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EphB Receptors Trigger Akt Activation and Suppress Fas Receptor-Induced Apoptosis in Malignant T Lymphocytes

Alison Maddigan,*1 Luke Truitt,*1 Ryan Arsenault,‡ Tanya Freywald,† Odette Allonby,† Jonathan Dean,* Aru Narendran,§ Jim Xiang,* Andrew Weng,‖ Scott Napper,‡ and Andrew Freywald†

Treatment of hematopoietic malignancies often requires allogeneic bone marrow transplantation, and the subsequent graft-versus-leukemia response is crucial for the elimination of malignant cells. Cytotoxic T lymphocytes and NK cells responsible for the immunoelimination express Fas ligand and strongly rely on the induction of Fas receptor-mediated apoptosis for their action. Although cancer cells are removed successfully by graft-versus-leukemia reactions in myeloid malignancies, their efficiency is low in T cell leukemias. This may be partially because of the ability of malignant T cells to escape apoptosis. Our work shows that Eph family receptor EphB3 is consistently expressed by malignant T lymphocytes, most frequently in combination with EphB6, and that stimulation with their common ligands, ephrin-B1 and ephrin-B2, strongly suppresses Fas-induced apoptosis in these cells. This effect is associated with Akt activation and with the inhibition of the Fas receptor-initiated caspase proteolytic cascade. Akt proved to be crucial for the prosurvival response, because inhibition of Akt, but not of other molecules central to T cell biology, including Src kinases, MEK1 and MEK2, blocked the antiapoptotic effect. Overall, this demonstrates a new role for EphB receptors in the protection of malignant T cells from Fas-induced apoptosis through Akt engagement and prevention of caspase activation. Because Fas-triggered apoptosis is actively involved in the graft-versus-leukemia response and cytotoxic T cells express ephrin-Bs, our observations suggest that EphB receptors are likely to support immuno evasion of T cell malignancies and may represent promising targets for therapies, aiming to enhance immunoelimination of cancerous T cells. The Journal of Immunology, 2011, 187: 000–000.

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eukemias and lymphomas of the T cell origin are often aggressive malignancies that are associated with a wide range of molecular defects in the T cell lineage (1, 2). In pediatric T cell acute lymphoblastic leukemia (T-ALL), for example, the most common molecular abnormalities are presented by mutations or silencing of the cdkn2a/2b genes that block expression of the p15INK4 and p16INK4 cell cycle suppressors (3–5) and prevent production of the p14ARF activator of p53 (1).

Other prominent flaws in T-ALL molecular machinery also include Notch1 receptor-activating mutations (6, 7), inactivating mutations of Notch negative regulator, Fbxw7 (8–10), and of the inhibitor of PI3K signaling, the PTEN phosphatase (11). Alternatively, among lymphoid malignancies, cutaneous T cell lymphomas are characterized by frequent mutations in the nav3 gene (12), by the constitutive activation of STAT3 (13), by the overexpression of the CCR10 receptor (14), of JunB (15), and of the antiapoptotic protein Mcl-1 (16). In addition, the phosphorylation/inactivation of the proapoptotic factor Bad (16) is also common in lymphoma cells. Despite substantial progress in the understanding of the molecular biology of T cell malignancies and despite recent advances in their treatment, a large number of patients develop resistance to currently used multiagent chemotherapies and relapse (1, 17–19). These patients are typically offered an allogeneic bone marrow transplant and their best hope resides in the immunotherapeutic effect of the graft-versus-leukemia/lymphoma reaction (17, 19, 20). The graft-versus-leukemia/lymphoma response strongly relies on Fas receptor (FasR)-triggered cell death induced in malignant T cells by the Fas ligand expressed on allogeneic cytotoxic T lymphocytes and NK cells (21–23). The immunotherapeutic response may be additionally augmented by donor lymphocyte infusions (17, 20). Unfortunately, although this strategy works well in myeloid leukemias, it has proved to be of a limited efficiency in a significant portion of T-ALL patients, presumably because of the ability of malignant T cells to evade immunoelimination (2, 17, 21, 24). The evading ability may partially depend on the molecular mechanisms that reduce sensitivity to Fas stimulation and, thus, support survival of cancerous T cells.

Accumulating observations show that tyrosine kinase receptors of the Eph family are involved in the regulation of cell survival in nonhematopoietic malignancies, including the antiapoptotic action of the EphA2 receptor in breast cancer (25) and of the EphB4 receptor in colorectal cancer (26), ovarian cancer (27), head and neck carcinomas (28), and melanoma (29). Accumulating evidence suggests that EphB receptors play a role in the regulation of apoptosis in hematopoietic malignancies. This is exemplified by the expression of EphB2 in chronic myeloid leukemia (30), EphB3 in acute myeloid leukemia (31), EphB4 in chronic lymphocytic leukemia (32), and EphB6 in acute lymphoblastic leukemia (33).

