Human Natural Killer Cell Expansion Is Regulated by Thrombospondin-Mediated Activation of Transforming Growth Factor-β1 and Independent Accessory Cell-Derived Contact and Soluble Factors

By Bryce A. Pierson, Kalpna Gupta, Wei-Shou Hu, and Jeffrey S. Miller

Natural killer cells (NK) were studied to determine factors important in their expansion. Fluorescence-activated cell sorter (FACS) purified CD56+/CD3− NK cells cultured alone for 18 days in IL-2 containing medium (1,000 U/mL) showed enhanced cytotoxicity but only minimal expansion. NK expansion was increased (12.5 ± 1.6-fold) by coculturing NK with soluble factors produced by irradiated peripheral blood mononuclear cells (PBMC) in which the two populations were separated by a microporous membrane. However, for 18 days in IL-2 containing medium stromal cells to separate the contact-induced proliferation. Experiments were designed using fixed M2-10B4 on signals provided by the bone marrow (BM) microenvironment inhibited a similar contact-dependent increase in NK expansion with marrow stroma was similar to PBMC. Fibroblast cell lines (M2-10B4, NRK-49F, NIH-3T3) and human umbilical vein endothelial cells (HUVEC), all homogeneous populations and devoid of monocytes, also exhibited a similar contact-dependent increase in NK expansion. Experiments were designed using fixed M2-10B4 stromal cells to separate the contact-induced proliferative stimuli from soluble factors. NK plated directly on ethanol/acetic acid-fixed M2-10B4, which leaves stromal ligands (cell membrane components and ECM) intact, resulted in increased NK expansion compared with medium alone. We further show that the combination of independent contact and soluble factors is responsible for maximal late NK expansion (days 28 through 40) but paradoxically inhibits early NK expansion (day 7). The proliferation inhibitory effects were verified by 3H-thymidine uptake and could be detected at days 2 through 6 but no longer 14 days after the initiation of the culture. We show that both laminin and thrombospondin inhibit early NK proliferation, whereas only thrombospondin was capable of also stimulating late NK expansion. The effect of thrombospondin on early NK proliferation is related to activation of transforming growth factor-β1 (TGF-β) because anti-TGF-β neutralizing antibody completely abrogated thrombospondin-mediated inhibition of early NK proliferation. Although inhibitory early in culture, active TGF-β added only at culture initiation increases late NK expansion similar to thrombospondin. TGF-β was not present in the thrombospondin preparation but latent TGF-β in serum, or TGF-β transcripts identified in IL-2-activated NK could explain paracrine or autocrine mechanisms for the regulation of NK proliferation. Finally, anti-TGF-β neutralizing antibody only minimally affects stroma-mediated inhibition of early NK proliferation suggesting that aside from thrombospondin/TGF-β, additional contact factors are important for the regulation of NK proliferation.

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WE HAVE previously shown that monocyte-produced soluble factors and direct cell-cell contact are required for natural killer cell (NK) expansion suggesting that both direct contact with accessory cells and soluble factors other than IL-2 are important NK stimuli.1-3 Lymphoid progenitors require direct contact between cells and stroma for survival and proliferation. B-cell differentiation is dependent on signals provided by the bone marrow (BM) microenvironment4-6 and in the absence of marrow stroma, rapid apoptosis of immature B-cell progenitors is observed.7 T-cell maturation is dependent on contact between T-cell precursors and thymic epithelium, which is required for proliferation and differentiation.8 We and others have shown that differentiation of NK from primitive marrow progenitors requires direct contact with stroma and that interrupting contact prohibits differentiation.9-11 Although more committed CD34+/CD7+ progenitors do not require direct contact with stroma, expansion of their NK progeny is greatly enhanced when contact was permitted suggesting that similar contact-mediated stimuli may be involved in blood NK activation and expansion.

Several extracellular matrix (ECM) components may be responsible for the observed increase in NK expansion. Fibronectin and laminin are ubiquitously expressed components of ECM that have importance in lymphocyte interactions and may stimulate NK. Thrombospondin is contained within α-granules of platelets and is also expressed by many cell types including monocytes,12 fibroblasts13 and endothelial cells.14 We show that all of these cell types stimulate expansion of NK. Additionally, thrombospondin is localized surrounding tumors and near sites of inflammation and has been implicated in cell migration and tumor metastases.15 Because NK are implicated in tumor surveillance, it is possible that thrombospondin could influence NK activity and proliferation.

