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Essential roles for the Tec family kinases Tec and Btk in M-CSF receptor signaling pathways that regulate macrophage survival[1]

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Abstract

Tec family kinases have important roles in lymphocytes, however little is known about their function in monocytes/macrophages. Here we report that Tec family kinases are essential for macrophage colony stimulating factor (M-CSF)-induced signaling pathways that regulate macrophage survival. Compared to wildtype bone marrow-derived macrophages (BMM) cultures, *Tec−/−Btk−/−* BMM cultures displayed increased cell death that correlated with a severe drop in macrophage numbers. In addition, macrophages deficient in either Tec or Btk showed expression and activation of caspase-11. Elucidation of M-CSF receptor (M-CSFR) signaling pathways revealed that the total tyrosine phosphorylation pattern upon M-CSF stimulation was altered in *Tec−/−Btk−/−* macrophages despite normal expression and phosphorylation of the M-CSFR. Further, Tec and Btk are required for proper expression of GM-CSF receptor alpha (GM-CSFRα) chain in macrophages but not dendritic cells, implicating Tec family kinases in the lineage-specific regulation of GM-CSFRα expression. Taken together, our study shows that Tec and Btk regulate M-CSFR signaling-induced macrophage survival and provides a novel link between Tec family kinases and the regulation of caspase-11 and GM-CSFRα expression.

Keywords

Monocytes/macrophages; Protein kinases/Phosphatases; cell differentiation; transgenic/knockout

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Introduction

Macrophages are large phagocytic mononuclear cells that play important roles in innate and adaptive immunity. Their progenitors, the monocytes enter the blood stream from the bone marrow (BM) and migrate to tissues where they mature into resident tissue macrophages (1). The differentiation, proliferation and survival of macrophages are regulated by the M-CSF. In fact, M-CSF receptor (M-CSFR)-deficient mice or mice with an inactivating mutation of M-CSF have pleiotropic phenotypes including decreased macrophage numbers *in vivo* (2, 3). In mice, impaired M-CSF signaling has also been implicated in the pathogenesis of several disorders (for a detailed review see (4) and references therein). Thus, a better understanding of M-CSFR signaling may also be of medical relevance.

Members of the Tec kinase family (Bmx, Btk, Itk, Rlk and Tec) constitute the second largest family of non-receptor tyrosine kinases and are preferentially expressed in the haematopoietic system. A large number of studies have shown important roles for these kinases in the lymphoid system. Furthermore, mice with combinatorial deletions of Tec family kinases revealed both unique and redundant functions in B cells (Tec, Btk) and T cells (Rlk, Itk). Although the Tec family kinase members Tec, Btk and Bmx are expressed in monocytes/macrophages (5-7), little is known about their function in this lineage. Several studies implicated Tec family kinases in the LPS-induced signaling in macrophages leading to the induction of TNFα production. Btk-defective X-linked immunodeficient (*Xid)* macrophages have impaired secretion of the pro-inflammatory cytokines TNFα and IL-1β after stimulation with LPS (8), and are also incapable of producing efficient bursts of reactive oxygen intermediates (9). In line with this, *xid* macrophages show impaired p65 phosphorylation and transactivation upon LPS stimulation, while IkBα-degradation is normal (10). Meanwhile, another study could not find any differences in TNFα expression between control and *xid* macrophages after LPS stimulation (5), which may reflect differences in the genetic backgrounds or different macrophage populations used in these studies. However, the importance of Tec family kinases for monocyte function has been confirmed through the analysis of Btk-deficient human monocytes. Blood monocytes isolated from X-linked agammaglobulinemia (XLA) patients that lack a functional *Btk* gene have impaired phagocytic functions and altered chemotactic responses (11) and are impaired in the production of TNFα and IL-1β upon stimulation of toll-like receptor (TLR) 2 or 4 (12), although another study reports that Btk is not essential for LPS/TLR4 signaling (13). Overexpression of Btk in wildtype human monocytes leads to the stabilization of TNFα mRNA and therefore to an increase in TNFα production (5, 12). Interestingly, incubation of XLA monocytes with M-CSF leads to an increase in the expression of Tec and restores their ability to produce TNFα upon LPS stimulation (5). This suggests a compensatory role for Tec, similar to the situation observed in murine B cells (14), which may also explain why XLA patients show normal innate immune responses (5).

In this study, we aimed to further dissect the role of Tec family kinases in monocytes/macrophages. We employed a genetic approach to study macrophages lacking various members of the Tec kinase family and generated combinatorial Tec family kinase knockout mice. We could show that Tec and Btk regulate survival of BMM by controlling M-CSFR signaling. A severe drop in cell numbers in *Tec−/−Btk−/−* macrophage cultures was observed, and this correlated with increased cell death of macrophages. Despite normal expression and M-CSF-induced autophosphorylation of the M-CSFR, M-CSF stimulation of *Tec−/−Btk−/−* macrophages resulted in an altered tyrosine phosphorylation pattern. Since Btk was activated upon M-CSF stimulation of primary BMM, our study thus shows that Tec family kinases play an important role in M-CSFR signaling pathways that lead to macrophage survival. Interestingly, *Tec−/−* or *Btk−/−* macrophages showed constitutive expression of caspase-11, an inducible member of the caspase family (15). Finally, we found

that Tec and Btk are required for proper expression of GM-CSF receptor alpha chain (GM-CSFRα) in macrophages but not dendritic cells, therefore implicating Tec kinases also in the lineage-specific regulation of GM-CSFRα expression.

Material and Methods

Mice

Tec-deficient mice (14), Btk-deficient mice (16) (purchased from the Jackson Laboratory) and Bmx-deficient mice (17) were intercrossed and maintained in the animal facility of the Medical University of Vienna. The mice used in this study were of mixed 129/Sv × C57BL/6 background. C57BL/6 Ly 5.1 mice were obtained from the European Mouse Mutant Archive (EMMA). All animal experiments were performed according to protocols approved by the Federal Austrian Ministry for Education, Science and Art.

Flow cytometry and antibodies

The spleen was removed from euthanized mice and placed into 60-mm tissue culture dishes containing staining buffer (PBS, 2% FCS, 0.1% sodium azide). Peritoneal cells were obtained by lavage of the peritoneum with 10 ml PBS. Single-cell suspensions were made by passing the tissue through a 70-μm nylon cell strainer. BM cells were harvested from reconstituted mice by flushing femur and tibiae with PBS containing 2% FCS. After hypotonic lysis of red blood cells (RBCs) with ACK lysis buffer (0,15 M NH_4Cl, 1,0 mM $KHCO_3$, 0.1mM Na_2EDTA, pH 7,2), 1-5×10^5 cells were incubated on ice for 5 min with Fc-block (BD Pharmingen) and subsequently stained with respective antibodies for 30 min on ice in staining buffer. Afterwards, the cells were washed once with staining buffer and analysed. The following monoclonal antibodies were used: FITC-anti-mCD11b, PE-anti-Gr1, APC-anti-B220 from Caltag. Flow cytometric analysis was performed on FACSCalibur (BD Biosciences) and data were analyzed with CellQuest Pro software.