Although cancer cells are removed successfully by graft-versus-leukemia reactions in myeloid malignancies, their efficiency is low in T cell leukemias. This may be partially because of the ability of malignant T cells to escape apoptosis. Our work shows that Eph family receptor EphB3 is consistently expressed by malignant T lymphocytes, most frequently in combination with EphB6, and that stimulation with their common ligands, ephrin-B1 and ephrin-B2, strongly suppresses Fas-induced apoptosis in these cells. This effect is associated with Akt activation and with the inhibition of the Fas receptor-initiated caspase proteolytic cascade. Akt proved to be crucial for the prosurvival response, because inhibition of Akt, but not of other molecules central to T cell biology, including Src kinases, MEK1 and MEK2, blocked the antiapoptotic effect. Overall, this demonstrates a new role for EphB receptors in the protection of malignant T cells from Fas-induced apoptosis through Akt engagement and prevention of caspase activation. Because Fas-triggered apoptosis is actively involved in the graft-versus-leukemia response and cytotoxic T cells express ephrin-Bs, our observations suggest that EphB receptors are likely to support immuno evasion of T cell malignancies and may represent promising targets for therapies, aiming to enhance immunoelimination of cancerous T cells. The Journal of Immunology, 2011, 187: 000–000.
The Eph family of receptor tyrosine kinases is represented by 16 receptors that are assigned to EphA (EphA1–10) and EphB (EphB1–6) groups based on their structural organization and ligand-binding preferences. In mammals, EphA receptors (EphA1–8, EphA10) promiscuously bind ephrin-A proteins (ephrin-A1–6), and EphB receptors (EphB1–4, EphB6) interact in a similar manner with three ephrin-Bs (ephrin-B1–3) (31). This rule, however, is not without exceptions, because EphA4 is capable of binding ephrin-Bs, and EphB2 can be activated by ephrin-A5 in addition to their conventional ephrin-A and ephrin-B ligands (32–36). In contrast, EphB6 interactions appear to be restricted to ephrin-B1 and ephrin-B2 (34, 37, 38). All ephrins are membrane-attached proteins; ephrin-As are GPI-anchored, whereas ephrin-Bs have transmembrane and cytoplasmic domains, and their membrane localization appears to be crucial for their ability to trigger Eph signaling (39). Therefore, cell–cell contact formation between Eph-and ephrin-expressing cells is typically required for Eph receptor activation (40). Ligand-activated Eph receptors initiate a complex array of signaling events and control multiple aspects of cell behavior both in developing embryos and in adult organisms (41). Eph and ephrin proteins also appear to control cellular responses in human malignancies (31, 42, 43). It has been reported that some Eph receptors and ephrin genes have their promoter regions methylated in leukemic cells, and that overexpressed EphB4 inhibits Akt activation, thus inducing apoptotic cell death in Raji B-ALL cells (44). Nevertheless, expression of some Ephs and ephrins, including EphA1, EphA2, EphA3, EphA7, EphA10, EphB6, ephrin-A4, ephrin-B1, and ephrin-B2, has been observed in ALL cell lines and chronic lymphocytic leukemia patient samples (45–51). Moreover, ephrin-A4, EphA3, and ephrin-B1 are actively involved in the regulation of cell attachment and invasive properties in chronic lymphocytic leukemia, B cell ALL, and T-ALL cells (47, 51–53). Complementing each other, these observations suggest that a disbalance in Eph receptor signaling caused by distorted expression patterns, rather than its complete blockage, is likely to contribute toward the malignant behavior of leukemic cells.

Activation of both Eph receptors and FasRs occurs in the area of cell–cell contact formation (40, 54), and cytotoxic CD8 T cells express not only Fas ligand, but also ephrin-B proteins on their membranes (23, 55–57), suggesting that Eph receptors and FasR are likely to be costimulated in the course of the graft-versus-leukemia response and raising a possibility that a combination of ephrin-B–interacting Eph receptors expressed in malignant T cells may interfere with FasR signaling and FasR-mediated apoptotic cell death.

We report in this article that the EphB3 receptor is expressed by all tested malignant T lymphocytes, where it is usually accompanied by another EphB receptor, EphB6, and show that stimulation with their common ephrin-B ligands strongly suppresses FasR-initiated apoptosis in these cells. This prosurvival response is associated with ephrin-B–induced activation of apoptosis suppressor, Akt. In agreement with previously reported Akt properties (58), ephrin-B stimulation also inhibits FasR-mediated activation of both caspase-8 and -3 proteases that are responsible for the propagation of the apoptotic signal (59). Moreover, inhibition of Akt, but not of the Ras-MAPK pathway or Src kinases, blocks the prosurvival action of EphBs, suggesting that these receptors are likely to rely on Akt signaling in their antiapoptotic responses in cancerous T lymphocytes.

Overall, our work demonstrates a new role and a new mechanism of action for EphB receptors in the suppression of FasR-mediated cell death. Our observations also indicate that EphB receptors may significantly contribute toward the immunoevasive properties of T cell leukemias and, therefore, may serve as promising targets for novel therapeutic approaches, aiming to enhance the efficiency of the graft-versus-leukemia response.

