B-cell precursor acute lymphoblastic leukemia cells use tunneling nanotubes to orchestrate their microenvironment

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KEY POINTS

- Primary BCP-ALL cells use tunneling nanotubes to signal to mesenchymal stromal cells and thereby trigger cytokine secretion.

- Inhibiting tunneling nanotube signaling is a promising approach to induce apoptosis and sensitize BCP-ALL cells towards prednisolone.
ABSTRACT

Acute lymphoblastic leukemia (ALL) cells reside in the bone marrow microenvironment which nurtures, and protects cells from chemotherapeutic drugs. The disruption of cell-cell communication within the leukemic niche may offer an important new therapeutic strategy. Tunneling nanotubes (TNTs) have been described as a novel mode of intercellular communication, but their presence and importance in the leukemic niche are currently unknown. Here, we show for the first time that primary B-cell precursor ALL cells use TNTs to signal to primary mesenchymal stromal cells (MSCs). This signaling results in secretion of pro-survival cytokines, such as IP10/CXCL10, IL8 and MCP-1/CCL2. A combination of TNT disrupting conditions allows us to analyze the functional importance of TNTs in an *ex vivo* model. Our results indicate that TNT signaling is important for the viability of patient-derived B-cell precursor ALL cells and induces stroma-mediated prednisolone resistance. Disruption of TNTs significantly inhibits these leukemogenic processes and re-sensitizes B-cell precursor ALL cells to prednisolone. Our findings establish TNTs as a novel communication mechanism by which ALL cells modulate their bone marrow microenvironment. The identification of TNT signaling in ALL-MSC communication gives insight into the pathobiology of ALL and opens new avenues to develop more effective therapies that interfere with the leukemic niche.
INTRODUCTION

Acute lymphoblastic leukemia (ALL) cells reside in the local microenvironment of the bone marrow and are able to disrupt normal hematopoietic stem cell niches. The disrupted, so called leukemic, niche is essential in initiating and facilitating leukemogenesis. In addition, the leukemic niche protects leukemic cells from elimination by immune responses and chemotherapeutic agents, and can facilitate the development of drug resistance of leukemic cells. Therefore, the disruption of the ALL-leukemic niche interaction offers a promising new therapeutic strategy. However, it is still largely unclear how crosstalk occurs within the leukemic niche, and how this drives leukemic cell survival and chemotherapy resistance.

Recently, tunneling nanotubes (TNTs), or membrane nanotubes, have been described as a novel mode of communication between eukaryotic cells. TNTs are thin membrane protrusions consisting of F-actin, that connect cells and facilitate the transport of several types of cargo, including organelles, pathogens, calcium fluxes, death signals, and membrane bound proteins. These intercellular membrane conduits have been observed in several cell types, like cancer cells, complex tissues and organisms. The pathophysiological importance of TNTs has become evident by studies showing that prions and HIV-1 particles use TNTs to promote disease spread. However, the presence of TNTs within the leukemic niche and hence their role in communication between leukemic cells and the bone marrow microenvironment has not yet been addressed. Here, we study the role of TNT signaling in communication between primary B-cell precursor ALL (BCP-ALL) cells and MSCs and the contribution of TNT signaling to mesenchymal-mediated survival and resistance to the chemotherapeutic drug prednisolone.
METHODS

Cell lines

BCP-ALL cell lines, NALM6 (B-Other) and REH (TEL-AML1), were obtained from DSMZ (Braunschweig, Germany). Only low cell passages were used, and the identity of cell lines was routinely verified by DNA fingerprinting. Immortalized hTERT-MSCs were a kind gift from Prof. Dr. D. Campana, St. Jude Children’s Hospital, Memphis, TN, USA.

Primary patient-derived material

Bone marrow aspirates were obtained from children with newly diagnosed BCP-ALL prior to treatment. Mononuclear leukemic cells were collected and processed as previously described. All samples used in this study contained ≥97% leukemic blasts (supplemental Figure 7). Mesenchymal stromal cells (MSCs) were isolated from bone-marrow aspirates obtained from newly diagnosed BCP-ALL patients (before treatment) and healthy controls. MSCs were processed as described previously. Primary MSCs were characterized using positive (CD44/CD90/CD105/CD54/CD73/CD146/CD166/STRO-1) and negative surface markers (CD19/CD45/CD34) (supplemental Figure 6). Multilineage potential of MSCs was confirmed for adipocyte (Oil Red O staining), osteocyte (Alizarin Red S staining), and chondrocyte (Col2a1/Thionine/Alcian Blue staining) differentiation.

Dye transfer experiments

Cells were stained with 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI; yellow), 3,3'-dioctadecyloxacarbocyanine (DiO; green), 1,1'-dioctadecyl-3,3',3'-tetramethylindodicarbocyanine perchlorate (DiD; red), or Calcein red-orange AM (all from Life Technologies) according to the manufacturer’s protocol. Target populations were analyzed
before and after co-culture with flow cytometry (BD Bioscience, San Jose, CA, USA) or confocal microscopy (see below).

