lysates were prepared from non-lytic and lytic TIL, separated on SDS-PAGE, and analyzed by immunoblotting with anti-Rab 27a Ab as described in Materials and Methods. B, Rab 27a colocalizes with lytic vesicles in nonlytic and lytic TIL. TIL were isolated and analyzed for localization of granzyme B and Rab 27a on conjugate formation with cognate tumor cells in vitro by confocal microscopy as described in Materials and Methods. Rab 27a was detected using FITC-conjugated secondary Ab, and granzyme B was detected using PE-conjugated secondary Ab. Arrows, Colocalization of granzyme B and Rab 27a. Magnification, ×200. This experiment has been repeated three times.

FIGURE 10. Recovery of lytic function requires proteasome function. TIL were isolated and allowed to recover in vitro for 6 h in the presence or absence (DMSO) of the indicated proteasome inhibitors (Freshly-isolated). Some TIL were first recovered by plating for 18 h and subsequently incubated with or without inhibitors for 6 h (Recovered). After all incubations, TIL were washed in complete medium and used as effector cells in standard redirected chromium release assays (at an E:T ratio of 10:1 with Na251CrO4-labeled P-815 cells in triplicate wells) as described in Materials and Methods. Viability of TIL after treatment with inhibitors was equal to that of TIL receiving vehicle only (DMSO).

Discussion

Defective lytic function of T cells infiltrating human (9) and rodent (10) tumors has been recognized for years, although the physiological basis for this phenotype has not been described. Typically, freshly isolated TIL do not lyse cognate tumor cells or MHC-matched tumor cell lines but can acquire lytic function after purification and propagation in vitro with exogenous IL-2.

We have analyzed the nature of the TIL lytic defect in murine tumors. After purification and brief culture in vitro, TIL demonstrate substantial tumor-specific, perforin-mediated cytolysis showing that TIL are transiently inhibited in lytic function. In keeping with the activated phenotype demonstrated by expression of various cell surface markers, TIL express high levels of FasL (12). However, after isolation and recovery in vitro, tumor cell lysis is exclusively due to exocytosis of lytic granules. FasL-mediated killing does not appear to be operative in tumors, because tumor cells are not detectably apoptotic in situ (12).

We have investigated a variety of considerations for defective TIL lysis including: TIL are immature (i.e., lacking expression of perforin/serine esterases); packaging of serine esterases into lytic granules is defective; or TIL are defective in granule exocytosis. Importantly, TIL contain high levels of perforin and serine esterase proteins that are packaged into lytic granules. These findings argue against the possibility that, after homing to the tumor site, TIL require further maturation defined by synthesis and packaging of serine esterases into granules.

TIL were considered to possibly resemble memory T cells in lymphoid organs which are usually nonlytic and require in vitro stimulation over many days to regain CTL activity (35). However, TIL recover lytic activity after only 6 h in vitro showing that their phenotype is distinct from memory cells. We further considered that TIL may resemble T cells that infiltrate normal nonlymphoid tissues in terms of their nonlytic phenotype. The best evidence in support of the notion that peripheral tissue T cells do not behave like TIL is provided by a recent report from Masopust et al. (36).
showing that Ag-specific T cells can exist in an activated form in tissues and be directly lytic upon isolation. The observation that TIL are mature CTL that recover lytic function after removal from contact with cells within the tumor bed implies that the tumor microenvironment induces the lytic dysfunction. This supposition is supported by the observation that tumor-draining lymph node CD8+ CTL have a high level of tumor-specific lytic capability (data not shown) which is obviously lost on entrance to the tumor bed. Despite containing granules loaded with lytic enzymes, mobilization of the lytic granules to the immunological synapse is defective as shown in immunofluorescence microscopic analyses of TIL:tumor cell conjugates formed in vitro. Defective localization of lytic granules was further shown to be due to the inability to recruit the MTOC to the immunological synapse, an activation-dependent event that is required for the proper localization of granules and subsequent exocytosis and target cell lysis (24, 37, 38). Defective MTOC mobilization is not likely due to some inherent defect in expression of tumor Ags by tumor cells in situ, because lytic granules do not migrate when conjugates are formed between TIL and Ag-irrelevant P-815 cells by redirected assay. This finding suggests either that some signaling event required for MTOC function is lacking in TIL or that MTOC function is repressed in nonlytic TIL.

Signaling requirements for CTL degranulation have been studied by others where, on the basis of pharmacological inhibitor or Ag dependency studies, it was shown that TCR-mediated signaling is required for release of serine esterases (39). On recognition of cognate Ag, CTL activate the ras/MAPK/ERK pathway and therein the PI3 kinase pathway, both of which are apparently required for cytolytic function (31). Activated PKC has been shown to be required for lytic function in certain T cell clones by influencing MTOC mobilization to the immunological synapse (28). TIL have defective mobilization of the MTOC despite normal TCR-mediated calcium flux and PKC activation. Therefore, although there is a precedent in the literature describing a requirement for both calcium flux and PKC function in both MTOC function and T cell cytolysis, the specific defect in TIL must be downstream of calcium flux and PKC activation.

Pharmacological inhibitor studies have shown that ERK activation is required for lytic function in murine MLR CTL (40). Supporting the notion that ERK is involved in degranulation, Wei et al. (29) have shown that in vitro lytic function of human NK cell lines and freshly isolated large granular lymphocytes is dependent on both contact with cognate target cells and activation of ERK2, but not ERK1. Our experiments using TIL, or as controls CTL obtained from primary MLR cultures, show a similar requirement for ERK2 activation in lysis of cognate tumor cells. ERK protein is present in nonlytic TIL and exists in the nonphosphorylated form; on TCR ligation, TIL and MLR CTL rapidly activate p42 ERK2 but not p44 ERK1. The lack of detectable activated ERK2 in lytic T cells suggests that, although ERK1 is required for T cell development (41), the effector phase function of T cell killing uses only ERK2. These experiments show that TCR-mediated signaling in TIL is intact and the lytic defect in TIL is downstream of ERK2 activation.

Degranulation of human NK cells was shown to require activation of PYK-2 (32). We have examined the activation-dependent recruitment of PYK-2 to the immunological synapse and found, in contrast to that observed in NK cell degranulation, that PYK-2 does not associate with the immunological synapse in fully lytic TIL, thereby excluding a defect in PYK-2 activation as the basis for the TIL lytic defect (data not shown).

After purification of TIL and brief culture, both localization of cytolytic granules to the immunological synapse and lytic activity are demonstrated. Recovery of TIL lytic function and granule localization does not require either RNA or protein synthesis, suggesting that acquisition of the lytic defect does not involve inactivation of a factor(s) required for granule mobilization. Rather, we suggest that a protein(s) is induced in TIL the expression of which prevents MTOC mobilization to the immunological synapse. This suggestion is further supported by our observation that inhibition of proteasome function during recovery in vitro blocks recovery of lytic function. Because TCR-mediated signal transduction is functional in nonlytic TIL, at least to the point of PI3 kinase-PKC-ERK activation (and therein calcium flux), a putative repressor of MTOC mobilization must act downstream of PI3 kinase-PKC-ERK activation. Such a repressor could act directly on the MTOC or may have an unknown target that is required for MTOC activation. Supporting this possibility is the observation that in a rat basophil cell line Ab to CD81 inhibits degranulation without inhibiting tyrosine phosphorylation or calcium mobilization (42), the phenotype demonstrated by nonlytic TIL. That precedent suggests that a similar regulatory mechanism may exist in TIL, a notion that we are at present pursuing.

References