**Materials and Methods**

**Cell culture**

T-ALL cell lines H9 and E6.1 were purchased from ATCC (Manassas, VA). Molt-16 cells were kindly provided by Dr. John DeCoteau (Department of Pathology, College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK, Canada). DND-41, ALL-SIL, TALL-1, and KOPT-K1 cells were from Dr. Andrew Weng’s collection and were previously characterized (6). All cell lines were cultured in RPMI-1640 (HyClone, Logan, UT) supplemented with FBS and Pen-Strep (Invitrogen, Carlsbad, CA) at 37°C, 5% CO2. Primary T-ALL samples were obtained from two independent pediatric patients with T-ALL at the time of diagnostic marrow aspiration after informed consent and with approval from local Institutional Review Board (Alberta Children’s Hospital, Calgary, AB, Canada). Mononuclear cells from bone marrow aspirates were prepared on Percoll (Pharmacia, New York, NY) gradient as directed by the manufacturer. The diagnoses of T-ALL in these cases were confirmed by flow cytometry as a part of clinical diagnostic workup. T-ALL cells were cultured in the LGM-3 medium (Lonz, Walkersville, MD).

**Reagents**

The anti-Fas-activating Ab, 7C11, was purchased from Beckman-Coulter (Mississauga, ON, Canada). Resazurin stain, anti–phospho-Akt (Ser473), ephrin-B1–Fc, and ephrin-B2–Fc were from R&D Systems (Minneapolis, MN). Anti-human Fc was purchased from Pierce (Rockford, IL). Abs for caspase-3, EphB3, and Erk2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The Ab for caspase-8 was from Cell Signaling Technology (Danvers, MA). The Akt inhibitors Akti-1/2 and perifosine were purchased from Calbiochem (La Jolla, CA) and BioVision (Mountain View, CA), respectively. The MEK1/2 inhibitors U0126 and PD98059 were very kindly provided by Dr. Michael Sherman (Department of Biochemistry, Boston University School of Medicine, Boston, MA). SU6656, an Src family inhibitor, and anti-EphB6 were from Sigma-Aldrich (Oakville, ON, Canada). Random primers, Superscript II reverse transcriptase, and Elongase Enzyme Mix were obtained from Invitrogen.

**RT-PCR assay**

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Mississauga, ON, Canada) according to the manufacturer’s instructions. The first-strand cDNA was obtained and PCR was performed as previously described (51). The PCR primers for EphB2, EphB3, EphB4, EphB6, and β-actin were described previously (51, 60, 61) and synthesized by Sigma-Genosys (Oakville, ON, Canada). EphB1 (5′-CAAGACCTGTGGCAACCATCACC-3′), ephrin-B1 (5′-GGTACTGACTTATCTGGACC-3′), and 5′-CGCTGCCAGAAGGAGAGGAGC-3′. and 5′-CTGTGCCTGCGAGGCCACGACC-5′) primers were also synthesized by Sigma-Genosys. PCR products were resolved by agarose gel electrophoresis, stained with ethidium bromide, visualized in ultraviolet light, and quantitated in triplicates using the Carestream Molecular Imaging Software densitometry tool. Expression of the Eph receptor transcripts in each cell line is presented as a percentage relative to matching β-actin controls.

**Cell survival assays**

The activating anti-FasR Ab, 7C11, in combination with ephrin-B1–Fc, ephrin-B2–Fc, or human IgG (IgG; Sigma) was immobilized in 96-well plates overnight, at 4°C. IgG was used to maintain a constant protein concentration and to provide a specificity control for the Fc fragment of the ephrin fusion proteins. The wells were rinsed with serum-free medium and seeded with 6 × 10⁴ cells/well to achieve a single-cell layer. ALL-SIL cells were seeded at 3 × 10⁴ cells/well. Cells were incubated at 37°C and 5% CO2 in serum-free RPMI-1640. To monitor survival, we stained cells with resazurin, following the manufacturer’s instructions, and fluorescence was measured using a microplate reader (Excitation 530 nm, Emission 590 nm). Error bars represent SD based on the analysis of triplicates. In some experiments, H9 and Molt-16 cells were incubated in the...
presence of inhibitors of Akt (Akti-1/2 or perifosine), MEK1/2 (U0126 or PD98059), or Src kinases (SU6656), as previously described (62–65). Matching volumes of corresponding solvents were used as controls.

**Cell stimulations**

Ephrin-B2–Fc was precomplexed with goat anti-human Fc for 30 min on ice (3 μg/ml ephrin-B2–Fc with 4.5 μg/ml anti-human Fc), and cells were stimulated at 37°C. Control cells were incubated at 37°C with precomplexed hIgG. In some experiments, ephrin-B2–Fc or hIgG was coimmobilized with activating anti-FasR Ab onto 24-well plates overnight at 4°C. Wells were washed with serum-free RPMI-1640; cells were loaded and incubated at 37°C for the indicated times. After stimulation, cells were lysed with 0.2% NP-40 lysis buffer and lysates were cleared by centrifugation. Alternatively, cells were lysed in boiling 1× SDS sample buffer to produce total lysates. Cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose (Amer sham, Arlington Heights, IL) for Western blotting.