Experiments were designed to determine the mechanisms of contact and soluble stimuli on NK expansion. Soluble factors, derived from conditioned medium, were separated from the contact stimuli by metabolically inactivating (with...
EtOH/acetic acid) the accessory cell feeder (M2-10B4), which preserves ligands important in cell communication.16 Interestingly, the combination of accessory cell derived contact and soluble factors that stimulates maximal NK expansion late in culture, paradoxically inhibits NK proliferation early in culture. Purified thrombospondin reproduced the paradoxical early inhibition and late stimulation of NK proliferation observed with EtOH/acetic acid-fixed M2-10B4 accessory cells. Moreover, the thrombospondin effects were indirect and mediated by thrombospondin activation of latent transforming growth factor-β1 (TGF-β1). However, other mechanisms besides those explained by thrombospondin and TGF-β are operant in NK interactions with stromal ligands showing the complex interactions of NK with local microenvironments.

**MATERIALS AND METHODS**

**Patient population.** Forty healthy adult volunteers ages 21 to 45 years were studied. Peripheral blood (PB) was collected in syringes containing heparin. The guidelines approved by the Committee on the Use of Human Subjects in Research at the University of Minnesota were followed in obtaining all samples.

**Purification of PB NK cells.** PBMC obtained by Ficoll-Hypaque (specific gravity 1.077) (Sigma, St Louis, MO) density centrifugation (30 minutes at 37°C and 400 g) were depleted of T cells by anti-CD3 and anti-CD5 mouse monoclonal antibodies (MoAbs) (Becton Dickinson, Mountain View, CA) and goat antimouse immunomagnetic beads (15 beads/cell) (PerSeptive Diagnostic, Cambridge, MA). The CD3+CD5-depleted PBMC were labeled with fluorescein isothiocyanate-conjugated anti-CD3 MoAb and phycoerythrin-conjugated anti-CD56 MoAb (250 ng/10^6 cells) (Becton Dickinson) and sorted on a FACS Star-Plus flow cytometer (Becton Dickinson) equipped with a Consort 32 computer (Hewlett Packard 340, Palo Alto, CA) into a CD56^+CD3^- NK population as previously described.13 The CD56^bright and CD56^dim NK subpopulations were sorted using established criteria for CD56^bright cells.17

**NK cultures.** NK were cultured in RPMI 1640-based NK culture medium (see Figs 1 and 2) or an improved supplemented 2:1 (vol/vol) Dulbecco’s Modified Eagle’s Medium (DMEM)/Ham’s F12-based NK medium (see Figs 3 through 9, Table 1), which significantly increases NK expansion.16 To account for differences in baseline NK expansion between the two media, all experiments were internally controlled. The RPMI 1640-based NK medium (GIBCO Laboratories, Grand Island, NY) was supplemented with 2 mmol/L L-glutamine (GIBCO) and the DMEM/F12 NK medium (GIBCO) was supplemented with 24 mmol/L 2-mercaptoethanol, 50 μmol/L ethanolamine, 20 mg/L L-ascorbic acid, and 5 μg/L sodium selenite. Both the RPMI 1640-based and the DMEM/F12-based NK medium were supplemented with 1,000 U/mL recombinant interleukin-2 (IL-2) (a generous gift from Amgen, Thousand Oaks, CA), 10% heat-inactivated human AB serum (North American Biologics, Miami, FL) and 100 U/mL penicillin with 100 U/mL streptomycin (GIBCO).

Sorted CD56^+CD3^- NK (10^6) were plated in 1 mL NK medium in 24-well tissue culture plates in untreated wells, wells containing viable accessory cells either in direct contact with the accessory cells (soluble + contact) or physically separated from accessory cells using a Transwell insert (Costar, Cambridge, MA) with a 0.4-μm microporous collagen type IV-coated membrane (soluble alone), or in tissue culture wells containing EtOH/acetic acid-fixed M2-10B4, fibronectin, laminin, or thrombospondin. At day 7, the medium volume in wells containing NK plated in direct contact with accessory cells, fixed stroma, or ECM components was doubled to 2.0 mL with fresh NK medium whereas wells containing NK plated physically separated from accessory cells (Transwell) had 700 μL from the bottom compartment discarded and replaced with fresh medium. On days 10 and 14, half-medium changes were performed on all cultures. For the cultures lasting 28 to 40 days, half-medium changes were performed every 4 days beginning on day 18. In some experiments, NK were plated in M2-10B4 conditioned medium that was transferred daily from irradiated M2-10B4 feeder layers. NK-containing wells were split 2:1 as needed to maintain cell concentrations below 2 x 3 x 10^6 cells/mL. At indicated time points, cultures were terminated and cells were enumerated with a hemocytometer and analyzed for cytotoxicity and phenotype as described.1,15 NK fold expansion was determined as (total cultured cells at day 18)/(initial NK plated at day 0). All cultures contained greater than 98% CD56^+ CD3^- NK at the termination of culture.