Confocal laser scanning microscopy

For high resolution images, differentially stained cells were cultured on a glass slide coated with 10 μg/mL fibronectin (Sigma) at 37 °C and 5% CO₂. Cells were fixated as previously described33. Confocal images were acquired with sequential scanning of different channels at a resolution of 1024 x 1024 pixels in the x x y plane and 0.15 μm steps in z-direction (Leica SP5). For time-lapse confocal imaging, cultures were maintained at 37°C on a heated stage at 5% CO₂ and images were acquired with sequential scanning of different channels at a resolution of 512 x 512 pixels in the x x y plane and 0.5 μm steps in z-direction. 3D image stacks were acquired by optical sectioning using the LAS software provided with the instrument. The system was equipped with a 63× plan-apochromat oil 1.4 NA DIC objective. The pinhole diameter was set to 1 airy unit (95.5 μm). DiO and DiI were excited with a 488-nm Argon laser and a 561-nm Diode-Pumped Solid-State laser, respectively. Phalloidin-FITC (Sigma) was excited with the 488-nm Argon laser. Image processing was done with Fiji software34.

TNT inhibition

TNTs were inhibited using actin inhibition by latrunculin B (125 – 500 nM; Sigma) or cytochalasin D (250 nM – 1 μM; Sigma)20, by mechanical disruption via gentle shaking of cell cultures (250 rpm)21,35, or by physical separation of leukemic cells (cultured in a 3.0 μm pore-sized insert) and MSCs (cultured in the bottom compartment of a transwell system; Corning, NY, USA)21,36.
Cell viability assays

Primary patient cells (1 x 10^6 cells) were co-cultured with or without primary MSCs (5 x 10^4) for five days in a 24-well plate at 37 °C and 5% CO₂. The percentage of viable leukemic cells was determined by staining with Brilliant Violet 421 anti-human CD19 or CD45 antibody (Biolegend), FITC Annexin V (Biolegend), and Propidium Iodide (PI; Sigma), after which the percentage of AnnexinV<sup>neg</sup>/PI<sup>neg</sup>/CD19<sup>pos</sup>/CD45<sup>pos</sup> cells within the MSC negative fraction (see Figure 6A for gating strategy) was determined by flow cytometry (BD Biosciences). In supplemental Figure 10, viable leukemic cells were counted (PI<sup>neg</sup>/CD19<sup>pos</sup>) using a MACSQuant analyzer (Miltenyi Biotec, Gladbach, Germany).

Multiplexed fluorescent bead-based immunoassay (Luminex)

Primary leukemic cells and leukemic cell lines were co-cultured with primary MSCs with or without TNT inhibition (shaking or transwell condition) for indicated time points at 37 °C and 5% CO₂. Next, the supernatant was collected and cell viability of leukemic cells was assessed as described above. The concentration of 64 cytokines/chemokines in supernatants of ALL-MSC co-cultures was analyzed using a fluorescent bead-based immunoassay (Luminex Human Cytokine/Chemokine Panel I and II; Merck Millipore) according to the manufacturer’s protocol.

Statistical analysis

Student’s t-test was used as a statistical test and a Student’s paired t-test was used when applicable (indicated in figure legends). Bar graphs represent the mean of biological replicates. Error bars show as standard error of the mean (SEM).
For more details see supplemental methods.
RESULTS

BCP-ALL cells use TNTs to effectively signal to MSCs

TNT formation within the hematopoietic niche was studied by confocal microscopy and flow cytometry. TNTs are thin membrane tethers and can be visualized using lipophilic carbocyanine dyes\(^ {20,21} \). These dyes stain lipophilic structures in the entire cell, and exhibit very low cell toxicity, while passive transfer of these dyes is negligible. Therefore, these dyes are widely used in live cell tracking experiments\(^ {1,37} \). Interestingly, it has been shown that organelles and membrane components stained by these dyes can be actively transported via TNTs\(^ {20} \). Therefore, these dyes were used to visualize intercellular communication via TNTs within the leukemic niche.

Differential staining of NALM6 BCP-ALL cells (stained with DiI-yellow) and mesenchymal stromal cells (hTERT-MSCs; stained with DiO-green) revealed that TNTs were formed within 3 hours of co-culture (Figure 1A, supplemental Figure 1M-P). 3D reconstruction of these images shows that nanotubular structures between cells do not connect with the substratum (i.e. the fibronectin-coated glass slide; see supplemental Video 1A-B and supplemental Video 2). As expected, staining with Phalloidin-FITC shows the presence of F-Actin filaments in these nanotubular structures (see supplemental Figure 1Q-R). Importantly, bidirectional transfer of lipophilic dyes was observed, indicating active crosstalk within the leukemic niche (Figure 1A).