**Flow cytometry**

To monitor FasR-mediated apoptosis, the TUNEL-based APO-BRDU Apoptosis Detection Kit (BD Biosciences, Mississauga, ON, Canada) was used according to the supplier’s directions. The activating anti-FasR Ab in combination with ephrin-B2–Fc or hIgG, or IgG alone, were coimmobilized onto 24-well plates overnight at 4°C. Plates were rinsed with serum-free RPMI-1640; cells were loaded and incubated at 37°C for the indicated times. After stimulation, cells were lysed with 0.2% NP-40 lysis buffer and lysates were cleared by centrifugation. Alternatively, cells were lysed in boiling 1× SDS sample buffer to produce total lysates. Cell lysates were resolved by SDS-PAGE and transferred to nitrocellulose (Amersham, Arlington Heights, IL) for Western blotting.

To monitor the cell surface expression of the FasR, we treated cells with precomplexed ephrin-B2–Fc or hIgG for 5 or 18 h. Stimulated cells were stained with FITC-conjugated anti-FasR (BD Biosciences) or matching isotype control (BD Biosciences). Isotype control staining was confined to the first log. The presence of the FasR was determined by flow cytometry as described above.

**Peptide-based kinome analysis**

H9 cells (3 × 10^7) were stimulated with 3 μg/ml precomplexed ephrin-B2 or hIgG for 4 min or 2 h. Molt-16 cells (5.6 × 10^7) were stimulated in the same manner as H9 for 10 min. After stimulation, cells were pelleted and snap-frozen in liquid nitrogen. Cell pellets were lysed with 80 μl lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM Na3VO4, 1 mM NaF, 1 μg/ml leupeptin, 1 g/ml aprotinin, 1 mM PMFS), incubated on ice for 10 min, and then spun in a microcentrifuge at maximum speed for 10 min at 4°C. An 80-μl aliquot of this supernatant was mixed with 10 μl of the activation mix (50% glycerol, 500 μM ATP, 60 mM MgCl2, 0.05% v/v Brij-35, and 0.25 mg/ml BSA) and incubated on the chip for 2 h at 37°C in a humidity chamber. After incubation, slides were washed once in PBS-Triton, then submerged in stain (PRO-Q Diamond Phosphoprotein Stain; Invitrogen) with agitation for 1 h. Arrays were then washed in tubes containing destain (20% acetonitrile [EMD Biosciences, VWR distributor, Mississauga, ON, Canada] and 50 mM sodium acetate [Sigma] at pH 4.0) for 10 min three times with the addition of new destain each time. A final wash was done with distilled water. Arrays were dried and read using a GENEPIX professional 4200A microarray scanner (MDS Analytical Technologies, Toronto, ON, Canada) at 532–560 nm with a 580-nm filter to detect dye fluorescence. Images were collected using the GENEPIX 6.0 software (MDS Analytical Technologies). Signal from images was then captured and statistical analysis performed using a variation of previously published microarray analysis techniques (66, 67).

**Human phospho-kinase array**

E6.1 cells were treated for 15 min with precomplexed ephrin-B2–Fc (3 μg/ml) or hIgG as a control and analyzed using the Human Phospho-Kinase Array kit (R&D Systems) according to the manufacturer’s instructions. In brief, treated cells were lysed and the lysates were cleared by centrifugation. Cleared lysates were loaded onto the provided

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**FIGURE 1.** The EphB3 receptor is consistently expressed by malignant T cells. A, Expression of ephrin-B–interacting Eph receptors was assessed by reverse transcription-PCR in the indicated human malignant T cell lines and in two independent T-ALL patient samples (patients 1, 2). Water controls were used to ensure the absence of contamination, and β-actin primers were included as a consistency control. PCR products were resolved by agarose gel electrophoresis, stained with ethidium bromide, and visualized in ultraviolet light; loading of β-actin controls was reduced 2-fold to optimize visualization. Amount of cDNA in each sample was quantitated by densitometry in triplicates, normalized on a matching actin control, and values are presented in arbitrary units (AU). B and C, Expression of the EphB3 and EphB6 receptors in malignant T cell lines was analyzed by Western blotting with anti-EphB3 and anti-EphB6 Abs. Erk2 presence was monitored as a loading control.
membranes precoated with capture Abs, and the presence of bound phospho-proteins was determined by Western blotting with a mixture of detection Abs. The signal intensities for Akt and GSK3 phosphorylation were determined in triplicates by densitometry and normalized on the provided positive control. The status of protein phosphorylation in ephrin-B2–treated cells is reported relative to the IgG-treated control.

**Results**

**EphB receptors are expressed by malignant T cells and protect them from FasR-mediated cell death**

Rapidly accumulating observations suggest that both ephrin membrane proteins and EphA receptors play an important role in

![Graph](image)

**FIGURE 2.** EphB receptors suppress FasR-mediated cell death. A and B, The anti-FasR activating Ab, 7C11, (αFasR) and ephrin-B2–Fc (eB2) were immobilized overnight at 4˚C onto 96-well plates at the indicated concentrations. hlgG was immobilized, where required, to keep protein concentration constant and as a specificity control for the Fc portion of an eB2 fusion protein. H9 (A) and Molt-16 (B) cells were loaded into the precoated plates (6 × 10^4 cells/well) and incubated for 18 (H9) or 24 h (Molt-16) at 37˚C. Cells were stained with resazurin for 2 h at 37˚C, and survival was measured using a microplate reader. Data represent the analysis of triplicate readings and are shown as a percentage relative to a respective hlgG control.