**Preparation of accessory cells and fixed stroma.** Unseparated PBMC were obtained as described above and irradiated (2,000 rad) before use as accessory cells. Irradiated allogeneic BM stroma monolayers were prepared as previously described.9 The murine stromal cell lines M2-10B4 (a kind gift from Dr C. Eaves, Vancouver, Canada), was subcloned in 24-well tissue culture plates and irradiated with 6,000 rad once confluent. In some experiments, accessory cell-derived soluble factors were obtained by cell-free transfer of M2-10B4 conditioned medium.

M2-10B4 monolayers, preirradiated with 1,000 U/mL IL-2 for 30 to 40 hours, were fixed in a 3:1 vol/vol mixture of ethanol (EtOH)/acetic acid for 15 minutes followed by a 5-minute EtOH rinse, washed four times with phosphate-buffered saline solution, and incubated with Bovine Modified Dulbecco’s Medium and 20% fetal calf serum (HyClone Laboratory Inc, Logan, UT) overnight at 37°C before use.

**3H-Thymidine uptake assay.** Sorted CD56^+CD3^- NK (6 x 10^3 or 1 x 10^4) were cultured in 96-well plates and pulsed at the indicated time points with 16.3 μCi/well 3H-thymidine (DuPont, Wilmington, DE). Wells were then harvested using a Skatron Instruments Cell Harvester (Skatron Instrument Inc, Sterling, VA) onto 96-partition filter papers. The radioactive counts per minute (cpm) were then measured by a 1205 Betaplate Liquid Scintillation Counter (Pharmacia LKB Nuclear, Gaithersburg, MD).

**Purified extracellular matrix components and TGF-β.** Fibronectin (a kind gift from Dr J. McCarthy, University of Minnesota) was purified from human plasma as a byproduct of factor VIII production using sequential ion exchange and gelatin chromatography as previously described.22 Laminin (a kind gift from Dr A. Skubitz, University of Minnesota) was purified from the Engelbreth-Holm-Swarm (EHS) tumor by gel filtration chromatography as previously described.23 Thrombospondin (a kind gift from Dr R. Hebbel, University of Minnesota) was purified from the released products of thrombin-stimulated human platelets by a heparin-agarose column affinity chromatography procedure.23 Fibronectin and laminin were coated on tissue culture plates by overnight incubation in 500 μL of Voller’s carbonate buffer (pH 9.6). Fibronectin was added at 0.1, 0.3, 0.9, and 2.7 μg/well (0.4, 1.2, 3.6, and 10.8 pmol/well) in 96-well plates and at 40 μg/well (160 pmol/well) in 24-well plates. Laminin was added at 0.1, 0.3, 0.9, and 2.7 μg/well (0.25, 0.75, 2.25, and 6.75 pmol/well) in 96-well plates and at 40 μg/well (100 pmol/well) in 24-well plates. Thrombospondin was coated on tissue culture plates by 3-hour incubation in 250 μL of Voller’s buffer containing 0.3 mmol/L Ca^{2+} (pH 9.6). Thrombospondin was added at 0.05, 0.15, 0.45, or 1.35 μg/well (0.11, 0.33, 1.0, and 3.0 pmol/well) in 96-well plates and at 2 μg/well (4.4 pmol/well) in 24-well plates. All culture wells were washed three times with PBS before use in cell culture. Recombinant human TGF-β1 (R&D Systems, Minneapolis, MN), anti-TGF-β neutralizing antibody (R&D Systems) and normal
Total RNA was extracted from each population according to the method with 1,000 U/mL IL-2 for 48 hours before KT-PCR transcript analysis. Neutralizing antibody concentration curves showed saturation at 25 to 50 μg/mL anti-TGF-β antibody (data not shown).

Antibody blocking. Antibodies against the β1, β2, and β3 integrins were added at the initiation of culture and again on days 4 and 7 to cultures where NK were cocultured directly on viable M2-10B4 accessory cells to assess effects on late NK expansion. NK proliferation was measured by direct cell counts after 18 days of culture. Experiments to assess the effect on early NK proliferation were performed by coculture of NK on EtOH/acetic acid-ixed M2-10B4 and MoAbs were added only at the initiation of culture. Early NK proliferation was measured after 4 days of culture using the 4-day ^3H-thymidine uptake assay. The specific MoAbs against the β1 (P4C10 clone asces; GIBCO), β2 (P4H9 clone asces; GIBCO), and β3 (S2.21 clone purified; AMAC, Inc, Westbrook, ME) integrins were used at dilutions of 1:1,000, 1:1,000, and 1:100, respectively. Purified-mouse IgG (Sigma), IgG, clarified ascites (Sigma, MOPEC-21), and IgG, clarified ascites (Sigma, FLOPEC-21) were used at dilutions of 1:1,000, 1:500, and 1:500, respectively (all at 10 μg/mL final concentration) as nonspecific controls.

