Activation of the lymphotoxin-beta receptor induces NFκB-dependent interleukin-6 and MIP-2 secretion in mouse fibrosarcoma cells

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ABSTRACT. Activation of the lymphotoxin beta-receptor (LTβR), a member of the tumor necrosis factor receptor family, plays a crucial role in lymphoid organogenesis and tumor development. Lymphotoxin $\alpha_1\beta_2$ ($LT\alpha_1\beta_2$) and LIGHT have been identified as membrane anchored ligands for the LTβR. While LTβR is expressed on a wide range of cell types e.g. fibroblasts and monocytes, the ligands are expressed only on activated lymphocytes and NK cells. In order to characterize LTβR expression and the biological consequences of LTβR activation rat anti-mouse LTβR monoclonal antibodies were generated. These antibodies recognized a mouse LTβR-Ig fusion protein as well as endogenous LTβR on a variety of mouse fibroblast and fibrosarcoma cell lines. Specificity was demonstrated by the lack of binding to LTβR-deficient embryonic fibrobasts. Competitive binding studies revealed that three different epitopes were recognized by the monoclonal antibodies. Two of the monoclonals activated the LTβR and induced activation of NFκB and secretion of MIP-2 and IL-6 in L929 mouse fibroblast cells. MIP-2 and IL-6 secretion was NFκB-dependent because IκB-transfected cells released significantly reduced amounts of both mediators.

Keywords: NFκB; MIP-2; IL-6

INTRODUCTION

The LTβR, a member of the tumor necrosis factor (TNF) family, activated by its functional ligands lymphotoxin $\alpha_1\beta_2$ ($LT\alpha_1\beta_2$) or LIGHT [1,2], plays an important role in lymphoid organogenesis [3,4]. LTβR is expressed on fibroblasts and stromal cells and at low levels in some myeloid cell lines [5]. In contrast, the specific ligands $LT\alpha_1\beta_2$ and LIGHT are found on activated lymphocytes, especially T cells but also on B cells and NK cells [6,7,8]. Currently, the biological functions of LTβR activation are poorly characterized. Recent studies in LTβR-deficient mice indicated that the LTβR is crucially involved in lymphoid organogenesis [3]. In the adult animal, signaling through the LTβR seems to be necessary for maintaining the lymphoid architecture [9], which provides an explanatory link to the involvement of LTβR signaling in the generation of adaptive humoral immune responses [3, 4]. Furthermore, it has been shown that signaling through the LTβR induces cell death in some adenocarcinoma tumor lines in the presence of IFN-γ [10], despite the absence of a classical death domain in the intracellular part of the receptor molecule. Recently we reported growth promoting effects of LTβR activation in a mouse tumor model mediated by pro-angiogenic chemokine production [11]. Cell type-restricted production of certain chemokines has been reported earlier to be a consequence of LTβR activation [12, 13, 14]. Several molecules of the TRAF family and the transcription factor NFκB are involved in the intracellular signaling pathways of LTβR activation [15, 16, 17]. In order to gain more insight into the expression pattern of the LTβR and also to investigate molecular mechanisms induced by LTβR activation, monoclonal antibodies (mAbs) directed against the mouse LTβR (anti-mLTβR mAbs) were generated and functionally characterized.

METHODS

Reagents and cell lines

CHO cells stably expressing the mLTβR (CHOmLTβR) or mock transfected CHO cells (CHOneo) were generated by transfection of either the full length cDNA of the mLTβR in the expression vector pcDNA3 (Invitrogen, Groningen, The Netherlands) or by transfection of the empty expression vector using DOTAP (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Transfected cells were selected and maintained in G418 (0.8 mg/ml) (PAA Laboratories, Linz, Austria). Wild type embryonic fibroblasts and LTβR-deficient mice were kindly provided by K. Pfeffer (Institute of Microbiology, Hygiene and Immunology, TU Munich, Germany).

For prokaryotic expression, the extracellular domain of the mLTβR was inserted into the pET15b vector (R&D

Systems, Wiesbaden, Germany) immediately upstream of a sequence coding for the HIS-tag. The construct was used for expression in *E. coli* and purification under native conditions was performed according to the manufacturer's instructions.

For eukaryotic expression in Drosophila S2 cells, the mLTβR extracellular domain was fused to the Ig-Fc part of human IgG1 and inserted into the pMT/BiP/V5-His vector (Invitrogen, Groningen, The Netherlands). A V5-epitope and HIS-tag were added to the C-terminus of the secreted mLTβR-Ig fusion protein for purification and detection. The fusion protein was purified under native conditions according to the instructions given in the Xpress™ System manual (Invitrogen, Groningen, The Netherlands).

Recombinant soluble mouse LIGHT (rsmLIGHT) was generated as reported earlier for recombinant human LIGHT [14], expressed in Drosophila S2 cells and purified as described above.