C, The activating anti-FasR Ab was pre-immobilized on 24-well plates in the presence of either eB2 or hlgG. H9 and Molt-16 cells or cells from T-ALL patient 1 sample were loaded into precoated plates and incubated for 6, 7, or 24 h, respectively. Cells were fixed in 1% formaldehyde, permeabilized with 70% ethanol, and TUNEL stained. Apoptotic response was measured by flow cytometry. Cell debris was excluded by gating. Cells treated with hlgG alone were used as a reference. D and E, Indicated cells were loaded into precoated 96-well plates as in A, incubated at 37˚C, stained with resazurin, and assessed for survival. E6.1 cells were incubated for 32 h, whereas KOPT-K1 were incubated for 72 h. ALL-SIL were loaded at 3 × 10^4 cells/well and incubated for 72 h at 37˚C. F, DND-41 cells were loaded onto the anti-FasR Ab preimmobilized in combination with ephrin-B2 or hlgG. Cells were incubated for 21 h and apoptotic response was measured as in C. Statistical analyses: Student t test, *p < 0.05, for indicated points and their matching anti-FasR/hlgG controls. All analyses represent one of at least two independent experiments.
lymphoid malignancies, predominantly through the regulation of attachment and invasive properties of leukemic cells (47, 51–53). Meanwhile, little is known about the potential involvement of the ephrin-B–interacting Ephs, EphB1–4, EphB6, and EphA4 (34), collectively referred to as EphrinB receptors.

To monitor the expression of specific EphrinB receptors, we used the RT-PCR approach. Aliquots of the RT-PCR products were resolved by electrophoresis; the amount of cDNA in each sample was determined by densitometry and normalized on a matching β-actin control. The results of this analysis have demonstrated that all human T-ALL cell lines assessed and also the malignant lymphoblastic T cells, H9, express the EphB3 and EphB6 transcripts, whereas expression of other EphBs varies, and EphA4 is missing in six cell lines from seven tested (Fig. 1A). Pediatric T-ALL represents only approximately 10% of childhood ALLs (68), and a limited number of patient samples were available for our analysis. Nevertheless, EphB3 expression has been observed in both examined independent patient samples (patients 1, 2) and one of them lacked EphA4. The EphB6 transcript was present in one of two samples (Fig. 1A).

Because EphB3 and EphB6 were present in the majority of analyzed malignant T cells, their expression was also assessed on a protein level by Western blotting. Although, unfortunately, amounts of material in T-ALL patient samples were not sufficient for Western blot analysis, both proteins were easily detected with specific Abs in all T cell lines, thus supporting our RT-PCR data for Western blot analysis, both proteins were easily detected with amounts of material in T-ALL patient samples were not sufficient on a protein level by Western blotting. Although, unfortunately, analyzed malignant T cells, their expression was also assessed.

The ability of ligand-activated EphB receptors to inhibit FasR-induced apoptosis was further verified by the TUNEL assay, most probably because of the higher sensitivity of this approach (Fig. 2F).

Additional experiments with another EphB3 and EphB6 ligand, ephrin-B1 (34, 37), have, once again, shown the ability of EphB proteins to attenuate FasR cytotoxicity (Fig. 3A–C), indicating that ephrin-B1– and ephrin-B2–activated EphB receptors trigger the antia apoptotic action and may be potentially responsible for the immuno evasive behavior of malignant T lymphocytes.

**EphB receptors initiate Akt-dependent signaling in malignant T cells**

To determine whether EphB receptors may suppress FasR responses by downregulating its cell surface expression, we stained ephrin-B2–stimulated H9 and Molt-16 with anti-FasR Ab and monitored FasR presence by flow cytometry. Although untreated cells appeared to express FasR at a very high level, no effect of ephrin-B2 on its expression could be observed (Fig. 4A), sug-

**FIGURE 3.** FasR-induced apoptosis is inhibited by ephrin-B1 stimulation. A–C, H9 (A), Molt-16 (B), or ALL-SIL and KOPT-K1 (C) were loaded into 96-well plates precoated with the indicated concentrations of the anti-FasR–activating Ab and ephrin-B1–Fc. hIgG was used, where required, to keep protein concentration constant. Cells were incubated at 37˚C, stained with resazurin, and cell survival was assessed as in Fig. 2A. Data are shown as a percentage relative to the hIgG control. Statistical analyses: Student t test, *p < 0.05, for indicated points and their matching anti-FasR/hIgG controls. All analyses represent one of at least two independent experiments for each cell line.
gesting that EphBs were likely to affect FasR action at the level of cytoplasmic signaling.