Besides formation of TNTs between leukemic cells and MSCs, TNT networks and transfer of lipophilic dye was also observed in mono-cultures of BCP-ALL cells and MSCs (supplemental Figure 1A-L and supplemental Figure 2).

In order to quantify active crosstalk via TNTs we used flow cytometrical analysis of dye transfer from labeled donor cells to unlabeled recipient cells. First, CD19-positive BCP-ALL cell lines (NALM6 and REH) were stained with lipophilic dye DiI and co-cultured with unstained CD19-
negative hTERT-MSCs (supplemental Figure 3A-C for gating strategy). After 6 hours of culture, more than 50% of the MSCs were positive for DiI. This number increased to > 85% after 24 hours, highlighting the efficient dye transfer from leukemic cells to MSCs (Figure 1B and supplemental Figure 3D). In reciprocal experiments we also observed dye transfer from MSCs to BCP-ALL cells, but the magnitude of dye transfer was strikingly less than from ALL cells towards MSCs (175-fold, p-value ≤ 0.001) (Figure 1C-D). Transfer of lipophilic dyes can be mediated by several processes including TNT signaling and signaling via extracellular vesicles (ECV). To evaluate the contribution of TNT signaling to the observed lipophilic dye transfer between leukemic cells and MSCs, we inhibited TNTs using three independent experimental setups: 1) reducing TNT formation through actin inhibition, 2) mechanical disruption of TNT connections through gentle shaking of cell cultures, and 3) prevention of TNT formation by physically separating leukemic cells (cultured in a 3.0 μm pore-sized insert) and MSCs (cultured in the bottom compartment of a transwell system). For inhibition of the polymerized F-actin of which TNTs are composed, we used two classes of F-actin polymerization inhibitors: cytochalasin D and latrunculin B. In addition to F-actin elements, TNTs need prolonged cell contact to signal efficiently. Gentle shaking of ALL-MSC co-cultures reduces the lifespan of TNTs, while direct contact between BCP-ALL cells and MSCs is still possible. However, it is well established in literature that flow-derived shear forces (gentle shaking) induce integrin-mediated signaling. Therefore, we also physically separated ALL cells and MSCs using a transwell system, to exclude the effects of increased integrin signaling. We used a 3.0 μm transwell system to also investigate the contribution of extracellular vesicle signaling to lipophilic dye transfer. In this transwell system leukemic cells are physically separated from MSCs, while exchange of extracellular vesicles (30-1000 nm) is still possible. Cytochalasin D
and latrunculin B both caused a dose-dependent reduction of dye transfer after 6 hours of co-culture (p ≤ 0.01; Figure 1E-F and supplemental Figure 3E-F). Due to the short half-time of these actin inhibitors\textsuperscript{40,41}, TNT formation was restored within 24 hours (supplemental Figure 3E-F). Disruption of TNT structures by gentle shaking reduced dye transfer by more than 5-fold (p ≤ 0.01; Figure 1E-F and supplemental Figure 3G). Importantly, dye transfer from BCP-ALL cell lines to MSCs was nearly absent in transwell experiments (p ≤ 0.001), which indicates that dye transfer occurs mainly via TNTs and not via extracellular vesicles (Figure 1E-F and supplemental Figure 3H).

The TNT forming capacity was also evaluated in an \textit{ex vivo} niche model using leukemic cells and MSCs that were both freshly obtained from patients with newly diagnosed BCP-ALL (Figure 2A, supplemental Figure 4A-J, supplemental Figure 5A-D, supplemental Figure 6 and supplemental Figure 7). ***Ex vivo*** co-cultures revealed that TNTs rapidly (< 3 hours) form between primary BCP-ALL cells and primary MSCs, and efficiently transfer lipophilic dye (Figure 2B, supplemental Figure 4G-J, supplemental Figure 5). The source of MSC did not affect the efficacy of TNT signaling. Dye transfer was similarly efficient from ALL cells towards 10 different primary MSCs (n = 5 healthy and n = 5 leukemic primary patient-derived MSCs; Figure 2C-D and supplemental Figure 8A-C).

The dynamics of TNT formation between BCP-ALL cells and MSCs were investigated using time-lapse confocal microscopy. Leukemic cells initiated formation of TNTs and transferred lipophilic dye towards MSCs within minutes (Figure 3A; supplemental Videos 3 and 4A). Leukemic cells were able to form multiple TNTs and signal to several MSCs simultaneously (Figure 3B; and supplemental Videos 3 and 4A-B). TNTs formed between MSCs and leukemic cells were stable for multiple hours and could reach several cell diameters in length (Figure 3C,
supplemental Videos 3 and 4A). After 10 hours, the majority of MSCs became DiI-positive (supplemental Videos 3 and 4B), as confirmed by flow cytometry (Figure 1B).