Reverse transcriptase-polymerase chain reaction (RT-PCR) for TGF-β mRNA. Thirty-five thousand sorted cells were incubated with 1,000 U/mL IL-2 for 48 hours before RT-PCR transcript analysis. Total RNA was extracted from each population according to the method of Chomczynski and Sacchi. cDNA was generated using 200 U of murine Moloney leukemia virus (M-MLV) RT mixed with the following reagents: 0.1 mol/L diethanol, 14 U RNasin (GIBCO), 10 pmol antisense primer, 10 pmol dNTPs (Phar- macia, Piscataway, NJ), and total RNA from 3.5 × 10^6 cells resuspended in 50 μL reverse transcriptase buffer. After a 1-hour incubation at 42°C, PCR amplification of the human TGF-β and β-actin was performed using 2.5 U of Taq DNA polymerase (Promega, Madison, WI), 50 pmol sense and antisense primers (5'-3' TGF-β 20-mers: GACTATCCACCTGCAAGACT, GCTGTGTGACCTTGCTGTTGA; β-actin 19-mers: TACCTCATTGAGATCTTCCA, TCCGTGGATGCCCACGAGC), 30 pmol dNTPs (Pharmacia) and PCR buffer (50 mmol/L Tris, 50 mmol/L KCl, 1.5 mmol/L MgCl2; pH 8.4). PCR cycles consisted of: 95°C for 20 seconds, 55°C for 15 seconds, and 72°C for 1 minute for 40 cycles with a 72°C extension for 10 minutes (Perkin Elmer Cetus DNA Thermal Cycler). Twenty-five percent of the PCR products were electrophoresed on a 4.5% agarose gel and transferred to a Hybrid-N nylon membrane (Amersham, Arlington Heights, IL) for Southern analysis. Synthesized probes (5'-3' TGF-β 25-mer: GCTCCGCTTTCGACTGCTC; β-actin 19-mer: CACTCCTTGTCCGCGACT) were labeled by a 3' end-labeling technique with β-3P-dATP (Amersham). Southern transfers were probed at 42°C for 1 hour in Rapid hybridization buffer (Amersham) and then washed at 42°C with 5×, 1×, and finally 0.1× SSPE buffer (Sigma) plus 15% sodium dodecylsulfate. X-OPT-AR films (Eastman Kodak Co, Rochester, NY) were exposed for varying lengths of time. RNase- and DNase-free DEPC-treated water was used as a negative control for the RT-PCR reaction.

Statistics. Results of experimental points obtained from multiple experiments are reported as mean ± 1 SEM. Significance levels were determined by two-sided Student's t-test analysis.

RESULTS

Irradiated PBMC were used as a source accessory cells for NK expansion. Culture of FACSPurified CD56^-CD3^- NK in medium containing 1,000 U/mL rIL2 resulted in minimal activated natural killer cell (ANK) expansion after 18 days (4.0 ± 0.8-fold) and irradiated PBMC cultured alone did not proliferate over the same time period (n = 10, data not shown). Coculture of NK in direct contact with autologous irradiated PBMC, providing both contact and soluble factors, resulted in an accessory cell dose-dependent NK expansion (Fig 1). The decrease in NK expansion at high PBMC numbers (2 × 10^6) may be explained by the observation that these cultures became acidic compared to other PBMC doses suggesting that proliferation inhibitory

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![Figure 1](image-url)  
**Fig 1.** Sorted CD56^-CD3^- NK (5 × 10^5) were cocultured in direct contact with zero to 2 × 10^6 irradiated autologous PBMC accessory cells. Irradiated PBMC did not expand when cultured alone (data not shown). NK expansion was PBMC dose-dependent and significantly greater expansion was observed compared to NK cultured in the absence of PBMC (*P < .008; #P = .026).

![Figure 2](image-url)  
**Fig 2.** Maximal NK expansion requires direct contact with accessory cells. Sorted CD56^-CD3^- NK (10^5) were plated in direct contact with (W) or physically separated from (■) 5 × 10^5 autologous PBMC (n = 20), allelogenic human marrow stroma (n = 9) or with monolayers of the murine stromal cell line, M2-10B4 (n = 16). NK expansion with accessory cells was significantly greater than the media alone control (*P < .001) in all cases. NK cultured in direct contact with accessory cells expanded significantly greater than NK cultured physically separated from accessory cells in all cases (*P < .005, #P = .036).
factors or medium nutrients may become limiting at high cell concentrations.