Generation of BM-derived macrophages (BMM)

BMM were generated as described (18). Briefly, after RBC lysis, 10^7 BM cells were seeded onto 10 cm bacterial dishes in 10 ml of Dulbecco's modified Eagle medium (DMEM; Sigma) supplemented with 10% FCS (Gibco), 100 U/ml penicillin, 10 μg/ml streptomycin, 2 mM L-Glutamine, 10 mM HEPES (Sigma) and 50 μM β-mercaptoethanol (Invitrogen) in the presence of 20% L929-cell conditioned medium (LCM). The culture medium was changed on day 3 and 6, unless indicated otherwise. Cells were analyzed by flow cytometry and microscopy (Nikon Eclipse TS100). LCM was produced as described (18). For determining the effects of M-CSF and GM-CSF, and of wildtype and *Tec−/−Btk−/−* supernatant exchanges on macrophage numbers, culture medium changes were done on day 2 and 5. For the M-CSF and GM-CSF rescue experiments, BMM were removed on day 6 from the plate with citric saline (0,135 M potassium chloride and 15 mM sodium citrate) and reseeded in 6-well plates at 0.8 ×10^6 cells per well. Medium (total of 2 ml) containing M-CSF (10 and 30 ng/ml) or GM-CSF (500 and 1500 U/ml) or LCM was exchanged daily until the end of the culture. For the supernatant exchange experiments, 10^7 wildtype and *Tec−/−Btk−/−* cells were reseeded onto 10 cm dishes on day 6, and the corresponding supernatants were added daily to the culture. For the M-CSF titration experiments, cells were reseeded in 6-well plates at 0.8 ×10^6 cells per well on day 5 and different concentrations of M-CSF (15, 30, 60 and 90 ng/ml) were added.

Generation of BM-derived dendritic cells (BMDC)

BM cells were isolated and RBC were lysed as described above for the generation of BMM. To generate BMDCs, 2 × 10^6 BM cells were cultured on 10 cm dishes in RPMI medium

with 10% FCS, 100 U/ml penicillin, 10 μg/ml streptomycin, 2 mM L-Glutamine, 10 mM HEPES (Sigma), 50 μM β-mercaptoethanol (Invitrogen) and 700 U/ml recombinant murine GM-CSF (Peprotech) for 10 days (19).

Reverse transcription (RT)-PCR

Total RNA from the various cell types was isolated with Trizol reagent (Sigma), treated with DNase I (Boehringer Mannheim) and converted into cDNA by reverse transcription with oligo(dT)- and random primer according to the manufacturer's protocol (SuperScript II first-strand synthesis for RT-PCR, Invitrogen). The following primers were used for expression anaylsis: Tec-F: 5′-TAACCATGGTGACTCGTGGCCA; Tec-R: 5′-GGTATACATGGCTGGCACTCA: Btk-F: 5′-GAGTAACATTCTAGATGTGATGG; Btk-R: 5′-CAGTCTGTTAGGAGTCTTGAA; Bmx-F: 5′-GCAGCCCTATGACTTATATGAT; Bmx-R: 5′-CAGATAAACAGCACATAGACC; Hprt-F: 5′-GATACAGGCCAGACTTTGGTTG; Hprt-R: 5′-GGTAGGCTGGCCTATAGGCT; Csf2Ra-F: 5′-CCCCCACGGAGGTCACAAGGTCAA; Csf2Ra-R: 3′-CAGGGCAACAGGGGTCCAGTCACA.

BrdU incorporation

BM cells were differentiated into macrophages as described above. On day 5, cells were reseeded at 0.8×10^6 cells per well of a 6 well plate. At day 6, BMM cultures were incubated with 0.1 mM BrdU (Sigma-Aldrich) for a 1.5 hour period. Cells were removed from the plate with citric saline as described above, resuspended in 500 ml 0.15 M NaCl. 95% EtOH (−20°C) was added dropwise. After 30 minutes on ice, the cells were washed with PBS and resuspended in 1 ml of 1% paraformaldehyde/0.01% Tween 20 in PBS and incubated at 4°C overnight. The cells were incubated in 1 ml DNase I solution (50 Kunitz units / ml DNase I, 0.15 M NaCl, 4.2 mM $MgCl_2$, 10 mM HCl; Sigma) at 37°C for 30 minutes. The samples were washed with PBS, stained with FITC anti-BrdU or isotype control antibody (BD PharMingen) and analysed by FACScan (BD Biosciences).

Propidium iodide stainings – cell death analysis

Macrophages were harvested with citric saline as described above and resuspended in PBS. Propidium iodide (4 μg/ml in PBS) was added and the percentage PI-positive cells was determined by flow cytometry (FACSCalibur, BD Biosciences).

Bone marrow competition

BM cells from wildtype and *Tec−/−Btk−/−Bmx−/−* as well as from Ly 5.1-positive wildtype mice were isolated and RBC were lysed with ACK buffer. The cells were washed 3 times with PBS and counted. Ly 5.1 BM cells were mixed in the ratio of 1:1 with either wildtype BM or knockout BM. Mixed BM cells (1×10^6) were injected into the tail vein of lethally-irradiated Ly 5.1 mice (2 × 3500 milli-gray, Hille TH-150). Mice were treated with 25 μg/ml neomycin (Invitrogen) and 25.000 U/ml polymyxin B sulfate (Sigma) in the acidified drinking water for 1 week. After 6-8 weeks the reconstituted mice were sacrificed and analyzed by flow cytometry (FACSCalibur, BD Biosciences).

RNase protection assay

RNA from wildtype and *Tec−/−Btk−/−* BMM was isolated with Trizol Reagent (Sigma) at day 10. The multi-probe RNase protection assay was performed according the manufacturer's protocol (BD Biosciences). The hybridization products were separated on a 4,75% denaturating polyacrylamid gel. The gel was dried and exposed to autoradiography-films (Kodak) overnight at −80°C. Probes for the housekeeping gene probes L32 and GAPDH were used as normalization controls.