**TNTs are important players in signaling from BCP-ALL cells to MSCs**

The above-mentioned findings suggest that TNT signaling is a highly effective communication mechanism between ALL cells and MSCs. This was further illustrated by comparing TNT signaling to other intercellular communication mechanisms, including gap junctions, integrins and ECV (Figure 4A). Dye transfer from leukemic cells towards MSCs was minimal using a 3.0 µm transwell system, in which ECV signaling is possible (> 500 fold lower compared to normal co-culture after 24 hours; \( p \leq 0.001 \); Figure 4B-C). Transfer experiments with the gap junction-specific dye Calcein revealed that signaling from leukemic cells towards primary MSCs via gap junctions is highly ineffective compared to signaling via TNTs (> 90 fold lower after 24 hours; \( p \leq 0.001 \); Figure 4D-E). Integrin signaling has been implicated in the induction of TNTs\(^{42}\), raising the question whether TNTs function autonomously or in an integrin-dependent manner. To inhibit integrin signaling between BCP-ALL cells and MSCs, we used RGDS-peptides, reported to prevent the binding of integrins to membranes\(^{43}\). RGDS negatively affected the efficiency of lipophilic dye transfer via TNTs whereas the transfer in the presence of negative control peptides (GRADSP) remained unaffected (Figure 4F). The reduction in lipophilic dye transfer was limited to a maximum of 30% (\( p \leq 0.01 \)). Taken together, these data reveal that TNT signaling acts independently of other important intercellular communication mechanisms, i.e. signaling via extracellular vesicles, gap junctions, and integrins.

**BCP-ALL cells use TNTs to drive cytokine release within the microenvironment**
The question remains how TNT signaling from leukemic cells affect their microenvironment. Since cytokines and chemokines can greatly affect the survival of leukemic cells\textsuperscript{44,45}, we considered that the microenvironment responds to TNT signaling by secreting supportive soluble factors. Therefore we investigated the secreted levels of 64 known cytokines/chemokines in co-cultures of primary leukemic cells of two BCP-ALL patients with different sources of primary MSCs. Co-culture of these cells induced the secretion of several cytokines. The cytokine signature produced within co-cultures suggested that this secretion was leukemia-driven and independent of the MSC source (Figure 5). IP10/CXCL10 levels increased more than 1000-fold when patient ALL#7 cells were co-cultured with MSCs, but were undetectable in co-cultures from patient ALL#9 cells (Figure 5A and 5E). Likewise, MDC/CCL22 and TARC/CCL17, both undetectable in patient ALL#7 co-cultures, were induced 6-18 fold when patient ALL#9 cells were co-cultured with MSCs (Figure 5C-D and 5G-H). Interleukin-8 (IL8) levels were induced (2-7 fold) by primary ALL cells from both patients (Figure 5B and 5F). Cytokines that were induced less than 2 fold in co-culture are shown in supplemental Figure 9. Only a limited number of cytokines/chemokines were found to be significantly upregulated in patients’ ALL-MSC co-cultures compared to mono-cultures of both cell types. Also in a proliferative setting (using the BCP-ALL cell line NALM6), a limited number of known cytokines were upregulated in co-culture with two different primary MSCs: IL8 and VEGF levels were on average induced 2 and 3 fold respectively (n=3, \( p \leq 0.01 \), Supplemental Figure 9E-H). These MSC-independent and leukemia-consistent cytokine signatures suggest that leukemic cells, and not MSCs, are responsible for the active modulation of the tumor microenvironment. Induction of the observed cytokines was dependent on TNT signaling, as TNT inhibition significantly lowered the secreted levels of these factors (Figure 5A-H, Supplemental Figure 9E-
However, TNT inhibition only partly reversed the induction of cytokine levels in ALL-MSC co-cultures, suggesting that, next to TNT signaling, other intercellular signaling routes contribute to the induction of these secretomes.

**TNT signaling is important for the survival of primary BCP-ALL cells**

Several studies have shown the importance of the microenvironment for the survival of malignant cells, but without elucidating how\textsuperscript{46}. In order to study the effect of TNT signaling on leukemic cell viability, we used *ex vivo* co-cultures of primary BCP-ALL cells and primary MSCs (see table S1 for BCP-ALL subtype information, Figure 6A for gating strategy). Primary BCP-ALL cell survival significantly increased in 5-day co-cultures with primary MSCs compared to mono-cultures. When TNT formation was prevented by shaking of co-cultures or by transwell conditions, this increase significantly reduced 3.5- and 3.6-fold, respectively (n = 7, p ≤ 0.001; Figure 6B-D). The effect of TNT inhibition was consistent across leukemic cells from multiple cytogenetic BCP-ALL subgroups (TEL-AML1, BCR-ABL1-like, and B-Other).

