Dual Function of the Extracellular Matrix: Stimulatory for Cell Cycle Progression of Naive T Cells and Antiapoptotic for Tissue-Derived Memory T Cells

Andreas Sturm, Kimberley A. Krivacic, Claudio Fiocchi and Alan D. Levine

*J Immunol* 2004; 173:3889-3900; doi: 10.4049/jimmunol.173.6.3889
http://www.jimmunol.org/content/173/6/3889

**References**
This article cites 59 articles, 27 of which you can access for free at:
http://www.jimmunol.org/content/173/6/3889.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Dual Function of the Extracellular Matrix: Stimulatory for Cell Cycle Progression of Naive T Cells and Antiapoptotic for Tissue-Derived Memory T Cells

Andreas Sturm, Kimberley A. Krivacic, Claudio Fiocchi, and Alan D. Levine

Tissue T cells encounter Ag in a distinct microenvironment, where they are embedded in the interstitial extracellular matrix (ECM). In contrast, while naive T cells are exposed to Ag in the lymph node, immediately after naive T cells are activated they must extravasate into the ECM to function effectively. Because integrin-mediated adhesion to the ECM modulates cell cycle progression and survival in adherent nonimmune cells, we hypothesize that blood and tissue-derived T cells have similarly adapted their behavior to their first or continued encounter with ECM. T cells from peripheral blood (PBT) and tissue (the intestinal lamina propria T cell (LPT)) were stimulated with anti-CD3-coated beads in the presence or absence of native ECM derived from intestinal fibroblasts, plate-immobilized fibronectin, or collagen type I. Native ECM and collagen, but not fibronectin, induced in anti-CD3 activated PBT a 4- to 5-fold increase in the entry, progression, and completion of the cell cycle over that triggered by anti-CD3 alone. Neutralizing β1 integrin Abs abrogated this increase. None of these ECM proteins stimulated cell cycle progression in LPT. In contrast, anti-CD3 activation of LPT in the presence of native ECM and fibronectin reduced activation-induced cell death by 40%. These results demonstrate that naive and effector/memory T cells respond differently upon exposure to specific ECM components. When naive PBT encounter Ag in the context of ECM, their progression through the cell cycle is enhanced, favoring clonal expansion; while tissue T cell longevity may be mediated by interactions with the ECM. The Journal of Immunology, 2004, 173: 3889–3900.
Jurkat T cells (24). We recently reported that exposure to collagen during PBT activation rapidly generates an integrin expression profile and adhesive phenotype for ECM that mirrors the characteristics of tissue-derived T cells (25).

In the present study, we addressed these contradictory findings, by developing an in vitro model system that allows us to evaluate the constitutively active three-dimensional ECM derived from human intestinal fibroblasts (native ECM), purified fibronectin, or collagen on freshly isolated naive PBT, effector/memory PBT, and tissue-derived intestinal T cells, the prototypical extravascular effector/memory T cells. In addition, we recreated the three-dimensional geometry of Ag presentation in tissue by delivering the signals for TCR activation by anti-CD3-coated polystyrene beads. We performed a detailed analysis of the ability of $\beta_1$ and $\beta_2$ integrins to regulate the key events of cell cycle progression, and showed that naive and memory T cell populations respond differently to specific components of the ECM. Circulating PBT are costimulated by native ECM or collagen type I for entry into and progression of the cell cycle, while tissue T cells are protected against apoptosis by fibronectin. These results highlight the unique functional role exerted by the ECM in differentially modulating T cell responses, adjusting the immune outcome depending upon the origin and state of maturation of the responding T cell subset.

Materials and Methods

Reagents and Abs

For T cell isolation, the mucosal strips derived from surgically resected colonos were digested in collagenase type 3 (206 U/mg) and deoxyribonuclease type 1 (3228 U/mg), both from Worthington Biochemical (Lake-wood, NJ). Recombinant human IL-2 (Chiron, Emeryville, CA), and anti-CD3 mAb (OKT3; Ortho Diagnostics, Raritan, NJ) were used for T cell activation. Polystyrene, sulfate-coated beads (5.3 µm; Interfacial Dynamics, Portland, OR) were used for T cell stimulation. Fluorescein-conjugated granzin B1 was purchased from BD Pharmingen (San Diego, CA), anti-phosphohistone H3 Ab was obtained from Upstate Biotechnology (Lake Placid, NY), and secondary Alexa 350-labeled goat-anti-rabbit IgG was obtained from Molecular Probes (Eugene, OR). Propidium iodide (PI) was purchased from BD Pharmingen (San Diego, CA), anti-cyclin B1 was purchased from BD Pharmingen (San Diego, CA), anti-phosphohistone H3 Ab was obtained from Upstate Biotechnology (Lake Placid, NY), and secondary Alexa 350-labeled goat-anti-rabbit IgG was obtained from Molecular Probes (Eugene, OR). Propidium iodide (PI) was purchased from Calbiochem (San Diego, CA). All protease and phosphatase inhibitors used for Western blotting were purchased from Sigma-Aldrich (St. Louis, MO). The Abs against human retinoblastoma protein, cyclin A, p21, p27, and p53 were obtained from BD Pharmingen. For immunofluorescence, cells were stained with a mAb against human α1 (clone 1C10, Chemicon International, Temecula, CA). Anti-human integrin α2 mAb clone P1E6, anti-human integrin α3 mAb clone P1B5, anti-human integrin α4 clone P4C2, anti-human integrin α6 clone P1D6, and anti-human integrin β1 clone P4C10 were purchased from Invitrogen Life Technologies. Flow cytometry, the purification of PI (0.6 µg/ml, 30–60 Kunitz units; Sigma-Aldrich), incubated at 37°C for 15 min and then chilled on ice. One hundred twenty-five micro-liters of PI (200 µg/ml) were added before analysis by flow cytometry. Each analysis was performed on at least 25,000 events. Integrin surface receptor expression was determined after staining with Abs against human integrin α1, α4, and β1 for 45 min at 4°C, followed by an incubation with a goat-anti-rabbit AlexaFluor-350 Ab for 45 min at 4°C. Then, cells were washed twice with PBS, incubated with a polyclonal rabbit-anti-pH3 Ab for 45 min at 4°C, followed by an incubation with a goat-anti-rabbit AlexaFluor-350 Ab for 45 min at 4°C. Thereafter, cells were washed and incubated for 45 min at 4°C with cyclin B1-FITC-conjugated mAb. After the final wash, cells were resuspended in PBS and 5 µl of RNase (0.6 µg/ml, 30–60 Kunitz units; Sigma-Aldrich), incubated at 37°C for 15 min and then chilled on ice. One hundred twenty-five micro-liters of PI (200 µg/ml) were added before analysis by flow cytometry. Each analysis was performed on at least 25,000 events. Integrin surface receptor expression was determined after staining with Abs against human integrin α1, α4, and β1 for 45 min at 4°C, followed by FITC-goat anti-mouse Abs (BioSource International, Camarillo, CA). Cells were then washed twice in 1% BSA-PBS and fixed in 1% paraformaldehyde. Each analysis was performed on at least 10,000 events. The background level of immunofluorescence was determined by incubating cells with FITC- or PE-conjugated mouse IgG. Cell fluorescence was measured with the Elite ESP and Epics XL flow cytometers (Beckman Coulter, Miami, FL) using UV and/or 488-nm excitation and band pass filters optimized for individual fluorochromes. Flow cytometry data were analyzed with WinList (Verity Software House, Topsham, ME). As an alternate determination of apoptosis, cells were stained with a FITC-labeled mAb against annexin V (BD Pharmingen) to detect externalization of phosphatidylserine to identify necrotic cells. Fluorescence on a minimum of 15,000 cells was analyzed by flow cytometry using the CellQuest software program (BD Pharmingen).