Preparation of BMM lysates and immunoblot analysis

Cell lysates were prepared by washing the macrophages on the tissue culture dish with ice-cold PBS and followed by their lysis in 120 μl (for 10^7 cells) lysis buffer (1% NP-40, 20 mM Tris-HCl pH 8.0, 138 mM NaCl, 10 mM EDTA and 10% glycerol) supplemented with 1 mM orthovanadate and complete protease inhibitor mix (Roche). Cell lysates were cleared by centrifugation and protein concentrations were determined after Bradford (Bio-Rad). The cell lysates were analyzed by standard western blotting techniques using the following antibodies: anti-P-Y (PY99; sc-7020, Santa Cruz), rabbit-anti-P-Y223-Btk (#3531, Cell signaling), rabbit anti-Btk (# 556365, BD Pharmingen), rabbit anti-M-CSFR (sc-692, Santa Cruz), rabbit anti-phospho-M-CSFR (#3155, Cell signaling), rat anti-caspase-11 (#C1354, Sigma), rabbit anti-actin (# A2066, Sigma), HPRT-coupled anti-rabbit Ig (Jackson ImmunoResearch), HPRT-coupled anti-goat Ig (Jackson ImmunoResearch), HPRT-coupled anti-rat Ig (P0450, Dako) and rabbit anti-Tec (kind gift of Prof. Dr. H. Mano, Jichi Medical University, Japan). Immunoblot protein bands were visualized by enhanced chemoluminescence (ECL, Amersham).

Stimulation of BMM

BMM were incubated overnight in 10 ml medium without LCM at a density of 10^7 cells per 10 cm dish. The following day the adherent cells were stimulated directly on the dish with 100 ng/ml M-CSF (Peprotech) in a total volume of 4 ml for the indicated time periods at 37°C. To terminate the stimulation, the plates with the adherent cells were put on ice and the cells were washed with ice cold PBS. Protein lysates were harvested as described above.

Cell surface M-CSFR immunoprecipitation

The protocol for immunoprecipitation of cell surface M-CSFR was adapted from Lee et al. (20). In brief, day 8 BMM were reseeded at 10^7 cells per 10 cm dish and incubated overnight with medium without LCM. The next day, the adherent macrophages were stimulated with M-CSF as described above, washed 3x with ice cold PBS and incubated with 3 ml PBS containing 6 μg/ml sheep anti-M-CSFR antibody (AF3818, R&D Systems) specific for the extracellular domain of the M-CSFR for 15 minutes at 4°C. Unbound antibody was removed by washing the cells 5x with ice cold PBS. Macrophages were lysed as described above, and protein lysate (approx. 300 μg of protein) was incubated with protein G agarose beads (Roche) for 1 hour at 4°C. The beads were pelleted by centrifugation and washed 5x with lysis buffer. Proteins were removed from the beads by boiling in Laemmli buffer and the surface fraction of M-CSFR was determined by immunoblotting. Internal M-CSFR levels were measured in cell lysate aliquots taken after the incubation with protein G agarose beads. Total M-CSFR levels were determined in cell lysate aliquots after the stimulation with M-CSF.

Results

Normal myeloid cell development in Tec−/−Btk−/−Bmx−/− mice

Tec family kinases are broadly expressed in the hematopoietic system and Tec, Btk and Bmx have been detected in the murine and human monocyte/macrophage lineage (5-7). RT-PCR analysis showed that peritoneal macrophages (PM) expressed Btk, Tec and Bmx (Fig. 1A), while BM-derived macrophages (BMM) expressed Btk and Tec (Fig. 1A and B). Therefore, we focused our further studies on the analysis of *Tec−/−Btk−/−* BMM and *Tec−/−Btk−/−Bmx−/−* mice (Btk and Bmx map to the X-chromosome, thus the genotype of male knockout mice is *Y/−*. However, for simplicity we refer to Btk-deficient or Bmx-deficient mice as *Btk−/−* or *Bmx−/−* mice respectively throughout the manuscript independent whether they were male of female).

FACS analysis of various organs indicated that myeloid cell subsets are present in *Tec−/−Btk−/−Bmx−/−* mice under homeostatic conditions. However, since the lack of Tec and Btk leads to reduced peripheral B cell numbers (14), the relative percentage of the various sub-populations was different compared to wildtype mice (data not shown). To determine whether *Tec−/−Btk−/−Bmx−/−* myeloid cells display the same developmental kinetic, competitive BM reconstitution experiments were performed. Wildtype ($Ly5.1^+$) BM cells were mixed in a 1:1 ratio with either *Tec−/−Btk−/−Bmx−/−* ($Ly5.2^+$) or wildtype ($Ly5.2^+$) BM cells and transplanted into irradiated Ly 5.1^+ wildtype recipients. After 6-8 weeks of reconstitution, the ratio of Ly 5.1^+ and Ly 5.2^+ population of the various cell lineages in the bone marrow chimeras was determined. There was equal reconstitution of $Ly5.2^+$ to $Ly5.1^+$ macrophages (defined as $CD11b^{high}Gr1^{medium}$) (Fig. 1B, left panels). As expected, *Tec−/−Btk−/−Bmx−/−* BM cells were not able to reconstitute the B cell lineage (Fig. 1B, right panels) due to a severe block of B cell development in the combined absence of Tec and Btk (14).

Impaired survival of BM-derived macrophages in the absence of Tec and Btk

To generate BM-derived macrophages (BMM), BM cells of the various genotypes were isolated and differentiated using L929 cell-conditioned medium (LCM), an established source of M-CSF (21). Wildtype, *Tec−/−* and *Btk−/−* BM cell cultures resulted in a similarly confluent layer of macrophages after 10 days in culture (Fig. 2A). In contrast, *Tec−/−Btk−/−* macrophage cultures were less dense (Fig. 2A) and showed dramatically reduced cell numbers already at day 8 of culture (Fig. 2B). The drop in cell numbers occurred after day 6, since until this timepoint cell numbers were equal in the various macrophage cultures. *Tec−/−Btk−/−Bmx−/−* BMM cultures showed a similar reduction in cell numbers as *Tec−/−Btk−/−* cultures (data not shown). Although cell numbers were reduced in the absence of Tec and Btk, the differentiation kinetics of macrophages according to the expression of F4/80 and CD11b were similar in all genotypes analyzed (Fig. 2C).

The reduced cell numbers could be caused by increased cell death or reduced proliferation. BrdU labeling experiments revealed that there was no difference in the proliferation of *Tec−/−Btk−/−* macrophages one day 6 (Fig. 3A), while on day 8 and day 10 there was no detectable proliferation of wildtype and *Tec−/−Btk−/−* macrophages (data not shown). To determine whether increased cell death caused the reduction in cell numbers, the percentage of propidium iodide (PI)-positive *Tec−/−Btk−/−* and PI-positive wildtype macrophages was determined on day 6, 8 and 10 in BMM cultures. While there was no difference in the percentage of PI-positive cells around day 6 and 10, there was an approx. 60% increase in the percentage of PI-positive *Tec−/−Btk−/−* compared to PI-positive wildtype macrophages at day 8 (Fig. 3B).