Direct coculture of PBMNC with NK at a ratio of 5:1 resulted in NK expansion of 49.4 ± 5.9-fold. However, the effect of soluble factors alone measured when the same number of NK were cocultured physically separated from PBMNC by a microporous membrane was significantly less than when direct contact was permitted (12.5 ± 1.6-fold, n = 20; P < .001) suggesting that direct contact with accessory cell ligands may be an independent stimulus for NK expansion. We then examined if other accessory cells are capable of inducing NK expansion. Irradiated allogeneic human narrow stroma, containing fibroblasts, endothelial cells, macrophages, and adipocytes, and known to induce growth of primitive NK progenitors, was evaluated as an alternative feeder layer. NK cocultured in direct contact with marrow stroma resulted in significantly greater expansion than NK physically separated from the stromal layer, neither of which was significantly different from autologous or allogeneic irradiated PBMNC (Fig 2). Because PBMNC and marrow stroma contain multiple cell types, NK were cocultured with three fibroblast cell lines [M2-10B4 (murine), NIH-3T3 (murine), and NRK-49F (rat)] and human umbilical vein endothelial cells (HUVEC), all devoid of monocytes, to test their ability to support NK expansion. NK expansion was significantly greater when NK were cocultured in direct contact with any of the accessory cells compared to coculture of NK physically separated from the feeders. NK expansion in direct contact with all cell lines was similar to that observed with primary marrow stroma and irradiated PBMNC. Only the representative M2-10B4 fibroblast cell line is shown (Fig 2) and was chosen for further study to dissect mechanisms of NK expansion.

To determine if contact with specific cell membrane or ECM ligands could regulate NK expansion in the absence of accessory cell-produced soluble factors, we metabolically inactivated M2-10B4 fibroblasts prior to coculture with NK. M2-10B4 stroma was fixed with ethanol (EtOH)/acetic acid which left both stromal cellular and ECM components. PB NK were then cocultured with fixed M2-10B4 in fresh IL-2 containing nonconditioned medium. Coculture of NK with EtOH/acetic acid-fixed M2-10B4 significantly increased NK expansion (328% ± 41% of control, n = 20, P < .001) compared with medium alone showing an independent contact effect in the absence of soluble factors.

The effect of soluble factors on NK cultured for 21 days in a Transwell above viable M2-10B4 was similar to NK cultured in stroma-free wells fed by daily transfer of M2-10B4 conditioned medium (138.2 ± 24.2 v 130.3 ± 27.1-fold expansion; n = 6, P = NS). Therefore, the increased NK expansion observed in Transwell cultures was not dependent on the 1 mm distance between the NK and the underlying feeder layer and M2-10B4 produced soluble factors could be effectively transferred to NK present in a different culture vessel. To assess the independent and combined roles of direct contact and soluble factor-mediated effects on NK expansion, we plated sorted CD56+/CD3- NK in fresh nonconditioned medium alone (media), EtOH/acetic acid-fixed M2-10B4 with fresh medium (contact), M2-10B4 conditioned medium alone (soluble), or on fixed M2-10B4 with M2-10B4 conditioned medium (contact + soluble). We show that contact with stromal ligands and stromal conditioned medium synergistically induces late expansion (days 28 through 40) of mature blood NK, whereas contact-mediated signals alone (no stromal conditioned medium) or soluble factors alone are each capable of inducing suboptimal NK expansion (Fig 3). All cultured ANK populations contained greater than 98% CD56+/CD3- cells with less than 2% CD3+ T-cell contamination. Cultured ANK cytotoxicity against K562 and Raji targets was similar regardless of the NK culture conditions: medium alone, contact ligands alone, soluble factors alone, or contact + soluble factors (data not shown).

Although the combination of contact and soluble factors resulted in maximal NK expansion after 40 days of culture compared to medium alone (540 ± 127-fold v 8.1- ± 1.7-fold, n = 6, P < .001), paradoxically, the same conditions appeared to inhibit NK expansion after 7 days of culture compared to medium alone (1.6 ± 0.4-fold v 2.7- ± 0.5-fold, n = 6, P = .016). Because the low cell counts detected at day 7 may not be a sensitive measure of early NK proliferation, a 3H-thymidine uptake assay was used to assess NK proliferation during the first week of culture with M2-10B4 derived contact and soluble factors. CD56+/CD3- NK (10⁶) were plated in 96-well plates in medium alone, on EtOH/acetic acid-fixed M2-10B4 monolayers alone (contact), in M2-10B4 conditioned medium alone (soluble), or on EtOH/acetic acid-fixed M2-10B4 monolayers in M2-10B4 conditioned medium alone (contact + soluble) or on EtOH/acetic acid-fixed M2-10B4 with medium alone (soluble factors) and wells were obtained at different times in culture to determine NK fold expansion (n = 6). Contact and soluble factors alone significantly increased late NK expansion compared with media alone and the combination was synergistic late in culture.