Preparation of T lymphocytes from intestinal lamina propria and peripheral blood

Intestinal T cells were isolated from surgical specimen obtained from patients admitted for bowel resection, for malignant or nonmalignant conditions, including colon cancer, benign polyps, rectal prolapse, and diverticulosis. All specimens were histologically normal. Healthy volunteers served as the source of PBT. Lamina propria T cells (LPT) were isolated as previously described (26). Briefly, the dissected intestinal mucosa was freed of mucus and epithelial cells in sequential washing steps with DTT and EDTA, and digested overnight at 37°C with collagenase and deoxyribonuclease. Mononuclear cells were separated from the crude cell suspension by layering on a Ficoll-Hypaque density gradient. For LPT purification, macrophage-depleted lamina propria mononuclear cells were incubated for 30 min at 4°C with magnetically labeled anti-CD19, -CD14, and -CD16 Abs directed against B lymphocytes, monocytes, and neutrophils, respectively (MACS; Miltenyi Biotec, Auburn, CA). T cells were then collected by negative selection using MACS. PBT were isolated from heparinized venous blood using Ficoll-Hypaque density gradient and monocyte depleted. The same process of negative selection described for LPT was used to isolate PBT. As assessed by flow cytometry, the purified LPT and PBT population contained >90 and >95% CD3+ cells, respectively. For isolation of naive (CD45RA+) and memory (CD45RO+) PBT, PBMC were submitted to the same process of negative selection described for LPT in combination with CD45RO Abs to select the CD45RA+ population, or CD45RA Abs to select the CD45RO+ population, respectively (CD45 RA and RO Abs from Miltenyi Biotec). The purity of these naive and memory populations was >97%.

Isolation and culture of human intestinal fibroblasts (HIF)

HIF were isolated as described previously (27). Briefly, surgical specimens from patients undergoing bowel resection for colon cancer were washed in calcium- and magnesium-free HBSS (BioWhittaker, Walkersville, MD). Strips of normal mucosa, at least 10 cm from the margin, were dissected, cut in small fragments, and laid on the bottom of scored tissue culture dishes. Medium consisting of MEM supplemented with 10% heat-inactivated FCS, 2.5% t-glutamic acid, 1% mixture of penicillin, streptomycin, and Fungizone (PSF), and 25 mM HEPES buffer (all from BioWhittaker) for 10 min, followed by 0.25 N ammonium hydroxide (Fisher Scientific, Hanover Park, IL) for 2 min (28). The resultant native ECM was washed six times with 2 ml of DPBS.

Flow cytometry

Analysis of cell cycle phase distribution was performed after staining for cyclin B1, phosphohistone H3, and PI followed by flow cytometry as previously described (29). Briefly, cells were washed twice with PBS, adjusted to 1 × 10^6 cells/sample and fixed in 90% methanol at −20°C. After fixation, cells were washed twice with PBS, incubated with a polyclonal rabbit-anti-pH3 Ab for 45 min at 4°C, followed by an incubation with a goat-anti-rabbit AlexaFluor-350 Ab for 45 min at 4°C. Thereafter, cells were washed and incubated for 45 min at 4°C with cyclin B1-FITC-conjugated mAb. After the final wash, cells were resuspended in PBS and 5 µl of PI (200 µg/ml) were added before analysis by flow cytometry. Each analysis was performed on at least 25,000 events. Integrin surface receptor expression was determined after staining with Abs against human integrin α1, α4, and β1 for 45 min at 4°C, followed by FITC-goat anti-mouse Abs (BioSource International, Camarillo, CA). Cells were then washed twice in 1% BSA-PBS and fixed in 1% paraformaldehyde. Each analysis was performed on at least 10,000 events. The background level of immunofluorescence was determined by incubating cells with FITC- or PE-conjugated mouse IgG. Cell fluorescence was measured with the Elite ESP and Epics XL flow cytometers (Beckman Coulter, Miami, FL) using UV and/or 488-nm excitation and band pass filters optimized for individual fluorochromes. Flow cytometry data were analyzed with WinList (Verity Software House, Topsham, ME). As an alternate determination of apoptosis, cells were stained with a FITC-labeled mAb against annexin V (BD Pharmingen) to detect externalization of phosphatidylserine to identify necrotic cells. Fluorescence on a minimum of 15,000 cells was analyzed by flow cytometry using the CellQuest software program (BD Pharmingen).