Eph receptors are known to control cell behavior through the initiation of an intricate combination of signaling responses in both normal and malignant cells (31, 41, 69). To assess the overall effect of EphB receptors on the phosphorylation-dependent signaling, we stimulated H9 cells with ephrin-B2 and monitored the resulting responses using a human peptide array for kinome analysis recently developed in analogy to the previously described bovine-specific array (66, 67). The obtained data confirmed the ability of EphB receptors to initiate antiapoptotic signaling in malignant T cells; relevant array-based observations are summarized in Table I. Interestingly, although ephrin-B2 stimulation produces no effect on a central prosurvival event, PI3K-PDK1–dependent Akt activation after a short, 4-min stimulation in H9 cells, it induces a reduction in PTEN phosphorylation at the Tyr240 and Tyr308 residues, which is supposed to inactivate this inhibitor of the PI3K-PDK1-Akt signaling (Table I). Complementing PTEN inactivation, Akt phosphorylation on activating Thr308, which is mediated by the PI3K pathway, could be observed in these cells.

**FIGURE 4.** Ephrin-B2–stimulated EphB receptors trigger activating Akt phosphorylation. A, H9 and Molt-16 cells were treated with precomplexed ephrin-B2–Fc or hlgG for 5 or 18 h, stained with FITC-conjugated anti-FasR, and analyzed by flow cytometry. Staining with matching isotype control was confined to the first log (not shown). B, H9 cells were treated with 3 μg/ml soluble, precomplexed ephrin-B2 or hlgG for the indicated time periods. Cell lysates were prepared in NP-40 lysis buffer and analyzed for Akt phosphorylation by Western blotting with anti–phospho-Akt Ser473. Sample loading was monitored by blotting with anti-Erk2. C, H9 cells were stimulated as in B for the indicated times. Cells were lysed in boiling 1× SDS sample buffer and analyzed for Akt phosphorylation as in B. D, The anti-FasR–activating Ab (300 ng/ml), ephrin-B2 (10 μg/ml), or hlgG (10 μg/ml) was immobilized on 24-well plates. H9 cells, 4 × 10^5/well, were loaded, incubated at 37˚C for the indicated time periods, and lysed in boiling 1× SDS sample buffer. The lysates were resolved by SDS-PAGE and analyzed for Akt phosphorylation by Western blotting. Western blot images were quantitated by densitometry in triplicates. The results of phospho-Akt quantitations have been normalized on the Erk2 loading controls and are shown in arbitrary units (AU) relative to matching IgG points. Student t test, *p < 0.05. E, Lysates were prepared from ephrin-B2–stimulated H9 cells in NP-40 lysis buffer, and phosphorylation status of Src family members was assessed by Western blotting with anti–phospho-Src (Tyr418).
after a prolonged, 2-h treatment (Table I). Moreover, the results of the kinome arrays consistently indicate that EphB receptors support Akt activation through phosphorylation on the Ser473 residue, which is known to be performed by the mTOR kinase acting in the context of the mTORC2 complex (70). EphB receptors appeared to trigger predominantly the prosurvival responses, because no significant effect of ephrin-B2 stimulation on other signaling events central to the T cell lineage, including JNK2-, p38-, Lck- and Fyn-related phosphorylation (71–74), could be observed (Table I). In agreement, an independent assessment of ephrin-B2–stimulated Molt-16 cells in this array showed that EphBs trigger a 2.0-fold increase in Ser473 phosphorylation in malignant T cells both in the absence and presence of active FasR signaling (Fig. 4B–D). This phosphorylation could be easily observed after only 3 min of stimulation and was also detectable after 2 h of ephrin-B2 treatment (Fig. 4B, 4C). In agreement with the results of the kinome array, no effect of EphB receptors on Src family members could be detected using anti-

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Bold text indicates values belonging to a common pathway.

<sup>a</sup>Common name for substrate protein with a peptide for a phosphorylation site on the array.

<sup>b</sup>Position and name of target amino acid on the substrate protein.

<sup>c</sup>Fold changes in response to stimulation with ephrin-B2 were calculated by comparison with hIgG control.

<sup>d</sup>Curated PhosphoSite database provides names of molecules that alter phosphorylation status at the indicated residues. Where available, this information from PhosphoSite is presented here.

<sup>e</sup>PhosphoSite database maintains information on the effects of phosphorylation on a substrate protein.

<sup>f</sup>Reactome (http://www.reactome.org) database pathway: DOI: 10.3180/REACT_964.1.