Fig 3. Contact and soluble factors synergistically augment late NK expansion. Sorted CD56+/CD3- NK (10⁶) were plated in NK medium alone (6), on EtOH/acetic acid-fixed M2-10B4 monolayers (contact factor), in M2-10B4 conditioned medium (soluble factors) or on EtOH/acetic acid-fixed M2-10B4 with daily half medium changes of M2-10B4 conditioned medium (contact + soluble factors) or wells and were obtained at different times in culture to determine NK fold expansion (n = 6). Contact and soluble factors alone significantly increased late NK expansion compared with media alone and the combination was synergistic late in culture.
tioned medium (contact + soluble). NK cultured with M2-10B4 derived contact or soluble factors alone showed a similar 50% inhibition of \(^3\)H-thymidine incorporation compared to the fresh medium control and the combination of contact and soluble factors was more inhibitory than either factor alone (Fig 4). These inhibitory effects could be detected as early as day 2 of culture and persisted at days 4 and 6. However, by day 9 of culture, the \(^3\)H-thymidine uptake by NK cultured with either M2-10B4 contact or soluble factors alone was similar to the medium control, yet the combination of factors remained inhibitory. Later in culture (day 14) the inhibition induced by either soluble or contact factors or the combination could no longer be detected. These experiments show a paradoxical early inhibition (days 2 through 6) and late stimulation (beginning by day 14) of NK proliferation by stroma-derived factors.

The specific stromal ligands involved in the contact-mediated regulation of NK proliferation was further examined. Antibodies against the \(\beta_1\), \(\beta_2\), and \(\beta_3\) integrins were tested for their ability to block early inhibition and late stimulation of NK by stromal ligands. MoAbs were added at the initiation of culture and again on days 4 and 7 to NK cultured directly on viable M2-10B4. After 18 days of culture, no difference in late NK expansion was observed between cultures containing either test antibodies, control antibodies, or cultures in medium alone (data not shown). Likewise, no difference in early NK proliferation (day 4) was observed between cultures with or without blocking antibodies utilizing EtOH/acetic acid-fixed M2-10B4 feeder layers (data not shown). This suggests that the \(\beta_1\), \(\beta_2\), and \(\beta_3\) integrins do not play an important role in the regulation of early or late contact-mediated NK proliferation.

We then examined the effect of purified ECM components on early NK proliferation and late NK expansion. The 4-day \(^3\)H-thymidine proliferation assay was used first to test the influence of purified ECM components on early NK proliferation and cell number was used to measure the effect on late NK expansion. CD56\(^+\)/CD3\(^-\) NK were plated in 96-well tissue culture plates that were uncoated, or contained EtOH/acetic acid-fixed M2-10B4, fibronectin, laminin, or thrombospondin. EtOH/acetic acid-fixed monolayers significantly inhibited day 4 early NK proliferation (Table 1). While fibronectin had little effect at any concentration tested, laminin at a concentration of 2.7 \(\mu\)g/well inhibited early NK proliferation by 50% and thrombospondin at concentrations between 0.15 and 1.35 \(\mu\)g/well inhibited early NK proliferation by more than 50%.

Twenty-four well plates were next coated with fibronectin, laminin, or thrombospondin to determine if the early inhibition of NK proliferation observed with laminin and thrombospondin was coupled to late NK expansion. NK were cultured for 28 days with the addition of stroma-conditioned medium as a source of stroma-derived soluble factors necessary for maximal NK proliferation. Fibronectin and laminin had no effect on late NK expansion (Fig 5). In contrast, purified thrombospondin increased overall expansion.

<table>
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<tr>
<th>Tissue Culture Wells</th>
<th>ECM Component Density, (\mu)g/well (pmol/well)</th>
<th>(^3)H-Thymidine Uptake cpm Normalized to Uncoated Control, % (n = 3)*</th>
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<tr>
<td>Uncoated well</td>
<td>0</td>
<td>100</td>
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<td>EtOH/acetic acid-fixed M2-10B4 monolayer</td>
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* \(6 \times 10^3\) CD56\(^+\)/CD3\(^-\) NK were plated in 96-well tissue culture plates. Each condition was plated in triplicate and the mean of 3 donors is reported.

† Mean \(^3\)H-thymidine incorporation in the untreated well was 1,300 ± 630 cpm.
NK REGULATION BY CONTACT AND SOLUBLE FACTORS

Expansion of NK cells was observed with EtOH/lacetic acid-fixed M2-10B4 or wells containing EtOH/lacetic acid-fixed M2-10B4 compared to controls (P = .031). Expansion in thrombospondin-coated wells was similar to that seen in EtOH/acetic acid-fixed M2-10B4 containing wells (P = not significant) indicating that thrombospondin may at least in part be responsible for the inhibition of early NK proliferation and increased late NK expansion. NK cytotoxic function was similar for all culture conditions (data not shown).