Constitutive expression of caspase-11 in the absence of Tec family kinases

The increase in the percentage of PI-positive macrophages indicated increased cell death in the absence of Tec and Btk. To test whether the expression of pro- or anti-apoptotic genes is altered in *Tec−/−Btk−/−* macrophages, RNase protection assays were performed. The expression of most of the genes analyzed was similar between wildtype and *Tec−/−Btk−/−* macrophages (Fig. 4A), however we observed that caspase-11 expression was induced in *Tec−/−Btk−/−* macrophages. Two isoforms of 43 and 38 kDa exist that can be processed to an active form of 30 kDa (15). Expressed and activated forms of caspase-11 could be detected by immunoblot analysis not only in *Tec−/−Btk−/−* but also in *Tec−/−* and *Btk−/−* single knockout macrophages. This indicates a link between Tec family kinases and the regulation of caspase-11 expression (Fig. 4B).

Tec−/−Btk−/− BMM survival can be rescued by M-CSF

The reduced survival of *Tec−/−Btk−/−* BMM indicates that culture conditions that allow the survival of wildtype BMM are not sufficient for *Tec−/−Btk−/−* BMM. This could be the result of a cell intrinsic alteration. However, it is also possible that *Tec−/−Btk−/−* BMM secret a toxic factor, that a survival factor is missing or that a survival factor is faster depleted compared to wildtype cells. To distinguish between these possibilities, medium exchange experiments were performed. Daily replacement of macrophage culture medium (supplemented with LCM) led to a dense layer of *Tec−/−Btk−/−* macrophages (Fig. 5A, left panels) with normal macrophage numbers (Fig. 5B), indicating that the survival defect of *Tec−/−Btk−/−* macrophages is not caused by a cell autonomous process. Daily exchange of *Tec−/−Btk−/−* macrophage culture supernatant to wildtype cultures did not affect the survival of wildtype macrophages (Fig.5A, middle panel and Fig.5B). This argues against a toxic factor that is produced by *Tec−/−Btk−/−* macrophages or a faster depletion of a survival factor. Adding wildtype macrophage culture supernatant (i.e. without additional LCM) to *Tec−/−Btk−/−* cultures did not rescue the cell numbers of *Tec−/−Btk−/−* macrophages (Fig. 5A, middle panels and Fig. 5B), indicating that wildtype cells do not produce a survival factor that is missing in *Tec−/−Btk−/−* macrophages. As expected, daily supernatant replacement with culture medium not supplemented with LCM led to a severe reduction in cell numbers for both wildtype and *Tec−/−Btk−/−* macrophages (Fig. 5A, right panels).

The supernatant exchange experiments indicated that daily addition of new culture medium that is supplemented with LCM rescues the survival defect of *Tec−/−Btk−/−* BMM. LCM is viewed mainly as a source of M-CSF. Therefore, we tested whether daily addition of M-CSF can rescue macrophage numbers in *Tec−/−Btk−/−* BM cultures. Macrophage numbers were restored in a dose-dependent manner to a similar extent in wildtype and *Tec−/−Btk−/−* macrophage cultures (Fig. 5C). Next, we tested whether a sufficiently high concentration of M-CSF can be identified that, if added at day 5, would yield similar numbers of wildtype and *Tec−/−Btk−/−* macrophages. Thus, increasing amounts of M-CSF were added at day 5 to wildtype and *Tec−/−Btk−/−* cultures. This led to a dose-dependent increase both in wildtype and *Tec−/−Btk−/−* macrophage numbers. While at lower M-CSF concentrations the wildtype macrophage numbers were higher compared to *Tec−/−Btk−/−* cultures, at the highest M-CSF concentration the numbers were equal between wildtype and *Tec−/−Btk−/−* cultures (Fig. 5D). This indicated that similar numbers of *Tec−/−Btk−/−* macrophages compared to wildtype cells can be generated if the cells are generated under a sufficiently high concentration of M-CSF.

Impaired M-CSFR signaling in Tec−/−Btk−/− macrophages

Our data suggested that the M-CSFR signaling pathway is altered in *Tec−/−Btk−/−* macrophages. To test whether Tec family kinases are activated by M-CSFR stimulation, wildtype BMM were stimulated with M-CSF for 1, 5, 10 and 30 min. M-CSF stimulation resulted in the activation of Btk, indicated by the phosphorylation of Y223 (Fig. 6A), a known autophosphorylation site of Btk (22).

The M-CSFR showed normal tyrosine phosphorylation upon M-CSF stimulation in *Tec−/−Btk−/−* macrophages (Fig. 6B) and M-CSFR levels were similar in wildtype and the various knockout macrophages (Fig. 6C and data not shown). Daily addition of M-CSF rescued the cell numbers in *Tec−/−Btk−/−* BMM cultures (Fig. 5C). Thus it is possible that the decrease in cell numbers in the *Tec−/−Btk−/−* BMM cultures was caused by lower levels of M-CSF due to a more rapid endocytosis of the M-CSFR and therefore an increased utilization of M-CSF in the absence of Tec and Btk. However, surface M-CSFR disappeared with a similar kinetic in wildtype and *Tec−/−Btk−/−* macrophages (Fig. 6C), indicating a

similar turnover of the M-CSFR upon M-CSF stimulation. To investigate the activation of signaling pathways of *Tec−/−*, *Btk−/−*, *Tec−/−Btk−/−* and *Tec−/−Btk−/−Bmx−/−* macrophages to M-CSF in more details, the tyrosine phosphorylation pattern upon M-CSF stimulation was determined. *Tec−/−Btk−/−* and *Tec−/−Btk−/−Bmx−/−* macrophages displayed a changed tyrosine phosphorylation pattern as compared to wildtype, *Tec−/−* or *Btk−/−*cells (Fig. 6D). Tyrosine phosphorylation of proteins around 110-130 kDa was reduced or absent in macrophages lacking both Btk and Tec.

Myeloid subset-specific regulation of GM-CSFRa expression by Tec and Btk

By performing RNase protection assays, we also noted that *Csf2ra* (the gene encoding GM-CSFRα) expression was reduced in *Tec−/−Btk−/−* macrophages as compared to wildtype cells (Fig. 7A). In contrast, *Tec−/−Btk−/−* and *Tec−/−Btk−/−Bmx−/−* BM-derived DC (BMDC) showed normal expression levels of GM-CSFRa as compared to wildtype cells (Fig. 7B), indicating Tec/Btk-dependent expression of the *Csf2ra* gene in BMM but not in BMDC. The reduced expression levels of *Csf2ra* were only observed in *Tec−/−Btk−/−* BMM, but not in *Tec−/−* or *Btk−/−* BMM (data not shown). Unlike M-CSF, which upon addition restored cell numbers in *Tec−/−Btk−/−* BMM cultures to wildtype levels (Fig. 5D), GM-CSF addition restored cell numbers in *Tec−/−Btk−/−* BMM cultures only partially (Fig. 7C). The reduced expression of GM-CSFRα in the absence of Tec and Btk however may explain why the cell numbers upon GM-CSF addition were always lower in *Tec−/−Btk−/−* cultures compared to wildtype cultures (Fig. 7C). Culture of BM cells with GM-CSF instead of M-CSF leads to the generation of a confluent layer of adherent macrophages and the co-generation of loosely-attached $CD11c^+$ BMDC (23). The macrophage layer was observed in GM-CSF cultures (day 10) of wildtype but not of *Tec−/−Btk−/−Bmx−/−* BM cells (Fig. 7D). However, the generation of BMDC was not affected, since similar numbers of wildtype and *Tec−/−Btk−/−Bmx−/−* BMDC developed in the presence of GM-CSF (Fig. 7E).