Western blotting

Cells were washed twice in cold PBS, and lysed in cell lysis buffer (1% Triton-X, 0.5% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 5 mM EDTA, 50 mM phosphate and 50 mM protease inhibitor mixture, 1 mM PMSF, 100 µg/ml trypsin-chymotrypsin inhibitor, 100 µg/ml chymostatin in PBS). The concentration of proteins in each lysate was measured using the Bio-Rad protein assay (Hercules, CA). Equivalent amounts of protein (10 µg) were fractionated on a 4–20% Tris-glycine gel and electrotransferred to a nitrocellulose membrane (NOVEX, San Diego, CA). Membranes were blocked overnight at 4°C with 5% milk in 0.1% Tween 20-PBS (Fisher Scientific), followed by incubation for 60 min at room
FIGURE 1. Native ECM and collagen, but not fibronectin stimulate TCR-activated peripheral T cell entry and passage through the cell cycle. Freshly isolated PBT \( (3 \times 10^5) \) were stimulated with predetermined suboptimal concentrations of bead-bound anti-CD3 (25 \( \mu \)g/ml OKT3) in the absence or presence of native ECM, or graded concentrations of immobilized fibronectin or collagen type I. After 4 days, cells were stained for flow cytometric analysis of cyclin B1, phosphohistone H3, and DNA content \( (>2n, \text{ i.e. } S/G_2/M \text{ phases}) \). A. Representative dot plots depict the increase in cell cycle entry (DNA content, x-axis) and progression (cyclin B1, y-axis) induced by matrix. The mean percentage of cells positive for cyclin B1 (■), phosphohistone H3 (□) and in the S/G2/M phase (□) are shown for PBT activated in the presence of native ECM (B), fibronectin (C), and collagen (D).
temperature with the indicated primary Ab. The membranes were washed six times with 0.1% Tween 20-PBS and then incubated for 1 h with the appropriate HRP-conjugated secondary Ab (Santa Cruz Biotechnology), washed again, and incubated with a chemiluminescent substrate (Super Signal; Pierce, Rockford, IL) for 5 min. The membranes were then exposed to film (Amersham, Arlington Heights, IL).

**T cell stimulation**

Anti-CD3-coated beads were made by incubating $15 \times 10^6$ polystyrene, sulfate-coated beads (5.3 μm) overnight at room temperature, gently rocking with the following concentrations of OKT3 mAb: 0, 10, 25, 50, 75, or 100 μg/ml in 0.2 M carbonate-bicarbonate buffer, pH 9.5. Beads were then washed three times with DPBS, and resuspended in RPMI 1640, and used immediately. Flat-bottom 96-well plates were precoated overnight at 4°C with 0.5, 20, 100, or 500 μg/ml fibronectin in DPBS, or with collagen type I in 0.1 M acetic acid, and were then washed three times with DPBS. Freshly isolated T cells and anti-CD3-coated beads were then mixed at a 1:1 ratio in serum-free media and $1.3 \times 10^5$ T cells were added to each well of a 96-well plate or $3 \times 10^5$ cells in each well of a 24-well plate in the absence or presence of the indicated matrix, and incubated for 4 days at 37°C in a humidified incubator containing 5% CO2. Cells were then harvested, fixed in 90% methanol, and stained for flow cytometry. To measure proliferation, cells were cultured in 96-well plates at $2 \times 10^5$ cells/well at 37°C in a humidified incubator containing 5% CO2 for 96 h. One-half of a microcurie of [3 H]thymidine per well (DuPont/NEN, Boston, MA) was incorporated for the final 20 h of culture. Cells were harvested using a Harvester 96 (Tomtec, Orange, CT) onto glass filter mats and counted in a beta-scintillation counter (Wallac, Gaithersburg, MD). Suboptimal stimulation for PBT and LPT was observed for beads coated with 25 μg/ml OKT3. Maximal or optimal proliferation was induced with beads coated with 50 μg/ml, and a less than optimal response was noted with beads coated with 75 or 100 μg/ml OKT3.

![Figure 2](image)

**FIGURE 2.** Native ECM and collagen differentially regulate the expression or phosphorylation of cell cycle promoters and inhibitors. Fresh PBT were stimulated with predetermined suboptimal concentrations of bead-bound anti-CD3 (25 μg/ml OKT3) in the presence of BSA-coated plastic culture plates, 5 or 500 μg/ml fibronectin or collagen type I, or native ECM for 3 days, after which expression of cell cycle regulators was assessed by immunoblotting. Each panel is representative of four to six different experiments. GAPDH was used as an internal standard.

![Figure 3](image)

**FIGURE 3.** Native ECM-induced cell cycle progression is mediated through β1 integrins, and purified collagen stimulation is primarily mediated through α1,4,5. Fresh PBT were stimulated with 25 μg/ml bead-bound OKT3 and either native ECM (A) or 20 μg/ml collagen (B). After 4 days, the cells were analyzed by flow cytometry for cyclin B1 expression (□) and DNA content (■ S/G2/M phase). At the initiation of culture, saturating concentrations of blocking Abs to α1, α4, α5, and β1 were added as indicated. Each panel is representative of three different experiments.
Statistical analysis
Statistical analysis was performed using the paired Student t test (Microsoft Excel, Redmond, WA). Results are expressed as mean ± SEM, and significance was inferred with p values <0.05.