Among a variety of other functions, thrombospondin activates latent transforming growth factor-β1 (TGF-β) which could be responsible for both the inhibition of early NK proliferation as well as stimulation of late NK expansion observed when NK are cultured in plates coated with purified thrombospondin. To test this possibility, TGF-β at concentrations between 0.4 and 4 pg/mL was first added to the 3H-thymidine uptake assays that resulted in inhibition of early NK proliferation in a dose-dependent manner and no further inhibition was observed with concentrations greater than 4 pg/mL (Fig 6). This inhibition of early NK proliferation was similar to what is observed when NK are cultured with medium in thrombospondin-coated wells. TGF-β neutralizing antibodies were then added to CD56+/CD3- NK plated in fresh medium into (1) untreated tissue culture plates with or without active TGF-β, (2) wells coated with thrombospondin, or (3) wells containing EtOH/acetic acid-fixed M2-10B4 monolayers (Fig 7). There was a minor stimulatory effect of either anti-TGF-β or control antibodies on NK proliferation in the untreated culture wells which may be due to FcRyIII (CD16) induced NK activation. TGF-β neutralizing antibody completely abrogated the TGF-β inhibition validating the specific TGF-β and anti-TGF-β neutralizing antibody effects. When NK were plated on thrombospondin, the presence of anti-TGF-β neutralizing antibody completely abrogated the thrombospondin-induced inhibition of early NK proliferation whereas control antibody had no effect. In marked contrast, anti-TGF-β neutralizing antibodies did not completely reverse the early inhibition of NK proliferation when cultured on EtOH/acetic acid-fixed M2-10B4 suggesting that other contact-mediated factors besides TGF-β are also involved in inhibiting early NK proliferation.

We also evaluated the effect of TGF-β on late NK expansion. CD56+/CD3- NK (10^6) were plated in 1 mL fresh medium on uncoated wells with or without TGF-β. As originally described, 28 days later 3H-thymidine uptake was measured after 4 days of culture. All results are normalized to the 3H-thymidine cpm of NK cultured in untreated wells with medium alone (absolute cpm: 6,670 ± 2,270). NK proliferation with TGF-β at concentrations of 1 pg/mL or greater was significantly inhibited compared to medium alone (P < .001).

NK expansion measured as actual cell counts was significantly increased by culture on EtOH/acetic acid-fixed M2-10B4 (4.4 pmol/well) fibronectin, 40 μg/well) laminin, and in untreated 24-well culture plates, wells treated with Volker’s buffer, and wells containing EtOH/lacetic acid-fixed M2-10B4 containing wells (P = .029) or thrombospondin (P = .031). Experiments were performed in triplicate for each condition and data are presented as the mean ± SD.

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Fig 8. TGF-β stimulates late NK expansion in a dose-dependent manner. Sorted CD56⁺/CD3⁻ NK (10⁶) were cultured in 24-well plates with initial concentrations of 0.4 pg/mL to 4 ng/mL recombinant active TGF-β (n = 7). All medium changes were subsequently performed with fresh medium without addition of TGF-β. NK expansion was measured by cell counts after 28 days of culture. NK proliferation was significantly different from medium alone for TGF-β concentrations of 0.4 pg/mL to 0.1 ng/mL (P < .02).

These results suggest that TGF-β is almost entirely responsible for the modulation of NK proliferation when NK are cultured on thrombospondin. Thrombospondin must be activating TGF-β because TGF-β was not copurified with the thrombospondin preparation used where only a single band was found by silver stain after gel electrophoresis. This was further verified by Western blot analysis of the purified thrombospondin product in which no TGF-β was detected (data not shown). Although latent TGF-β is present in serum and was internally controlled for in these experiments, we show by RT-PCR that both IL-2 activated CD56⁺high and CD56⁺dim NK produce TGF-β mRNA and that small amounts of this factor may serve as an autocrine growth regulator (Fig 9).

**DISCUSSION**

We show that NK expansion is regulated by two independent stimuli from accessory cells: (1) direct contact with ligand(s) present on accessory cells and (2) soluble factor(s) produced by these cells. These contact and soluble factors are synergistic late in culture (days 28 through 40) and together stimulate maximal late NK expansion. Both contact-mediated and soluble factor-mediated NK expansion is induced by multiple cell types (monocytes, fibroblasts, and endothelial cells) and is not species-specific (human, rat, and murine) suggesting that ECM or another common ligand may mediate the contact-dependent effect. The contact-mediated stimulation of NK is consistent with observations by others. NK expansion was increased by coculture of adherent lymphokine-activated killer cells (A-LAK) with B-lymphoblastoid cell lines and A-LAK expansion in that system was dependent on direct contact between NK and B cells. Others showed that CD56⁺ NK proliferate when cultured with lymphocyte-conditioned medium and in direct contact with K562 cells. However, these studies do not isolate the independent contact factors responsible for the induction of NK expansion. Therefore, a fixed (metabolically inactivated), homogeneous, monolayer accessory cell line (M2-10B4) was chosen to examine the contact-mediated signals for NK proliferation independent of accessory cell-derived soluble factors. A noncrosslinking method of fixation, using EtOH/acetic acid-fixed leaves the secondary protein structure of cell-surface proteins, glycoproteins, and ECM components relatively unaffected. EtOH/acetic acid-fixed accessory cells and IL-2 markedly stimulates NK expansion and the addition of M2-10B4 derived soluble factors reconstitutes
the maximal NK expansion observed when NK are cocultured in direct contact with viable M2-10B4.