Discussion

In this study we performed a genetic approach to investigate in detail the role of Tec family kinases in murine macrophages. We observed reduced survival rates of *Tec−/−Btk−/−* bone marrow-derived macrophages. A severe drop in macrophage numbers correlating with increased numbers of dead cells occurred in the absence of Tec and Btk. Elucidation of M-CSFR signaling pathways revealed impaired total tyrosine phosphorylation pattern in *Tec−/−Btk−/−* macrophages upon M-CSF stimulation despite normal expression and phosphorylation of the M-CSFR. Thus, our data provide a novel link between Tec family kinases and M-CSF receptor signaling pathways that regulate macrophage survival. Finally, Tec and Btk are required for proper expression of GM-CSFRα in macrophages but not in dendritic cells, implicating Tec kinases in the lineage-specific regulation of GM-CSFRα expression.

The generation of BMM as assessed by surface marker expression was not influenced by the absence of Tec and Btk. This was also reflected by similar macrophage numbers at day 6 in wildtype and *Tec−/−Btk−/−* cultures. Therefore, Tec and Btk are not required for the differentiation of precursor cells into macrophages. The drop in cell numbers starting after day 6 could either be caused by reduced proliferation or reduced survival of differentiated macrophages. Since BrdU incorporation was the same in wildtype and *Tec−/−Btk−/−* macrophages, it is likely that Tec and Btk regulate macrophage numbers by promoting macrophage survival. This is supported by the observation that the drop in cell numbers correlated with an increase in propidium iodide-positive cells and also by the occurrence of a sub-N2 population in *Tec−/−Btk−/−* macrophages as revealed by DNA content analysis (data not shown). Tec family kinases have already been implicated in regulating cell survival and/or apoptosis in other cell lineages (24-26) and in the apoptosis of macrophages

after stimulation (9). Our findings indicate that Tec and Btk also regulate cell survival of BMM. Neither Tec nor Btk single-deficient BMM displayed a survival defect. Thus, our data also indicate redundant activities of Tec and Btk during macrophage generation, similar to the murine B cell lineage, where Tec and Btk are required for proper B cell development (14).

M-CSF is a crucial cytokine required for the differentiation, proliferation and survival of macrophages and is often provided in cultures of BM-derived macrophages with L929 cell-conditioned medium (LCM) (18). M-CSF starvation in macrophage cultures induces apoptosis (27). The observation that high levels of M-CSF (by daily addition of new LCM) can rescue *Tec−/−Btk−/−* macrophage numbers suggested that there are either differences in the utilization of M-CSF due to an increased internalization of the M-CSFR or an impairment of M-CSFR signaling, or both. However, since the kinetic of the internalization of the M-CSFR upon M-CSF stimulation was similar in wildtype and *Tec−/−Btk−/−* macrophages, these data point towards a M-CSFR signaling defect in the absence of Tec and Btk. This is supported by the observation that the tyrosine phosphorylation pattern upon M-CSF stimulation was changed in *Tec−/−Btk−/−* macrophages despite normal expression and phosphorylation of the M-CSFR. These data indicate that Tec family kinases are required for proper transmission of M-CSF signals in macrophages. Thus, sub-optimal concentrations of M-CSF that still allow macrophage survival in wildtype cells do not induce a sufficient signal to allow survival of *Tec−/−Btk−/−* macrophages. However, higher M-CSF concentrations (due to daily replacement of M-CSF) presumable provide a stronger (i.e. above “threshold”) signal to *Tec−/−Btk−/−* macrophages that rescues the survival defect. Thus, macrophages utilize Tec family kinases for proper “sensing” of M-CSF levels. In support of this hypothesis, we also observed a dose-dependent rescue of *Tec−/−Btk−/−* macrophage numbers that can reach wildtype cell numbers if sufficiently high amounts of M-CSF are added at day 5. Tec family kinase activation is a process involving their localization to the plasma membrane followed by a Src family kinase-mediated phosphorylation of a tyrosine residue in the activation loop of the kinase domain. Full activation of Tec family kinases is achieved after a subsequent autophosphorylation of a tyrosine residue in the SH3 domain of Tec kinases (28, 29). Membrane recruitment is mediated by the interaction of the pleckstrin homology domain of Tec family kinases with phosphatidylinositol (3,4,5)-trisphosphate (PIP_3), generated by PI3-Kinase (PI3-K) activity (30). Interestingly, it has been shown that PI-3K is required for macrophage survival (31), thus further supporting our finding of a novel link between Tec family kinases and M-CSFR signaling pathways that regulate macrophage survival. In a total phosphotyrosine blot, some tyrosine phosphorylated bands around 110-130 kDa are missing upon M-CSF stimulation in *Tec−/−Btk−/−* macrophages compared to wildtype cells. In preliminary experiments no difference could be observed in the phosphorylation status of several known signaling components of the M-CSFR pathway including SHIP1, Erk1,2, AKT, p38, JNK and Erk5 (data not shown). Therefore, further experiments including proteomics approaches are required to reveal the molecular nature of these factors that are not properly tyrosine phosphorylated in *Tec−/−Btk−/−* macrophages.

Another finding of our study was that *caspase-11* is expressed in Tec family kinase-deficient BMM. Caspase-11 can act as an upstream caspase for caspase-1 in inflammation and for caspase-3 in apoptosis (32). In contrast to other caspases, caspase-11 is generally not expressed in cells and tissues under homeostatic conditions (15). However, expression can be induced by stimuli like LPS, systemic inflammation or ischemic brain injury (33). Expression of caspase-11 in macrophages depends on NF-κB and STAT1 (34), p38 MAPK (35) and the transcription factor CHOP, a C/EBP family transcription factor (36). CHOP is implicated in endoplasmatic reticulum stress-mediated apoptosis providing a link between stress response and caspase-11 expression. However, we consider it unlikely that the up-

regulation of caspase-11 expression in Tec family kinase-deficient macrophages is linked with the increase in cell death, since caspase-11 is expressed in *Tec−/−Btk−/−*macrophages on day 10 when there are no differences in the percentage of PI-positive cells between wildtype and Tec/Btk-deficient cells. Furthermore, the observation that *Tec−/− or Btk−/−* macrophages show caspase-11 expression also argues against a direct link between caspase-11 and the drop in macrophage numbers. However, caspase-11 expression might indicate a stress response in Tec- or Btk-deficient macrophages. Finally, we noted that Tec and Btk are required for the expression of GM-CSFRα in BMM but not in BMDC, implicating Tec family kinases in the lineage-specific regulation of GM-CSFRα expression. This may also explain why the addition of GM-CSF does not rescue macrophage numbers to the same extent in *Tec−/−Btk−/−* cultures as compared to wildtype cultures. However, it remains possible that Tec and Btk are in addition also required for proper GM-CSFR signaling.