Results
Native ECM and collagen provide potent costimulation for cell cycle entry and progression by freshly isolated PBT
We have previously shown that activation of freshly isolated PBT in the presence of ECM components rapidly alters both the surface expression and functional status of integrins on PBT, thereby conditioning them to assume a more adhesive phenotype inherent to tissue-derived T cells (25). Therefore, we investigated the functional response of PBT when their TCR was cross-linked concurrently with their exposure to native ECM or two individual ECM components. PBT were activated by anti-CD3-coated polystyrene beads, used as surrogate APCs, and plate immobilized native ECM, fibronectin, collagen type I, or BSA as a control, and we assessed the role of integrin-dependent costimulation on cell cycle progression (Fig. 1). The aim of this in vitro model is to mimic the in vivo setting in which TCR and β1 integrin substrates are delivered to the T cell on two opposing surfaces, with the goal of addressing the ongoing controversy of whether integrins transduce signals required for cell cycle progression or simply provide the adhesive contact needed by the TCR to engage its cognate ligand (4–6). Resting T cells reside in the G0/G1 phase of the cell cycle and to progress through the cell cycle, they must enter and complete the S and G2/M phases. Progression through the S phase is controlled by cyclin A, and cyclin B1 drives the cell through late G2 phase up to mitosis, ensuring the initiation of cell division (30). The level of cyclin B1 was examined by flow cytometry in conjunction with PI staining to exactly localize its change within the cell cycle. Furthermore, we measured the phosphorylation of histone H3, a protein whose modification is exclusively detectable during unfolding of DNA in mitosis (31). When PBT were stimulated in the presence of native ECM or immobilized collagen type I, but not fibronectin, a significant stimulation of cyclin B1 expression was induced (Fig. 1; p < 0.01), indicating a potent costimulatory signal for PBT to enter the G2/M phase. PBT did not enter the cell cycle in the presence of ECM without engagement of the TCR/CD3 complex (data not shown). Confirmation of the entry of the cells into the S phase was obtained by pulsing the cells with [3H]thymidine for their final 16 h of culture. Both collagen and native ECM stimulated a 7- to 8-fold increase (20,000 cpm) in proliferation when compared with suboptimal CD3 cross-linking in the absence of matrix or CD3 cross-linking in the presence of

FIGURE 4. Collagen stimulates T cell homotypic clustering. Fresh PBT were stimulated with 25 μg/ml bead-bound OKT3 in the presence of BSA-coated plastic culture plates, 20 μg/ml fibronectin, 20 μg/ml collagen type I, or native ECM and on day 4, representative phase contrast microscopic images (×100 magnification) were recorded. Each panel is representative of four to six different experiments.

FIGURE 5. β1 integrin-mediated adhesion overcomes the requirement for cell-cell contact. Fresh PBT were stimulated with 25 μg/ml bead-bound OKT3 in the absence (A) or presence (B) of functional blocking Ab directed against the β2 chain. On day 4, representative phase contrast microscopic images from three donors (×100 magnification) were recorded (C). Parallel PBT cultures, activated with bead-bound OKT3 in the presence of a blocking Ab to β2 integrin, were incubated with increasing concentrations of collagen type I. Expression of cyclin B1 (□) and DNA content (■, S/G2/M phase) on day 4 was determined by flow cytometry. Each panel is representative of three different experiments.
fibronectin (2,500 cpm). Although the data in Fig. 1 illustrate the stimulation of cell cycle progression induced by collagen and native ECM at a fixed concentration of OKT3 on day 4, it is important to appreciate that cell cycle progression was monotonically increased over the 4-day period. In addition, the ability of collagen to costimulate T cell cycle progression was dose dependent and occurred at suboptimal, optimal, and superoptimal concentrations of TCR cross-linking in the presence and absence of serum (data not shown). We previously reported that collagen type I enhances the surface expression of its cognate integrin \(\alpha_4\beta_1\) on freshly isolated PBT (25), an observation consistent with our current finding that collagen stimulates entry of PBT into cell cycle progression.

**FIGURE 6.** Tissue-derived T cells are not stimulated by native ECM, fibronectin, or collagen. Freshly isolated LPT were stimulated with predetermined suboptimal concentrations of bead-bound anti-CD3 (25 \(\mu\)g/ml OKT3) in the absence or presence of native ECM, or graded concentrations of immobilized fibronectin or collagen type I. After 4 days, cells were stained for flow cytometric analysis of cyclin B1, phosphohistone H3, and DNA content. A, Representative dot plots demonstrating no change in cell cycle entry (S and G2/M phase, x-axis) and progression (cyclin B1, y-axis) induced by matrix are shown. The mean percentage of cells positive for cyclin B1 (■), phosphohistone H3 (■) and in the S/G2/M phase (■) are shown for LPT activated in the presence of native ECM (B), fibronectin (C), and collagen (D). Each panel is representative of four to six different experiments.
CD3 Abs, we investigated the levels of key regulatory molecules of the cell cycle in matrix-activated PBT. Immunoblot analysis showed that the phosphorylation of Rb, which is essential for the G_{1}/S phase transition and passage beyond the restriction point, increased when CD3-activated PBT were cultured with collagen or native ECM, but not fibronectin (Fig. 2). Similarly, strong up-regulation of cyclin A was induced in CD3-cross-linked PBT by collagen or native ECM, but not fibronectin (Fig. 2). In addition, expression of the key cell cycle regulator p21 increased after co-stimulation with collagen type I or native ECM, but not fibronectin (Fig. 2). In contrast, neither collagen, fibronectin, nor native ECM altered p27 protein expression. p53 expression and phosphorylation (pp53) increased only modestly in anti-CD3 activated PBT by collagen or native ECM, but not fibronectin (Fig. 2).