To further confirm the ability of EphB receptors to support activating Akt phosphorylation, we stimulated H9 cells with ephrin-B2 for both short and long time periods, and followed Akt phosphorylation by Western blotting with anti–phospho-Akt Abs, recognizing specifically the activating phosphorylation at Thr308 on Ser473. Surprisingly, despite the kinase array data showing an increase in Thr308 phosphorylation in response to the prolonged ephrin-B2 stimulation, we could not detect any similar effect by Western blotting (not shown), probably because of insufficient Ab sensitivity. However, our Western blotting with the anti–phospho-Akt(Ser473) Ab has demonstrated that EphB receptors are, indeed, responsible for the stimulation-dependent Akt(Ser473) phosphorylation by Western blotting with anti–phospho-Akt Abs, recognizing specifically the activating phosphorylation at Thr308 on Ser473. Surprisingly, despite the kinase array data showing an increase in Thr308 phosphorylation in response to the prolonged ephrin-B2 stimulation, we could not detect any similar effect by Western blotting (not shown), probably because of insufficient Ab sensitivity. However, our Western blotting with the anti–phospho-Akt(Ser473) Ab has demonstrated that EphB receptors are, indeed, responsible for the stimulation-dependent Akt(Ser473) phosphorylation in malignant T cells both in the absence and presence of active FasR signaling (Fig. 4B–D). This phosphorylation could be easily observed after only 3 min of stimulation and was also detectable after 2 h of ephrin-B2 treatment (Fig. 4B, 4C). In agreement with the results of the kinome array, no effect of EphB receptors on Src family members could be detected using anti-
phospho-Src, which specifically recognizes Src family kinases phosphorylated on the activating tyrosine (Fig. 4E). This, once again, suggested that the prosurvival action of EphB receptors may be mediated specifically by Akt.

FasR-triggered death signaling relies on the activation of the caspase proteolytic cascade, typically initiated by the cleavage of caspase-8 and leading to the processing of effector caspases, including caspase-3 (59). Because Akt has been shown to prevent FasR-mediated activation of caspase-8 and -3 in T cells (58), we monitored the effect of ephrin-B2 costimulation on the status of these proteins. Our analysis has shown that, in line with the ability of ephrin-B2–stimulated EphBs to activate Akt and inhibit FasR-initiated apoptotic response, costimulation with ephrin-B2 at least partially suppresses the proteolytic activation of both caspases (Fig. 5). This matches very well the partial protective effect of EphB receptors observed in the TUNEL assay and appears to be efficient in supporting cell survival (Fig. 2), suggesting overall, that by operating through Akt, EphB receptors may actively interfere with FasR proapoptotic signaling.

**FIGURE 5.** EphB receptors inhibit caspase activation in malignant T lymphocytes. A–D, The anti-FasR–activating Ab was immobilized onto 24-well plates in the presence of ephrin-B2–Fc or hlgG. H9 cells, $4 \times 10^5$/well, or Molt-16, $7 \times 10^5$ cells/well, were loaded, incubated at 37°C for the indicated time periods, and lysed in NP-40 lysis buffer. Cell lysates were resolved by SDS-PAGE, and caspase activation was examined by Western blotting.

**EphB-initiated Akt activation is required for the prosurvival function**

Akt activity is known to be crucial for cell survival and, in particular, for suppressing FasR-induced apoptosis (58). Because our experiments have shown that EphBs block the apoptotic cell death and prevent FasR signaling, while also promoting Akt activating phosphorylation on Ser$^{73}$, it appeared that EphB receptors may rely on Akt in their prosurvival signaling.

To examine the requirement for Akt for the prosurvival action of EphB receptors, we treated H9 and Molt-16 cells with the Akt inhibitors, Akti-1/2 and perifosine, or with inhibitors of signaling molecules known to play a crucial role in T cell biology, including MEK and Src family kinases. Although the MEK1/2 inhibitors, U0126 and PD98059, and the Src family inhibitor, SU6656, efficiently suppressed their related phosphorylation events (Fig. 6A, 6C, 6E, 6G), they failed to produce any consistent significant effect on the EphB receptor-induced antiapoptotic response (Figs. 6B, 6D, 6F, 7A). In contrast, treatments with both Akt inhibitors...
FIGURE 6. A. H9 cells were incubated at 37˚C in RPMI-1640 supplied with 15% serum in the presence of the MEK1/2 inhibitor, U0126 (10 μM), or an equal volume of DMSO, for the indicated times. Cells were lysed and Erk phosphorylation was analyzed by Western blotting with anti–phospho-Erk1/2. B. Activating anti-FasR and ephrin-B2–Fc or hlgG were immobilized onto 96-well plates overnight at 4˚C. H9 cells were loaded into the plates in the presence of U0126 (10 μM) or of an equal volume of DMSO and incubated at 37˚C for 24 h. Cells were stained with resazurin dye for 2 h at 37˚C, and survival was measured using a microplate reader. Data represent the analysis of triplicate readings and are shown as a percentage relative to anti-FasR/hlgG control. C. H9 cells were pretreated with the MEK1/2 inhibitor, PD98059 (25 μM), or DMSO for 30 min and incubated in RPMI-1640 supplied with 15% serum in the presence of the inhibitor or DMSO for the designated time periods. Erk phosphorylation was assessed as in A. D. The effect of 25 μM PD98059 on the survival of H9 was analyzed as in B. E and F. Molt-16 cells were treated with 10 μM U0126, and the effects of the inhibitor on Erk phosphorylation (E) and cell survival (F) were assessed as in A and B. G. H9 cells were incubated with the Src family inhibitor, SU6656 (40 μM), or matching volume of DMSO for 2 h in serum-free RPMI-1640. Molt-16 cells were incubated with SU6656 (10 μM) or DMSO for 30 min in serum-free RPMI-1640 and supplied with 15% serum for the indicated time periods. H9 and Molt-16 cells were lysed, and the phosphorylation of Src kinases on the activating tyrosine residue, Tyr418, was determined by Western blotting with anti–phospho-Src. Statistical analyses: Student t test, *p < 0.05, for indicated points and their corresponding solvent controls. All analyses represent one of at least two independent experiments for each cell line.
strongly suppressed the ability of EphBs to rescue H9 and Molt-16 cells (Fig. 7B, 7C), thus supporting the central role for Akt signaling in the antiapoptotic functions of EphB receptors in T cell malignancies.