**Inhibition of TNTs sensitizes BCP-ALL cells to prednisolone**

We investigated whether inhibition of TNT signaling also affects the response of leukemic cells to chemotherapeutic drugs. BCP-ALL patient cells were treated with the ALL spearhead drug prednisolone for 5 days. Prednisolone was less effective in inducing apoptosis of primary BCP-ALL cells in the presence of primary MSCs compared to mono-cultures of primary BCP-ALL cells (70% vs 5% reduced viability; p ≥ 0.001; Figure 7A), underlining the importance of MSCs in the induction of prednisolone resistance (Figure 7A). The protective effect of MSCs was significantly reduced by 2-3 fold (n = 4, p ≤ 0.001; Figure 7A) when TNT signaling was
inhibited. TNT inhibition alone was not sufficient to abrogate all microenvironment-induced drug resistance. In shaking and transwell conditions, which allow signaling via integrins, soluble factors or ECVs, microenvironment-induced resistance to prednisolone was still present (p ≥ 0.01). Since ex vivo cultured leukemic patient cells lose their propensity to proliferate, we also addressed the effect of TNT signaling on drug resistance in a proliferative setting using the BCP-ALL cell line NALM6. Similar to primary BCP-ALL cells, co-culture with MSCs induced prednisolone resistance of NALM6 cells (Figure 7B). Inhibition of TNT formation by shaking of co-cultures or transwell conditions significantly reduced this effect 4.5- and 8.5-fold, respectively (p ≤ 0.01; Figure 7B and supplemental Figure 10A-B). These data show that inhibition of TNT signaling in co-cultures sensitizes BCP-ALL cells to the anti-leukemic effects of prednisolone in both a primary non-proliferative and a proliferative setting.
DISCUSSION

The presented study identifies TNT formation as a novel regulator of interaction between BCP-ALL cells and their bone marrow niche, which facilitates signaling from leukemic cells towards MSCs and affects the release of cytokines and chemokines in the microenvironment. Disruption of TNTs inhibits this release, decreases the survival benefit that MSCs provide to primary BCP-ALL cells, and sensitizes BCP-ALL cells to the important anti-leukemic drug prednisolone (supplemental Figure 11).

Relapse of leukemia is caused by a small number of leukemic cells that are able to withstand chemotherapy and can cause the complete reconstitution of the tumor. Leukemogenic mouse models show the importance of signaling between leukemic cells and their bone marrow microenvironment and emphasize the pathophysiological relevance of cytokines within the leukemic niche.\textsuperscript{1,47-49} However, a major shortcoming in our knowledge about the leukemic niche is the lack of insight into the functional mechanism mediating crosstalk between leukemic cells and their local niche. Our data adds significant insight into this process and provides an opportunity to inhibit the leukemic niche. Importantly, primary BCP-ALL cells use TNTs to modulate their microenvironment, identifying the leukemic cell, and not MSCs, as the driver of niche modulation.

Several studies report that the leukemic niche can induce drug resistance for both classical chemotherapeutic agents and newly developed targeted therapies.\textsuperscript{9,44-46,52-54} The seminal papers by Strausmann et al.\textsuperscript{44}, and Wilson et al.\textsuperscript{45} revealed the widespread potential for growth-factor-driven resistance to kinase inhibitors in several tumor types. A recent study by Manshouri et al.\textsuperscript{54}, showed the induction of resistance against JAK2 inhibitors by bone marrow stroma-
secreted cytokines in JAK2-mutated primary hematopoietic cells. BCP-ALL cells use TNTs to induce a pro-inflammatory cytokine signature within their microenvironment. These cytokines have been reported to be involved in leukemia survival and resistance to therapy, like IP10/CXCL10, IL-8, and MCP-1/CCL2. When TNTs were inhibited, this signature was partly reversed and simultaneously leukemic cell survival was decreased. Since these cytokines also have a chemoattractive function, it is likely that migration towards the stromal compartments of the niche and subsequent induction of contact-dependent signaling modules, like integrins and gap junctions, also play a role in this process. Further, we observed TNT signaling from MSCs towards leukemic cells, which might also influence drug resistance of leukemic cells. For example, TNTs have been shown to transport drug-efflux pumps such as P-glycoproteins, and to transport mitochondria preferentially towards cancer cells.

Although less pronounced as seen between ALL cells and MSCs, TNTs are also used for communication between leukemic cells. This discovery may reveal a new aspect of tumor heterogeneity. In many cancer types, clonal evolution has been observed. A recent study in T-ALL by Blackburn et al. shows that leukemia subclones acquire mutations, that can mediate chemotherapy resistance even without prior drug exposure. Leukemic cells might exploit TNTs, which can transfer a broad spectrum of molecules and organelles like membrane-associated signaling molecules (e.g. H-Ras), to transfer mutant proteins and subsequently chemotherapy resistance between subclones.