Taken together, our analysis showed that Tec and Btk are crucially involved in macrophage survival by M-CSFR signaling. Our study provides a novel link between Tec family kinases and M-CSFR signaling as well as with the regulation of caspase-11 and GM-CSFRα expression. Future *in vivo* studies addressing the roles of Tec and Btk in inflammation and infection will be of interest since both caspase-11 and GM-CSF are already implicated in these processes.

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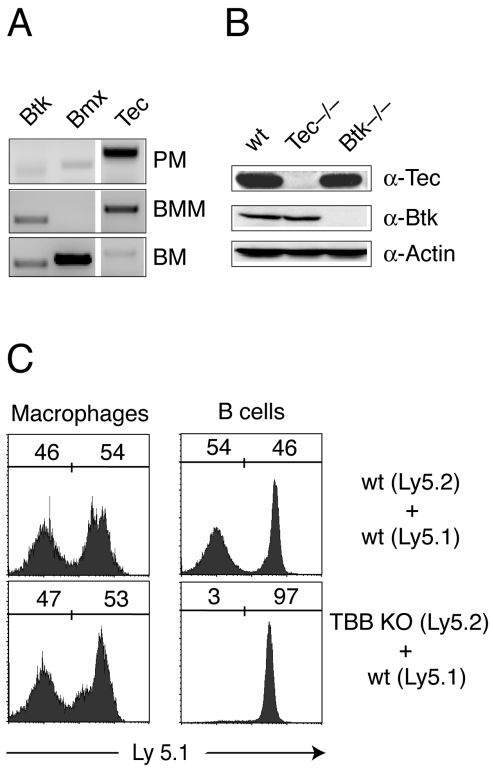

Figure 1. Myeloid cell development in *Tec−/−Btk−/−Bmx−/−* mice

(A) RT-PCR analysis of RNA isolated from peritoneal macrophages (PM), BM-derived macrophages (BMM), and BM showing expression of Btk, Bmx and Tec. Expression data are representative of two independent experiments.

(B) Immunoblot analysis showing expression of Tec and Btk in wildtype, *Tec−/−* and *Btk−/−* BMM. Expression data are representative of two independent experiments.

(C) Histograms showing Ly5.1 expression in macrophages ($CD11b^{high}/Gr1^{medium}$) or B cells ($B220^{high}$) isolated from the spleen of irradiated mice that were reconstituted with a 1:1 mixture of either $Ly5.1^+$ wildtype (wt) and $Ly5.2^+$ wildtype (upper panel) or $Ly5.1^+$ wildtype and $Ly5.2^+$ *Tec−/−Btk−/−Bmx−/−* (TBB, lower panel) BM cells. Numbers in the histogram indicate the percentage of cells in the indicated regions. One representative mouse from a total of 3 mice reconstituted with 2 different batches of BM cells is shown.

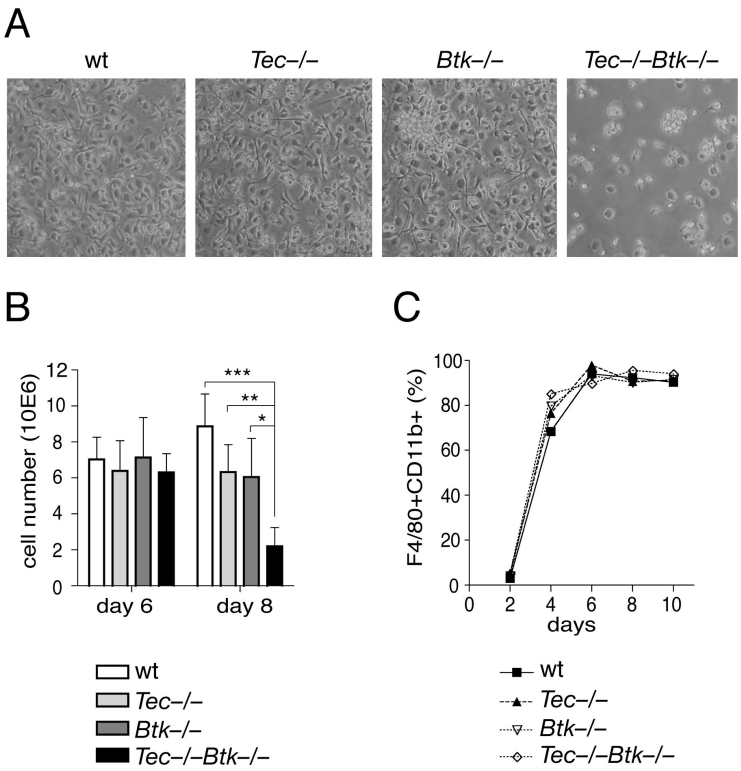

Figure 2. Impaired survival of *Tec*−/−*Btk*−/− BM-derived macrophages

(A) Pictures depicting day 8 BMM cultures. The data are representative of 10 independent experiments. Magnification 100x.

(B) Diagram showing BMM numbers in day 6 and day 8 cultures. The summary of 5 independent experiments is shown. Error bars show SD. The P-values were calculated using an unpaired student's t-test. The P-values shown are: *, 0.0104; ** 0.0011; *** <0.0001.

(C) Diagram showing the appearance of $F4/80^{+}CD11b^{+}$ macrophages (as determined by flow cytometry) in BM cultures. Numbers at the Y-axis indicate the percentage of the $F4/80^{+}CD11b^{+}$ population. Data are representative of three independent experiments.