**ECM-induced cell cycle progression in PBT is mediated by β_{1} integrins**

The ability of native ECM and collagen type I to costimulate cell cycle progression of CD3-activated PBT suggested an integrin-dependent mechanism. We have previously shown that PBT adhesion to native ECM and collagen type I is mediated by the β_{1} integrin family and that adhesion to collagen type I is almost exclusively mediated by the α_{1} subunit (25). Freshly isolated PBT were costimulated with anti-CD3-coated beads and either native ECM (Fig. 3A) or collagen type I (Fig. 3B) in the absence or presence of a mAb that blocks adhesion through the α_{1} or β_{1}
integrin chain. PBT entry into the cell cycle, as determined by the expression of cyclin B1 or the percentage of cells in the S/G2/M phases, was significantly inhibited by neutralizing the β1 family of integrins (p < 0.01). The presence of β1 integrin blocking Ab during PBT stimulation with bead-bound OKT3 alone did not inhibit cell cycling (data not shown), indicating the lack of a requirement for β1 integrin engagement in the absence of matrix. Furthermore, co-stimulation by type I collagen was largely mediated by the α6 subunit, in that >70% of PBT cyclin B1 expression and entry into the S/G2/M phases was inhibited by a neutralizing anti-α6 Ab (Fig. 3B, p < 0.01). Consistent with the identification of collagen-induced cell cycle progression being mediated by the α6β1 integrin, neutralizing Abs to α6 or α5 chains did not inhibit cyclin B1 expression and entry into the S/G2/M phases after co-stimulation (Fig. 3B). In contrast, neutralizing anti-α4 Ab did not inhibit PBT cell cycle progression induced by the native ECM, underscoring the importance of investigating a complex matrix of natural origin.

Collagen type I increases homotypic clustering of activated PBT

We routinely observed that high density T cell cultures (>1,200,000 cells/ml) required a lower concentration of bead-bound OKT3 to achieve the same percentage of cells progressing through the cell cycle, when compared with a low density culture (300,000–600,000 cells/ml; data not shown). Therefore, we assessed whether the presence of ECM components would collaborate with the cell-to-cell contacts likely to occur in a high density culture. Freshly isolated PBT were cultured on BSA-coated plastic, fibronectin, collagen type I, or native ECM in the presence of anti-CD3-coated beads for 4 days. In the presence of plate-immobilized collagen type I or native ECM, the size and number of PBT homotypic clusters, as observed by transmitted light microscopy, were decidedly increased compared with that of cells cultured in either BSA-coated plastic or fibronectin (Fig. 4). In fact, the number and size of PBT cell clusters in the presence of fibronectin were decreased when compared with those in the BSA-coated control wells (Fig. 4). PBT activated in the presence of fibronectin acquired a spread morphology and remained as isolated cells on the bottom of the wells, while PBT activated on native ECM or collagen assumed a spread morphology but formed large aggregates. Similarly, when PBT were activated in the presence of collagen or native ECM, but not fibronectin, cell size significantly increased, as determined by increased forward scatter on a flow cytometer (p < 0.05; data not shown).

Engagement of β1 integrins by the ECM compensates for the loss of β2-mediated cell-cell adhesion and supports PBT cell cycle progression

Because anti-CD3-induced cell cycle progression is dependent on T cell density, the interaction between the β1 and β2 integrin costimulatory pathways to promote S phase entry in quiescent T cells was investigated. Freshly purified PBT, stimulated by bead-bound OKT3 alone, rapidly formed homotypic clusters, which were entirely dependent on β2 integrin-mediated cell-cell adhesion, as shown by the complete inhibition of cell clustering in the presence of blocking β2 Ab (Fig. 5, A and B). Similarly, saturating concentrations of β2 Abs inhibited cell cycle progression of activated PBT (Fig. 5C). The inhibition of PBT cell cycle progression by blocking β2-mediated cell clustering could be reversed by activating T cells in the presence of various concentrations of collagen type I (Fig. 5C), but not fibronectin (data not shown). These findings demonstrate that under conditions where β2 integrins are inhibited, ligation of the β1 subset of integrins by collagen type I during TCR triggering provides the critical alternate second signal necessary for PBT cell cycle progression in vitro.

Native ECM, fibronectin, or collagen type I do not enhance the entry or progression of freshly isolated LPT into the cell cycle

The ability of collagen to induce cell cycle progression in activated PBT is somewhat paradoxical in light of previous reports suggesting that collagen is sequestered from the T cells within the secondary lymph nodes by reticular cells (21, 33). Therefore, consistent with our previous demonstration of distinctive adhesion properties of tissue T cells to the ECM (25), we hypothesized that PBT are first exposed to collagen after extravasating into their final tissue of residence. In support of this possibility, it was previously reported that mitogen-stimulated PBT lines derived in vitro, used as models of effector/memory T cells, are more potently simulated by collagen type I than fibronectin (7). We chose to examine the effects of native ECM, fibronectin, and collagen on freshly isolated LPT, a population of effector/memory cells (34) that maintain a CD4+ to CD8+ ratio similar to that of PBT (35), which reside in continuous contact within the interstitial matrix and express collagen-binding β1 integrins (25). LPT were stimulated with anti-
CD3-coated polystyrene beads in the absence and presence of native ECM, fibronectin, and collagen type I. Surprisingly, native ECM, fibronectin, or collagen type I had no effect on anti-CD3 activated LPT cell cycle progression (Fig. 6), even though a large percentage of LPT are positive for $\alpha_\beta_1$, $\alpha_\beta_2$, $\alpha_\beta_1$, and $\alpha_\beta_2$ integrins (see Fig. 8 below) (25). Similarly, native ECM, fibronectin, or collagen type I had no effect on LPT proliferation as measured by $[^{3}H]$thymidine incorporation (data not shown).

Native ECM and collagen type I differentially costimulate naive and effector/memory PBT

The insensitivity of LPT to ECM-induced T cell proliferation may be due to their residency in the interstitial matrix of the lamina propria or their effector/memory phenotype. To address this point, PBT were fractionated by negative selection into naive lamina propria or their effector/memory phenotype. To address this issue, LPT were stimulated with predetermined suboptimal concentrations of bead-bound anti-CD3 (25 $\mu$g/ml OKT3) in the absence or presence of native ECM, 20 $\mu$g/ml fibronectin, or 20 $\mu$g/ml collagen type I. After 4 days, cells were stained for flow cytometric analysis of DNA content. The percentage of cells containing subdiploid DNA content is indicated ($n = 3–4$).