Interestingly, we observed leukemia-specific cytokine patterns in MSC-ALL co-cultures that were affected by abrogation of TNT signaling. This observation opens the discussion how TNT signaling can lead to the upregulation of different soluble factors. The broad spectrum of
signaling molecules that are transported by TNTs potentially allows the ALL to convey specific messages to MSCs in order to differentially regulate the secretion of soluble factors by its microenvironment. In addition, leukemia is a highly heterogeneous disease consisting of different (cyto)genetic subtypes that also have individual heterogeneity with regards to their transcriptome and proteome\textsuperscript{58}. These factors are all likely to contribute to leukemia-unique demands for microenvironmental support.

Targeting TNT-directed communication between leukemic cells and their supportive niche may be a promising new approach to kill leukemic cells and prevent drug resistance in clinical practice. As of yet, no agents are available that induce specific inhibition of TNT signaling, but our data point to the importance to develop such agents. TNT signaling can be disrupted through shear stress, applying a physical distance between the cells, actin inhibition, and in some cases tubulin inhibition. In additional experiments, we observed that tubulin inhibition did not inhibit TNT signaling between BCP-ALL cells and MSCs (data not shown). The common building block for all TNTs reported in literature is F-actin, making it an obvious target for TNT disruption. Actin inhibitors are derived from fungi, plants and sponges, which developed these toxins as a defense mechanism. Consequently, these compounds inhibit TNT formation (Figure 1E-F) and induce cell death (data not shown). Therefore, it is important to develop more specific and less-toxic small molecule inhibitors that target elements of the actin cytoskeleton important for TNT formation. We propose our primary patient-derived \textit{ex vivo} model system as a highly suitable platform to identify such inhibitors. Once identified, these TNT-specific agents will allow us to investigate TNT signaling also \textit{in vivo}. 
In conclusion, the discovery of TNT signaling between BCP-ALL cells and mesenchymal stromal cells adds significant insight into the mechanisms of communication in the leukemic niche. BCP-ALL cells use TNT networks to modify their healthy microenvironment and hereby create a leukemic niche that induces survival and drug resistance. Current chemotherapeutic regimens are primarily focused on combating tumor intrinsic properties. Our data provide a new concept to develop alternative therapeutic strategies that include targeting of the leukemic niche in B-cell acute lymphoblastic leukemia.
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AUTHOR CONTRIBUTIONS

Co-first authors R. Polak and B. de Rooij contributed equally to the study and are listed alphabetically. R. Polak and B. de Rooij designed the study, performed the experiments, collected and analyzed all data, and wrote the paper. M.L. den Boer designed the study, analyzed data, and wrote the paper. R. Pieters analyzed data and wrote the paper. All authors discussed the results and approved the submitted manuscript.

CONFLICT OF INTEREST DISCLOSURE

The authors declare no competing financial interests.
REFERENCES


FIGURE LEGENDS

Figure 1. TNT signaling between BCP-ALL cells and MSCs

(A) Representative confocal images (Z-stack) showing TNT networks (white arrowheads) between BCP-ALL cell line REH (DiI, yellow) and hTERT-immortalized MSCs (DiO, green) after co-culture for 3 hours. Bidirectional exchange of lipophilic dye via TNTs was observed (green arrow for MSC to ALL, yellow arrow for ALL to MSC). Leukemic cells also formed TNT-like structures towards the fibronectin-coated substratum (orange arrowhead).

(B) Graph showing quantification of dye transfer from DiI-stained NALM6 cells towards unstained hTERT-MSCs (cultured in 4:1 ratio) in time. Figure shows representative experiment (n = 3).

(C) Graph showing quantification of dye transfer after 24 hours of co-culture (cultured in 1:1 ratio). Left panel shows dye transfer from DiI-stained hTERT-MSCs towards unstained NALM6 cells. Right panel shows the reciprocal experiment (also performed in a 1:1 ratio). White and grey histograms represent staining intensity at the start of each experiment.

(D) Quantification of dye transfer in time of experiment as exemplified in (C), performed with two different BCP-ALL cell lines (REH and NALM6). Dye transfer from MSCs towards ALL was compared to dye transfer from ALL towards MSCs (n = 4; two-tailed t-test, unpaired).

(E) Graph showing quantification of dye transfer from DiI-stained NALM6 cells towards unstained hTERT-MSCs with (red histograms) or without (grey histograms) TNT inhibition. Cells were co-cultured in 4:1 ratio for 6 hours. Three independent TNT inhibiting conditions were used: actin inhibition by cytochalasin D or latrunculin B, physical disruption by gentle shaking, or culture in a 3.0 μm transwell system.

(F) Quantification of dye transfer experiment exemplified in (E), performed with two different BCP-ALL cell lines (REH and NALM6) (n = 4; one-tailed t-test, unpaired).
Data are means ± SEM; ** p ≤ 0.01, *** p ≤ 0.001. See also supplemental Figures 1 and 3, and supplemental Videos 1A-B and 2.

**Figure 2. Primary BCP-ALL cells signal to MSCs via TNTs**

(A) Representative confocal images (Z-stack) showing TNT formation (white arrowhead) between a primary CD34-positive cell (DiI, yellow) and a hTERT-immortalized MSC (DiO, green) after co-culture for 3 hours. White arrow indicates transfer of dye to recipient cell.