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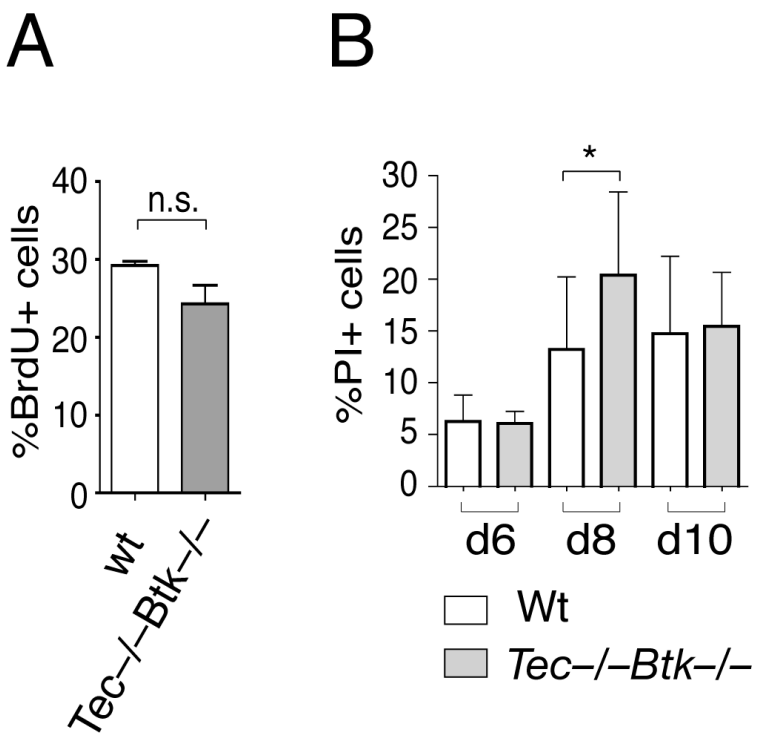

Figure 3. Normal proliferation but increased apoptosis in *Tec*−/−*Btk*−/− macrophage cultures

(A) Diagram showing the percentage of BrdU-positive cells in wildtype (wt) and *Tec*−/−*Btk*−/−macrophages cultures (day 6) after 90 minutes incubation with BrdU. Data are representative of four different experiments. Error bars show SD.

(B) Diagram showing the percentage of wildtype (wt) and *Tec*−/−*Btk*−/− propidium iodide-positive macrophages in day 6, 8 and 10 cultures. The summary of 4 (day 6 and 8) and 3 (day 10) independent experiments each performed in triplicates is shown. Error bars show SD. The P-value was calculated using a paired student's t-test. The P-value shown is: *, 0.0299.

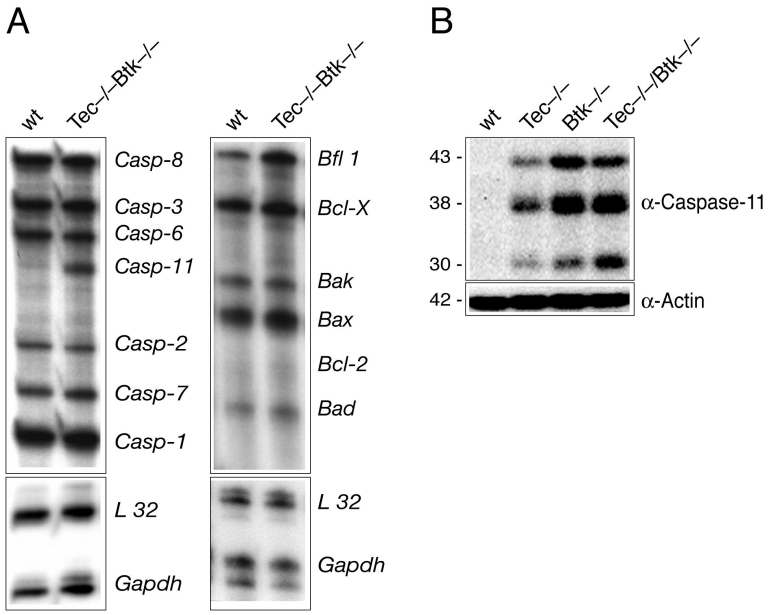

Figure 4. Expression of caspase-11 in the absence of Tec or Btk

(A) RNase protection assay using RNA isolated from day 10 cultures of wildtype and *Tec−/−Btk−/−* macrophages. One representative of two independent experiments is shown.

(B) Immunoblot analysis of wildtype (wt), *Tec−/−*, *Btk−/−* and *Tec−/−Btk−/−* macrophages showing caspase-11 expression. Actin was used as loading control. Expression data are representative of two independent experiments.

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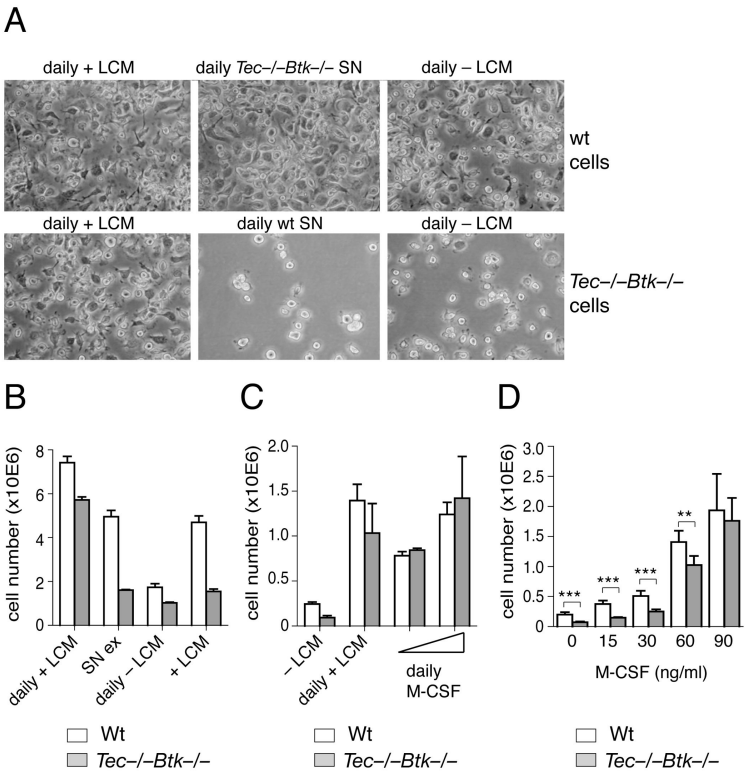

Figure 5. Effects of M-CSF and GM-CSF on macrophage survival

(A) Pictures depicting day 10 wildtype (wt, upper panels) and *Tec−/−Btk−/−* (lower panels) BMM cultures. The culture medium has been replaced daily with new LCM-containing medium (daily + LCM; left panels), with supernatant (SN) from *Tec−/−Btk−/−* (daily *Tec−/−Btk−/−* SN; upper middle panel) or wildtype (daily wt SN; lower middle panel) cultures, or with medium not supplemented with LCM (daily – LCM; right panels). Magnification is 100x.

(B) Diagram showing macrophage numbers in day 10 cultures after medium exchange experiments shown in A. Error bars show SD. Data are representative of two independent experiments. SN ex, indicates SN exchange between wt and *Tec−/−Btk−/−* cultures; + LCM, indicates culture conditions in which LCM was added on day 1, 3 and 6.

(C) Diagram showing macrophage numbers in day 10 cultures after daily addition (from day 6 on) of either LCM or M-CSF (10 and 30 ng/ml). Error bars show SD. Data shown are representative of two independent experiments.