Native ECM and specifically fibronectin inhibit AICD in tissue-derived memory T cells

We have previously reported that freshly isolated unstimulated LPT undergo a higher spontaneous rate of apoptosis in culture than do CD45RA$^+$ and CD45RO$^+$ PBT (38). In addition, the proportion of apoptotic T cells increases dramatically following anti-CD3 stimulation, whereas there was no significant increase in apoptosis in stimulated PBT (38). It is well established that most mesenchymal cells receive survival signals when adhered to an ECM and undergo apoptosis on the loss of integrin-mediated cell attachment (18). Because LPT are not costimulated to enter the cell cycle by native ECM or its components, we hypothesized that a major function of the interstitial matrix is to provide survival signals for those effector/memory T cells that are embedded within the ECM. Therefore, we assessed whether the presence of ECM during LPT activation would reduce apoptosis. Fresh PBT and LPT were stimulated by OKT3 Ab in the presence of native ECM, fibronectin, or collagen type I, and the degree of apoptosis quantified by PI staining, as previously described (39) (Fig. 9). One half of LPT underwent apoptosis following CD3 stimulation in the absence of ECM (BSA control). In contrast, activating LPT in the presence of native ECM or fibronectin afforded a significant protection against apoptosis (40%). No protection from AICD was provided by collagen. Not surprisingly, very few PBT underwent AICD (<7%), and this low level of apoptosis was unaltered in the presence of ECM. To confirm and extend these observations, we evaluated the protection afforded by fibronectin from AICD-induced apoptosis to LPT by measuring the expression of phosphatidylinerine (annexin V staining) on the outer leaflet of the dying T cell. Fibronectin, but not collagen, protected LPT from apoptosis (by 45–50%) even in the presence of neutralizing Abs against the $\beta_1$ and $\alpha_5$ through $\alpha_5$ integrin subunits. These findings demonstrate that upon activation, tissue T cells, which are in continuous contact with components of the $\beta_1$ integrin subunit with distinct levels of expression (Fig. 8). In contrast, naive CD45RA$^+$ PBT showed a bimodal expression of the $\beta_1$ chain, consisting of both a bright and dim population of cells (36), which occurs from an earlier report attributing the bimodal distribution to CD45RO$^+$ cells (37). However, the overall percentage of $\beta_1$ integrin expression and the mean fluorescence intensity is similar among all T cell populations examined. Expression of individual $\alpha$ integrin chains showed that a larger percentage of LPT express $\alpha_1$ and $\alpha_3$ on their surfaces, but were almost devoid of $\alpha_5$ surface expression when compared with that of both CD45RA$^+$ and CD45RO$^+$ PBT. There are also modest differences in $\alpha$ integrin expression between CD45RA$^+$ and CD45RO$^+$ PBT, in that a higher percentage of naive PBT express $\alpha_1$ and a higher percentage of effector/memory PBT express $\alpha_1$ when compared with one another. However, the overall level of expression among these T cell subsets was similar, as noted by minimal differences in the mean fluorescence intensity of the integrin-positive cells. In summary, the expression of the ECM-specific $\beta_1$ integrin family differs between naive and effector/memory circulating T cells and clearly differs between effector/memory circulating PBT and tissue LPT. However, differences in integrin surface expression do not account for the distinct costimulatory responses in these various T cell populations, as collagen costimulation promoted cell cycle progression of both the $\beta_1$ bright and dim population of PBT. These results indicate that not only the phenotype of the T cell (effector/memory vs naive), but also the origin of the cell, including its prior exposure to the surrounding interstitium, determine its integrin surface expression.

**Figure 9.** Native ECM and fibronectin, but not collagen, inhibit apoptosis in TCR-activated tissue-derived T cells. Freshly isolated PBT were stimulated with predetermined suboptimal concentrations of bead-bound anti-CD3 (25 $\mu$g/ml OKT3) in the absence or presence of native ECM, 20 $\mu$g/ml fibronectin, or 20 $\mu$g/ml collagen type I. After 4 days, cells were stained for flow cytometric analysis of DNA content. The percentage of cells containing subdiploid DNA content is indicated ($n = 3–4$).

Distinctive $\beta_1$ integrin expression profiles among naive PBT, effector/memory PBT, and LPT

Our findings that native ECM and collagen type I promote cell cycle progression of naive, but not effector/memory PBT or LPT, suggest that the surface expression of $\beta_1$ integrins on these T cell populations may differ. These differences may account for their divergent response to the ECM. Therefore, we compared the expression of the integrin subunits $\alpha_1$-$\alpha_5$ and $\beta_1$ on freshly isolated CD45RA$^+$ PBT, CD45RO$^+$ PBT, and LPT, which are almost exclusively CD45RO$^+$ (34). Both effector/memory PBT and LPT were composed of a homogeneous population of cells expressing the $\beta_1$ integrin subunit with distinct levels of expression (Fig. 8). In contrast, naive CD45RA$^+$ PBT showed a bimodal expression of the $\beta_1$ chain, consisting of both a bright and dim population of cells (36), which occurs from an earlier report attributing the bimodal distribution to CD45RO$^+$ cells (37). However, the overall percentage of $\beta_1$ integrin expression and the mean fluorescence intensity is similar among all T cell populations examined. Expression of individual $\alpha$ integrin chains showed that a larger percentage of LPT express $\alpha_1$ and $\alpha_3$ on their surfaces, but were almost devoid of $\alpha_5$ surface expression when compared with that of both CD45RA$^+$ and CD45RO$^+$ PBT. There are also modest differences in $\alpha$ integrin expression between CD45RA$^+$ and CD45RO$^+$ PBT, in that a higher percentage of naive PBT express $\alpha_1$ and a higher percentage of effector/memory PBT express $\alpha_1$ when compared with one another. However, the overall level of expression among these T cell subsets was similar, as noted by minimal differences in the mean fluorescence intensity of the integrin-positive cells. In summary, the expression of the ECM-specific $\beta_1$ integrin family differs between naive and effector/memory circulating T cells and clearly differs between effector/memory circulating PBT and tissue LPT. However, differences in integrin surface expression do not account for the distinct costimulatory responses in these various T cell populations, as collagen costimulation promoted cell cycle progression of both the $\beta_1$ bright and dim population of PBT. These results indicate that not only the phenotype of the T cell (effector/memory vs naive), but also the origin of the cell, including its prior exposure to the surrounding interstitium, determine its integrin surface expression.