(B) Graphs showing quantification of dye transfer in co-cultures of BCP-ALL patient cells with primary MSCs (cultured in 4:1 ratio). Inhibition of TNTs was performed by gentle shaking of co-cultures, or co-culture in a 3.0 μm transwell system (red histograms).

(C) Graphs showing quantification of dye transfer from DiI-stained NALM6 cells towards 10 different unstained primary MSCs (cultured in 4:1 ratio) obtained from leukemia patients (n = 5) and healthy controls (n = 5).

(D) Graphs showing quantification of dye transfer from DiI-stained NALM6 cells towards 3 different primary MSCs with (red histograms) or without (grey histograms) TNT inhibition.

See also supplemental Figures 4-8.

**Figure 3. Dynamic nature of TNT formation between BCP-ALL cells and MSCs**

(A-C) Time-lapse confocal images (3D image stacks) showing TNT formation (white arrowhead) between NALM6 cells (DiI, yellow) and primary MSCs at multiple time points. White arrow indicates transfer of dye to recipient cell. Time indicated in the right lower corner is
the duration from start of the experiment. Orange Hot look-up table (LUT) and transmission overlays were used to illustrate dye transfer towards MSCs. Scale bars represent 10 µm. (A and C) Depicted images are close-ups of the upper left corner of supplemental Video 3. (B) Depicted images are close-ups of supplemental Video 4A. Data is representative of three independent experiments. See also supplemental Figure 5 and supplemental Videos 3 and 4A-B.

Figure 4. TNTs are important players in signaling from BCP-ALL cells to MSCs

(A) Model of crosstalk between leukemic cells and MSCs in the hematopoietic niche.

(B) Graphs showing quantification of dye transfer from DiI-stained NALM6 cells towards unstained hTERT-MSCs. Blue and grey histograms represent staining intensity at the start of each experiment. Red histogram represents signaling efficiency via TNTs (normal co-culture; left panel) and via extracellular vesicles (ECV) (3.0 µm transwell; right panel).

(C) Bar graphs of experiment shown in (B) after 6 hours (left panel) and 24 hours (right panel) of co-culture (n = 4; two-tailed t-test, unpaired).

(D) Graphs showing quantification of dye transfer from BCP-ALL cell line NALM6, stained with either DiI or calcein, towards primary MSCs after 24 hours of co-culture. Blue and grey histograms represent staining intensity at the start of each experiment. Red histogram shows signaling efficiency via TNTs (left panel) and via gap junctions (right panel).

(E) Bar graphs of experiment shown in (D) after 8 hours (n = 6; left panel) and 24 hours (n = 4; right panel) of co-culture (two-tailed t-test, unpaired).
Bar graphs representing dye transfer from DiI-stained REH cells towards unstained primary MSCs with and without integrin blocking. Integrin signaling was blocked by addition of RGDS peptide, and compared to addition of the integrin non-binding peptide GRADSP (n = 5; one-tailed t-test, paired).

Data are means ± SEM; * p ≤ 0.05, ** p ≤ 0.01, *** p ≤ 0.001.

**Figure 5. BCP-ALL cells use TNTs to drive cytokine release within the microenvironment**

(A) IP10/CXCL10 supernatant levels in co-culture of primary leukemic patient ALL#7 cells with primary MSC#1 (left panel), MSC#2 (middle panel), or MSC#3 (right panel). TNT signaling was inhibited by gentle shaking or culture in a transwell system (one-tailed t-test, unpaired).

(B) Same as (A) for IL8 levels.

(C) Same as (A) for MDC (CCL22) levels.

(D) Same as (A) for TARC (CCL17) levels.

(E) IP10/CXCL10 supernatant levels in co-culture of primary leukemic patient ALL#9 cells with primary MSC#4 (left panel), or MSC#5 (right panel).

(F) Same as (E) for IL8 levels.

(G) Same as (E) for MDC (CCL22) levels.

(H) Same as (E) for TARC (CCL17) levels.

Data are means ± SEM; * p ≤ 0.05, ** p ≤ 0.01. nd = not detectable (below detection level).

See also supplemental Figure 9.

**Figure 6. TNT signaling is important for the survival of primary BCP-ALL cells**
(A) Flow chart and flow cytometrical gating strategy used to study the effect of TNTs on BCP-ALL cell survival. Co-cultures of CD19\textsuperscript{positive} leukemic cells and CD19\textsuperscript{negative} MSCs were stained with Brilliant Violet 421\textsuperscript{TM} anti-human CD19 antibody, FITC Annexin V, and Propidium Iodide (PI). MSCs were excluded (red gate), and the percentage of viable BCP-ALL blasts (Annexin\textsuperscript{V}\textsuperscript{neg}/PI\textsuperscript{neg}/CD19\textsuperscript{pos} cells) was determined within the MSC-negative fraction.