Discussion

FasR-initiated cell death plays an important role in the elimination of residual malignant T cells by cytotoxic T lymphocytes and NK cells in the course of the graft-versus-leukemia/lymphoma response after bone marrow transplantation (19, 21–23). This response is very effective in some malignancies, including myeloid leukemia; however, it has proved to be inefficient in T-ALL patients (2, 17, 21). Our work shows that malignant T cells may be at least partially protected from FasR-triggered apoptosis by the action of ephrin-B–activated Eph receptors. These receptors are typically represented by EphB receptors (EphB1–4, EphB6) and EphA4 (34). Our analysis suggests that malignant T cells consistently express EphB3 and most often EphB6, whereas frequently missing EphA4, which, according to previously published data and our observations (Supplemental Table I), is expressed in normal human T lymphocytes, in addition to EphB3 and EphB6 (46, 76, 77). Interestingly, EphB6 appears not to be strictly required for the antiapoptotic effect, because stimulation with ephrin-B2 rescues patient 1 T-ALL cells from FasR-induced death despite its absence, suggesting that EphB3 alone may be sufficient, especially when overexpressed (Figs. 1A, 2C). Nevertheless, our observations showing that EphB6 frequently accompanies the EphB3 receptor in T-ALL cells, and that stimulation with EphB6 and EphB3 common ligands, ephrin-B1 and ephrin-B2 (34), suppresses the apoptotic response, indirectly suggest that it may act in cooperation with EphB3 to enhance the antiapoptotic effect of ephrin-B stimulation. This model matches well the unusual properties of the EphB6 receptor, which is an atypical member of the Eph family, carrying alterations in the conserved motifs of its kinase domain and lacking tyrosine kinase activity (78, 79). Because of its kinase-deficient nature, EphB6 relies on the assistance provided by the catalytically active members of the EphB group and has been shown to interact with EphB1 and EphB4 (37, 80). Therefore, it is possible that EphB6 may form heteroreceptor complexes with EphB3 and support its antiapoptotic signaling in malignant T cells. It is also possible that the EphB3 and EphB6 receptors may have prosurvival functions in normal T cells, whereas EphA4, which also interacts with ephrin-B2 (34), may interfere with their antiapoptotic action, and EphA4 absence may provide a survival advantage to malignant lymphocytes. Interestingly, while this article was in preparation, another group reported a ligand-de-
pendent inhibitory effect of a different member of the EphA group, the EphA2 receptor, on prosurvival signaling in cancer cells (81). This presents a possibility that EphA4 may exhibit a similar behavior in T lymphocytes, thus moderating the efficiency of EphB receptor prosurvival response. These intriguing models of Eph receptor cross talks are currently under active investigation.

The antiapoptotic action of EphB receptors is accompanied by Akt activation in cancerous T cells through its phosphorylation on Ser473. Akt has been previously reported to interfere with the initiation of FasR signaling in T lymphocytes (58), and in agreement with the ability of ligand-activated EphB receptors to trigger Akt activation, ephrin-B2 stimulation also effectively suppresses FasR-dependent induction of the caspase cascade that plays a central role in FasR-mediated apoptotic response (59).

The initiation of FasR signaling can be also suppressed by the FLIP protein, which structurally resembles caspase-8, but lacks proteolytic activity (82). Therefore, we examined whether EphB receptors may block apoptotic cell death through the induction of FLIP expression; however, we failed to observe any effect of ephrin-B stimulation on the level of FLIP (not shown), which further supported a model where EphB receptors predominantly rely on Akt for the prosurvival action. In line with this, the general importance of Akt for the ability of EphBs to inhibit FasR-induced apoptosis was further confirmed by our observations that Akt, but not MEK or Src family inhibitors, block their prosurvival effect in malignant T cells.

Overall, our observations indicate that EphB receptors may act in malignant T lymphocytes, in particular, in pediatric T-ALL cells, to support their survival through Akt activation and, eventually, inhibition of FasR-initiated signaling and apoptotic cell death. Because FasR-mediated apoptosis plays a crucial role in the immunoeediting of T-ALL cells in the course of the graft-versus-leukemia response, our work also suggests that inhibitors of EphB receptors or EphB receptor-blocking Abs may represent promising new therapeutic approaches, especially when combined with allogeneic bone marrow transplantation. As it appears to be the case with other therapies targeting specific cell surface receptors or Ephs, the elimination of T-ALL cells in the course of the graft-versus-leukemia effect.

References

The authors have no financial conflicts of interest.

Disclosures

The Journal of Immunology 11