Distinctive $\beta_1$ integrin expression profiles among naive PBT, effector/memory PBT, and LPT

Our findings that native ECM and collagen type I promote cell cycle progression of naive, but not effector/memory PBT or LPT, suggest that the surface expression of $\beta_1$ integrins on these T cell populations may differ. These differences may account for their divergent response to the ECM. Therefore, we compared the expression of the integrin subunits $\alpha_1$-$\alpha_5$ and $\beta_1$ on freshly isolated CD45RA$^+$ PBT, CD45RO$^+$ PBT, and LPT, which are almost exclusively CD45RO$^+$ (34). Both effector/memory PBT and LPT were composed of a homogeneous population of cells expressing the $\beta_1$ integrin subunit with distinct levels of expression (Fig. 8). In contrast, naive CD45RA$^+$ PBT showed a bimodal expression of the $\beta_1$ chain, consisting of both a bright and dim population of cells (36), which occurs from an earlier report attributing the bimodal distribution to CD45RO$^+$ cells (37). However, the overall percentage of $\beta_1$ integrin expression and the mean fluorescence intensity is similar among all T cell populations examined. Expression of individual $\alpha$ integrin chains showed that a larger percentage of LPT express $\alpha_1$ and $\alpha_3$ on their surfaces, but were almost devoid of $\alpha_5$ surface expression when compared with that of both CD45RA$^+$ and CD45RO$^+$ PBT. There are also modest differences in $\alpha$ integrin expression between CD45RA$^+$ and CD45RO$^+$ PBT, in that a higher percentage of naive PBT express $\alpha_1$ and a higher percentage of effector/memory PBT express $\alpha_1$ when compared with one another. However, the overall level of expression among these T cell subsets was similar, as noted by minimal differences in the mean fluorescence intensity of the integrin-positive cells. In summary, the expression of the ECM-specific $\beta_1$ integrin family differs between naive and effector/memory circulating T cells and clearly differs between effector/memory circulating PBT and tissue LPT. However, differences in integrin surface expression do not account for the distinct costimulatory responses in these various T cell populations, as collagen costimulation promoted cell cycle progression of both the $\beta_1$ bright and dim population of PBT. These results indicate that not only the phenotype of the T cell (effector/memory vs naive), but also the origin of the cell, including its prior exposure to the surrounding interstitium, determine its integrin surface expression.
the ECM, depend on their interstitial microenvironment for survival, possibly to preserve their locally needed immune effector function.

Discussion

Immune surveillance by naïve T lymphocytes in search of Ag is achieved by their continuous recirculation from blood to secondary lymph tissue through the lymphatics to return to the blood stream. After their first exposure to cognate antigenic peptide, T cells acquire an effector/memory phenotype and a migration pattern that directs them to inflamed extravascular tissue (40, 41). Among a plethora of changes associated with Ag exposure, adaptation to the ECM microenvironment is among the most dramatic (25). In light of the well-documented costimulatory function played by integrins of the β1 family (LFA-1, Mac-1, etc) during TCR engagement (42), in this report we validate two fundamental hypotheses: 1) naïve T cell activation is modulated by its initial exposure to the interstitial ECM acting through the β1 family of integrins, and 2) the response to TCR ligation by tissue T cells, already adapted to the ECM, is fundamentally distinct from that of naïve T cells. We demonstrate that a three-dimensional, cell-derived ECM stimulates progression through the cell cycle of naïve T cells and inhibits apoptosis of tissue T cells. In addition, collagen and fibronectin, two major constituents of the ECM, play unique, discriminating roles in mediating these responses. Thus, initial and continued interaction with the ECM provides an additional regulatory checkpoint, which modulates the delicate balance between life and death in the activated T cell.

Clonal expansion is an essential component of a T cell response. Previous reports have investigated the role of both collagen and fibronectin engagement of surface integrins as potential accessory signals during T cell activation (4–6, 22). These studies were limited by the lack of availability of critical cell cycle reagents, and [3H]thymidine incorporation, which is believed to evaluate cell proliferation, was used as the sole indicator of cell growth. Because entry into and completion of the cell cycle is tightly regulated (43–45), we focused on individual checkpoints that control cell cycle progression (46). Resting T cells reside in the G0/G1 phase and must enter and complete the S and G2/M phases. Entry and progression through the S phase is controlled by Rb phosphorylation and cyclin A (47), while cyclin B1 regulates the late G2 phase up to mitosis, ensuring the initiation of cell division (30). Completion of cell division is marked by the phosphorylation of histone H3 (31, 48). In this report, we demonstrate that native ECM or collagen costimulation of PBT increases Rb phosphorylation and therefore stimulates cell cycle progression beyond the restriction point (47). Together, our results establish that engagement of the TCR in the presence of ECM enhances clonal expansion of an Ag-inexperienced PBT.