Maximal late NK expansion occurs when NK are cultured in the presence of both accessory cell-derived contact and soluble factors. However, we established that late NK expansion is accompanied by an early inhibition of NK proliferation as shown by cell counts and by thymidine uptake assays. The accessory cell-derived contact factors were added only once at the initiation of culture, suggesting that an early contact-mediated event is important not only for the early growth inhibition but also for the increased late NK expansion. Purified thrombospondin is the only ECM component tested that reproduces both the inhibition of early NK proliferation as well as the stimulation of late NK expansion. Further, thrombospondin regulates NK growth through the activation of latent TGF-β. However, unlike purified thrombospondin, EtOH/acetic acid-fixed M2-10B4 dependent inhibition of early NK proliferation is not inhibited by anti-TGF-β antibodies. Moreover, EtOH/acetic acid-fixed M2-10B4 mediated late NK expansion is significantly greater than that seen when NK are cultured on thrombospondin or in the presence of exogenous TGF-β.

These observations suggest that additional contact-mediated mechanisms are important in NK proliferation. Although it is well established that cell-cell and cell-ECM interactions are responsible for lymphocyte recognition, migration and activation, we were unable to show a role for the β1, β2, or β3 integrins in either inhibition of early NK proliferation or in stimulation of late NK expansion suggesting that other adhesive interactions may be responsible for regulating NK proliferation in a complex microenvironment.

It is well known that thrombospondin and latent TGF-β form complexes and this interaction results in activation of TGF-β. Although 1-5 ng/mL of latent TGF-β is present in 10% serum-containing medium, TGF-β is produced by NK themselves after stimulation with IL-2 and may be responsible for the observed effects on NK proliferation. By RT-PCR, we show the presence of TGF-β mRNA in IL-2 activated CD56+/CD3- cells, CD56+CD16+ and CD56+dim NK, which is consistent with other reports showing TGF-β mRNA in plastic adherent IL-2 activated NK. TGF-β is a multifunctional factor that aside from promoting transformed phenotypes in fibroblasts, inducing chondrogenic differentiation, and inhibiting myogenic differentiation can either promote or inhibit cell growth depending on the culture conditions. TGF-β has been shown to be antiproliferative for B cells, T cells, and thymocytes by lengthening or arresting the G1 phase of the cell cycle. However, TGF-β can also stimulate cell proliferation although the mitogenic effects appear to be secondary to other cellular responses. For example, NRK-49F fibroblast proliferation is stimulated by TGF-β in a semisolid medium or in a mitogen poor medium by inducing the production of a fibronectin-collagen-proteoglycan extracellular matrix to which the cells can adhere. In contrast, when NRK-49F cells are cultured as monolayers, TGF-β is growth inhibitory. TGF-β also has pleiotropic effects on both murine and human NK. Reports have indicated that TGF-β inhibits cytotoxic activity of NK after 4 days in culture but enhances NK function after 10 days of culture, an effect reminiscent of the actions of TGF-β on NK proliferation reported here. In a murine breast cancer model neutralizing antibodies against TGF-β infused in vivo decreased tumorigenicity and increased splenic NK function again showing the immune modulatory effects of TGF-β on NK function. In the human system, other studies showed that TGF-β inhibits NK-dependent killing of K562 targets and inhibits early IL-2-dependent NK proliferation. These studies did not measure the effect of TGF-β on late NK expansion or function. Although repeated addition of TGF-β at each medium change inhibited late NK outgrowth and virtually no cell proliferation was detected, a one-time addition of active TGF-β at the initiation of culture not only causes inhibition of early NK proliferation but also leads to late NK expansion, 28 to 40 days after the initiation of culture, with no effect on late NK function. As has been shown for other culture systems, the one time addition of low concentrations of TGF-β may influence late outgrowth by inducing production of ECM components, secondary autocrine cytokines or by decreasing cell death. The TGF-β, thrombospondin-mediated, time-dependent regulation of NK proliferation may provide insight into mechanisms by which NK interact with their local microenvironment.

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