(B) Percentage of viable primary leukemic patient cells (n = 3 TEL-AML1, n = 2 B-Other, n = 2 BCR-ABL1-like) in mono-culture (black bars) or co-culture with patient MSCs (white bars) after 5-day co-culture.

(C) The survival benefit for primary leukemic patient cells (n = 3 TEL-AML1, n = 2 B-Other, n = 2 BCR-ABL1-like) in co-culture with patient MSCs.

(D) The mean survival benefit for primary leukemic patient cells in co-culture with patient MSCs (n = 7; one-tailed t-test, paired).

Data are means ± SEM; *** p ≤ 0.001.

**Figure 7. Inhibition of TNTs sensitizes BCP-ALL cells to prednisolone**

(A) Leukemic cell survival of patient ALL\#6 cells cultured with or without MSCs after 5 days of prednisolone exposure (0.3 µg/mL). All graphs show percentage compared to untreated control. TNT signaling was inhibited by shaking or transwell conditions (one-tailed t-test, unpaired). Quadruplicates represent four different sources of MSCs (MSC\#1, MSC\#2, MSC\#6, MSC\#7).

(B) Leukemic cell survival of BCP-ALL cell line NALM6 cultured with or without MSCs after 5 days of prednisolone exposure. All graphs show percentage compared to untreated control. TNT signaling was inhibited by shaking or transwell conditions (one-tailed t-test, unpaired). Triplicates represent three different sources of MSCs (hTERT, MSC\#1, and MSC\#2).
Data are means ± SEM; * p ≤ 0.05, ** p ≤ 0.01. See also supplemental Figure 10.
Figure 1. TNT signaling between BCP-ALL cells and MSCs

A

Staining with Dyl (yellow) → Co-culture (3hr) → Fixation → Confocal microscopy

B

Dye transfer from Dil-stained BCP-ALL (NALM6) cells towards hTERT-MSC (BCP-ALL → MSC)

0 hr 1 hr 3 hr 6 hr 8 hr 24 hr

Count

Dil stain (PE)

C

Normalized count

Dil stain (PE)

D

MSC → BCP-ALL  BCP-ALL → MSC

Mean DiI-fluorescence

0 hr unstained cells

0 hr stained cells

Dye transfer after 24 hr co-culture

E

BCP-ALL → MSC

Cytochalasin D  Latrunculin B

Normalized count

Dil stain (PE)

F

BCP-ALL → MSC (6 hr co-culture)

Mean DiI-fluorescence

Normal  Shaking  Transwell  Cytochalasin D  Latrunculin B
Figure 2. Primary BCP-ALL cells signal to MSCs via TNTs

A

CD34+ MSC
Staining with DiI (yellow)
Co-culture (3 hr)
Fixation
Confocal microscopy

B

Primary BCP-ALL → Primary MSC

BCP-ALL (ALL#1) + Healthy MSC (MSC#7)

Dil stain (PE)
Normalized count

3 hr co-culture
+ TNT inhibition

0 hr
3 hr co-culture
6 hr co-culture
24 hr co-culture

MSC-DiO

C

Healthy MSCs

MSC#6
MSC#7
MSC#8
MSC#9
MSC#10

Normalized count

Dil stain (PE)

Leukemic MSCs

MSC#11 (B-Other)
MSC#1 (TEL-AML1)
MSC#12 (TEL-AML1)
MSC#13 (CML)

Normalized count

Dye transfer to MSCs (%)

0 hr
3 hr co-culture
6 hr co-culture
24 hr co-culture

Healthy
Leukemic

n.s.

D

Healthy MSC#7
Leukemic MSC#1 (BO)
Leukemic MSC#12 (TA)

Normalized count

Dil stain (PE)
Figure 3. Dynamic nature of TNT formation between BCP-ALL cells and MSCs
Figure 4. TNTs are important players in signaling from BCP-ALL cells to MSCs

A

B

C

D

E

F
Figure 5. BCP-ALL cells use TNTs to drive cytokine release within the microenvironment

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Figure 6. TNT signaling is important for the survival of primary BCP-ALL cells

A

B

C

D
Figure 7. Inhibition of TNTs sensitizes BCP-ALL cells to prednisolone

A

**Leukemic cell survival (%)**

- Normal
- Shaking
- Transwell

MSC | No MSC
--- | ---
+ Prednisolone | + Prednisolone | + Prednisolone

B

**Leukemic cell survival (%)**

- Normal
- Shaking
- Transwell

MSC | No MSC
--- | ---
+ Prednisolone | + Prednisolone | + Prednisolone

**NALM6 (BCP-ALL)**
B-cell precursor acute lymphoblastic leukemia cells use tunneling nanotubes to orchestrate their microenvironment

Roel Polak, Bob de Rooij, Rob Pieters and Monique L. den Boer