Previous reports by different investigators disagree on the individual abilities of collagen or fibronectin to modulate PBT proliferation (4–6, 22). Two investigations demonstrated that collagen displayed no costimulatory activity, while fibronectin did (5–7), which contrasts with our and other studies. In the majority of these reports, both anti-CD3 and the matrix protein were coinoculated onto the surface of a plastic culture dish (4–6, 22). It is likely that adhesion to fibronectin itself via α5β1, not costimulation by fibronectin, mediated the enhanced T cell proliferation, as suggested by the authors (5–7). In other reports, the investigators were unable to detect T cell proliferation with anti-CD3 alone (4, 5), an unlikely scenario. To address this controversy, we chose an in vitro model that separates events initiated by adhesion from those attributable to costimulation. Under physiological conditions, opposing surfaces of the migrating T cell encounter ECM and APC, therefore, we respected the geometry of the adherent PBT by engaging their TCR with beads coated with anti-CD3 Ab. At every concentration of beads tested, both native ECM and collagen type I exhibited a concentration-dependent costimulation of PBT cell cycle progression. Reviewing earlier reports revealed that when those investigators used anti-CD3-coated beads, instead of coinoculation of Ab and matrix, the T cell proliferative response to fibronectin was lost (5, 6), supporting our findings. Furthermore, other investigators, coinoculating anti-CD3 and matrix, did observe that collagen costimulated T cell proliferation (4, 6, 22), similar to our results. In light of our recent report that α5β1 is solely responsible for adhesion of PBT to fibronectin (25), an alternate interpretation of the earlier data, suggested and supported by Geginat et al. (20), is that increased adhesion to the plate via fibronectin expands the surface area of the T cell in contact with anti-CD3 and thus enhances the effective concentration of CD3 cross-linking in the culture.

Integrin-dependent cell cycle progression in response to growth factor stimulation is observed in anchor-dependent-cell types (49), and in anti-CD3 activated PBT that require α5β1-driven homotypic cell clustering. When T cells are activated, β2 integrin engagement is a requisite for the induction of G1 cyclins D2 and D3, activation of Cdk6, and phosphorylation of Rb (20). In agreement, we find that higher PBT cell density requires less activation to enter the cell cycle due to increased engagement of α5β1. However, costimulation by β1 integrins (i.e., collagen type I engagement) is not observed at high cell density, due to the increased cell-cell adhesion mediated by β2 integrins. In this light, earlier reports attributing a costimulatory activity to fibronectin after coinoculation with anti-CD3 may be reinterpreted as showing that fibronectin enhanced the capacity for β2-mediated cell-cell contact by increasing the local T cell density at the site of adhesion.

Loss of PBT cell cycle progression due to the inhibition of β2 engagement is partially reversed by increased β1 engagement by collagen or native ECM. This suggests three possible mechanisms by which collagen costimulates cell cycle progression: 1) engagement of β1 integrins provides the necessary costimulatory signals needed; 2) β2 integrin ligation increases the affinity/avidity of the β2 integrin family, and the resulting increase in homotypic clustering drives costimulation; 3) a combination of β1 integrin engagement by collagen and the increase in β2 integrin engagement by its ligand, ICAM, together deliver the necessary signals for cell cycle progression. These results illustrate three major effects of integrin engagement: 1) activation through the TCR/CD3 complex converts integrins from the inactive to the active conformation (25); 2) integrin cross-talk modulates T cell responses and they compensate for one another (50); and 3) collagen engagement of β1 integrins or cross-linking β2 integrins by cell-cell contact is essential for progression through the G1 phase of the cell cycle beyond the restriction point (20).

Only the naïve population of PBT responds to native ECM and collagen costimulation, yet these cells bear a similar integrin surface expression profile as nonresponding effector/memory PBT. Therefore, integrin expression alone cannot modulate T cell entry into the cell cycle. Instead, the combination of T cell phenotype coupled to ECM exposure during T cell activation determines a functional response. We previously reported that freshly isolated PBT, preconditioned with collagen, increase their surface expression of α1 and α1 (25) and acquire the ability to adhere strongly to collagen, which then costimulates cell cycle progression. In fact, blood T cells may never completely interact with all of the components of the interstitial ECM when migrating through a lymph node (LN) (33, 51), as it was proposed that collagen, in particular,
is sequestered from the blood T cell by a complex reticular network of dendritic cells that shield LN T cells from this protein (33, 51). This proposed structure of the LN suggests that in vivo the first contact of a CD45RA⁺ T cell with collagen may be directly within the inflamed tissue. It was reported that naive T cells reside exclusively in secondary lymphoid tissues, such as the spleen and lymph nodes, in mice that were not exposed to Ag (40). Upon Ag exposure, freshly activated naive T cells proliferate in the LN and then migrate to the lungs, liver, gut, and salivary glands (40), where they will be exposed to collagen embedded within the interstitial matrix. Based on our results, we hypothesize that the entry of these CD45RA⁺ T cells into only those tissues in which the cognate Ag is present will stimulate an additional round of proliferation during which costimulation by interstitial collagen further enhances cell cycle progression.

In contrast, LPT are completely embedded in the ECM (52), causing the cell to polarize and forcing the T cell to encounter its cognate peptide on one pole of the cell (53). Once T cells are removed from the interstitial matrix, they rapidly undergo AICD (38), which is reduced by the presence of native ECM or cognate peptide on one pole of the cell (53). Once T cells are activated during which costimulation by interstitial collagen further increases cell cycle progression.

We thank Gail West, R. Michael Sramkoski, and Megan Gottlieb for technical assistance. We also thank the Departments of Surgery and Pathology, University Hospitals of Cleveland, the Colorectal Surgery Department of the Cleveland Clinic Foundation, and the Departments of Surgery and Pathology, Charite, Campus Virchow Clinic (Berlin, Germany). The Cooperative Human Tissue Procurement Facility of the University Hospitals of Cleveland provided some tissue samples.

References


42. Rossetti, G., M. Collinge, J. R. Bender, R. Molteni, and R. Pardi. 2002. Integrin-