(D) Diagram showing macrophage numbers in day 10 cultures after addition (day 5) of increasing amounts of M-CSF (0, 15, 30, 60 and 90 ng/ml). Error bars show SD. Data show summary of two independent batches each performed in triplicates. The P-values shown are: ***, <0.0001; **, 0.029 (for 60 ng/ml M-CSF).

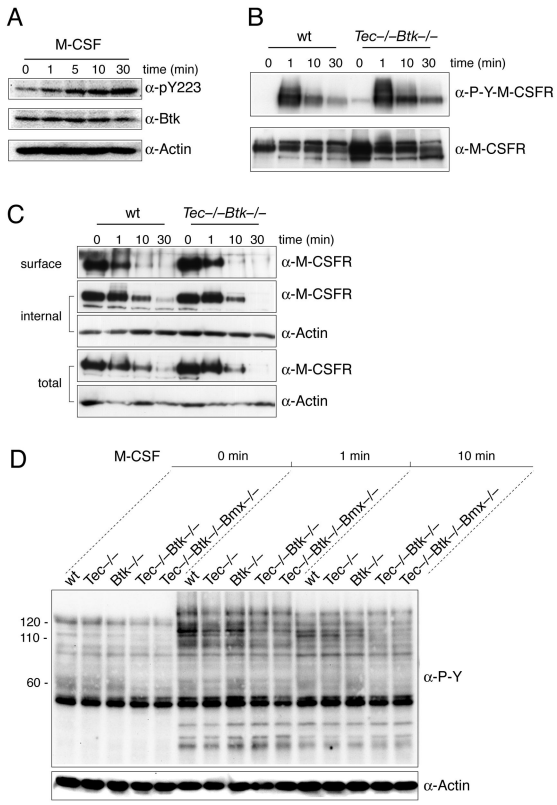

Figure 6. M-CSF receptor signaling in wildtype and *Tec−/−Btk−/−* macrophages

(A) Immunoblot analysis of wildtype BMM (day 9) showing Btk activation upon M-CSF stimulation for various timepoints.

(B) Immunoblot analysis showing the tyrosine phosphorylation of the M-CSFR in wildtype and *Tec−/−Btk−/−* BMM (day 9) upon M-CSF stimulation (upper panel). Total M-CSFR levels are shown as control (lower panel).

(C) Immunoblot analysis showing surface (first row), internal (second row) and total (forth row) expression levels of M-CSFR in wildtype and *Tec−/−Btk−/−* BMM (day 9) upon M-CSF stimulation (upper panel). Actin was used as a loading control (third and fifth row).

(D) Immunoblot analysis showing the tyrosine phosphorylation pattern of M-CSF stimulated wildtype, *Tec−/−*, *Btk−/−*, *Tec−/−Btk−/−* and *Tec−/−Btk−/−Bmx−/−* BMM (day 9). (A, B and D) Actin was used as loading control. (A, B, C and D) The cell lysate equivalent of 2 x 10^6 cells was used for each immunoblot. Data are representative of two independent experiments.

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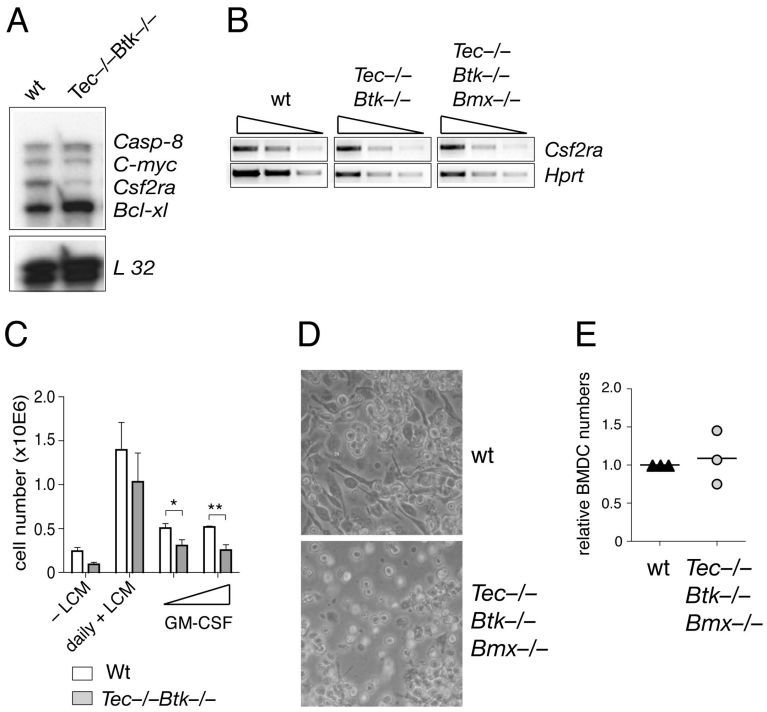

Figure 7. Reduced expression of GM-CSFRα in *Tec−/−Btk−/−* BMM

(A) RNase protection assay using RNA isolated from day 10 cultures of wildtype and *Tec−/−Btk−/−* macrophages. One representative of two independent experiments is shown.

(B) Semi-quantitative RT-PCR showing expression of *Csf2Ra* in BM-derived dendritic cells (BMDC) of the indicated genotype. *Hprt* expression was used as input control. Data are representative of two independent experiments.

(C) Diagram showing macrophage numbers in day 10 cultures after daily addition (from day 6 on) of GM-CSF (500 and 1500 U/ml). “daily + LCM” indicates culture conditions in which LCM was added daily; – LCM indicates control cultures without LCM. Error bars show SD. Data shown are representative of two independent experiments. The P-values shown are: *, 0.0168; **, 0.0017.

(D) Pictures depicting adherent GM-CSF-generated wildtype (wt, upper panel) and *Tec−/−Btk−/−Bmx−/−* (lower panel) BMM in day 10 GM-CSF cultures (after removal of non-adherent DC).

(E) Diagram showing the relative number of wildtype (wt) and *Tec−/−Btk−/−Bmx−/−* BMDC. BM cells were differentiated with GM-CSF and non-adherant BMDC were counted at day 10. The summary of three different experiments each performed in duplicates is shown. The percentages of *Tec−/−Btk−/−Bmx−/−* BMDC cell numbers compared to wildtype BMDC were 147%, 108% and 75% for experiments 1, 2 and 3, respectively. The absolute cell numbers ($\times 10^5$) were 4.6 ± 1.2 (wt) and 6.8 ± 0.9 (*Tec−/−Btk−/−Bmx−/−*, TBB) for experiment 1, 8.6 ± 1.0 (wt) and 9.3 ± 2.6 (TBB) for experiment 2, and 6.3 ± 1.5 (wt) and 4.7 ± 0.9 (TBB) for experiment 3.