L929, NIH 3T3, and BFS-1 mouse fibrosarcoma cells, as well as HeLa, WiDr and HT29 cells, obtained from the American Type Culture collection (ATCC, Rockville, MD, USA), were grown in RPMI-medium supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and penicillin/streptomycin (all from GIBCO-BRL, Gaithersburg, MD, USA).

Generation of specific mAbs to mLTβR

Wistar rats (Charles River, Sulzfeld, Germany) were immunized twice (i.m. and sc.; day 1 and day 21) with a mixture of Titer Max (Sigma, Deisenhofen, Germany) and 100 µg of mLTβR protein expressed in *E.coli*. A boosting injection (i.v.) of 100 µg mLTβR-Ig fusion protein expressed in S2 cells was given 3 days prior to the fusion of splenocytes from the immunized rats with mouse myeloma cells (SP2/0-Ag14) [18] using PEG 1500 (Roche Diagnostics, Mannheim, Germany) according to established protocols [19,20]. Hybridomas were selectively grown in hypoxantine-aminopterin-thymidine (HAT medium, Roche Diagnostics, Mannheim, Germany), in the presence of peritoneal exudate cells as feeder cells. Hybridoma supernatants were screened for binding to the LTβR-Ig fusion protein by ELISA. Positive hybridomas were subcloned by limiting dilution, and screened for stable immunoglobulin production. Monoclonal antibodies were purified from supernatants by Protein-G column affinity chromatography (Amersham-Pharmacia Biotech, Freiburg, Germany) and dialysed against PBS.

FACS analysis

Expression of mLTβR was detected by flow cytometry on a FACStar Plus (Becton Dickinson, San Jose, CA, USA) using the rat anti-mLTβR mAbs followed by a FITC-conjugated mouse anti-rat IgG at concentrations of 10 µg/ml.

NFκB activation

L929 mouse fibroblast cells stably transfected with an NFκB-dependent luciferase reporter plasmid were seeded at a density of $1x10^5$ cells per well. Cells were incubated with medium alone or with 10 µg/ml of anti-mLTβR mAbs, rsmLIGHT, or rat IgG (Sigma, Deisenhofen, Germany). Cells were washed twice with PBS 16 h later, lysed, and luciferase activity was assayed using the Luciferase Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Each reporter gene assay was performed in triplicate and repeated at least twice.

Immunoassays

MIP-2 and IL-6 expression was induced by incubation of L929 cells or L929 cells stably expressing IκB with 10µg/ml of anti-mLTβR mAb or rsmLIGHT for 16 h. MIP-2 levels were measured using the Quantakine kit according to the manufacturer's instructions (R&D Systems, Wiesbaden, Germany). All tests were performed in triplicate and repeated at least two times.

RESULTS AND DISCUSSION

Immunization and generation of rat anti-mouse LTβR mAbs

To generate antigen for immunization, the extracellular domain of the mLTβR was expressed either in *E. coli* or as a fusion protein with the Fc region of human IgG1 in Drosophila S2 cells. Both proteins contained HIS-tags for purification. For immunization of rats, the recombinant LTβR extracellular domain expressed in *E. coli* was administered twice and S2-expressed LTβR-Ig fusion protein was used for boosting, three days prior to the fusion of splenocytes from the immunized rats with myeloma cells. A total of 1,500 hybridoma clones were analyzed for their LTβR-Ig binding capacity by ELISA. Six independent clones were positive in ELISA and Western blotting, recognizing the recombinant extracellular domain of the mLTβR (data not shown).

Flow cytometric analysis revealed that four hybridomas produced anti-mLTβR mAbs recognizing the mLTβR expressed on the cell membrane of stably transfected CHO cells, but did not bind to mock-transfected cells. Thus, the mAbs C5, H11, G11, and D12 specifically recognized cell surface-expressed LTβR (Figure 1). All four mAbs belonged to the IgG2a isotype class.

The mouse fibrosarcoma cell line BFS-1, expressing LTβR as determined by RT-PCR (data not shown),

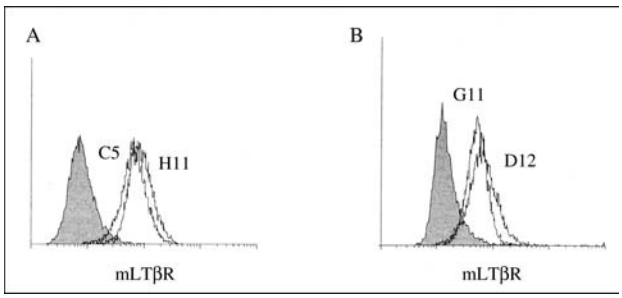

Figure 1
Flow cytometric analysis of mLTβR on CHO cells stably expressing the mLTβR.
CHO cells expressing mouse LTβR (CHOmLTβR) or mock-transfected cells (CHOneo) were stained with the mAbs C5 or H11 (A) or mAbs G11 or D12 (B).
As secondary antibody FITC labeled anti-rat Ig was used. The mock transfected cells (CHOneo) were used as negative control (shaded curve).

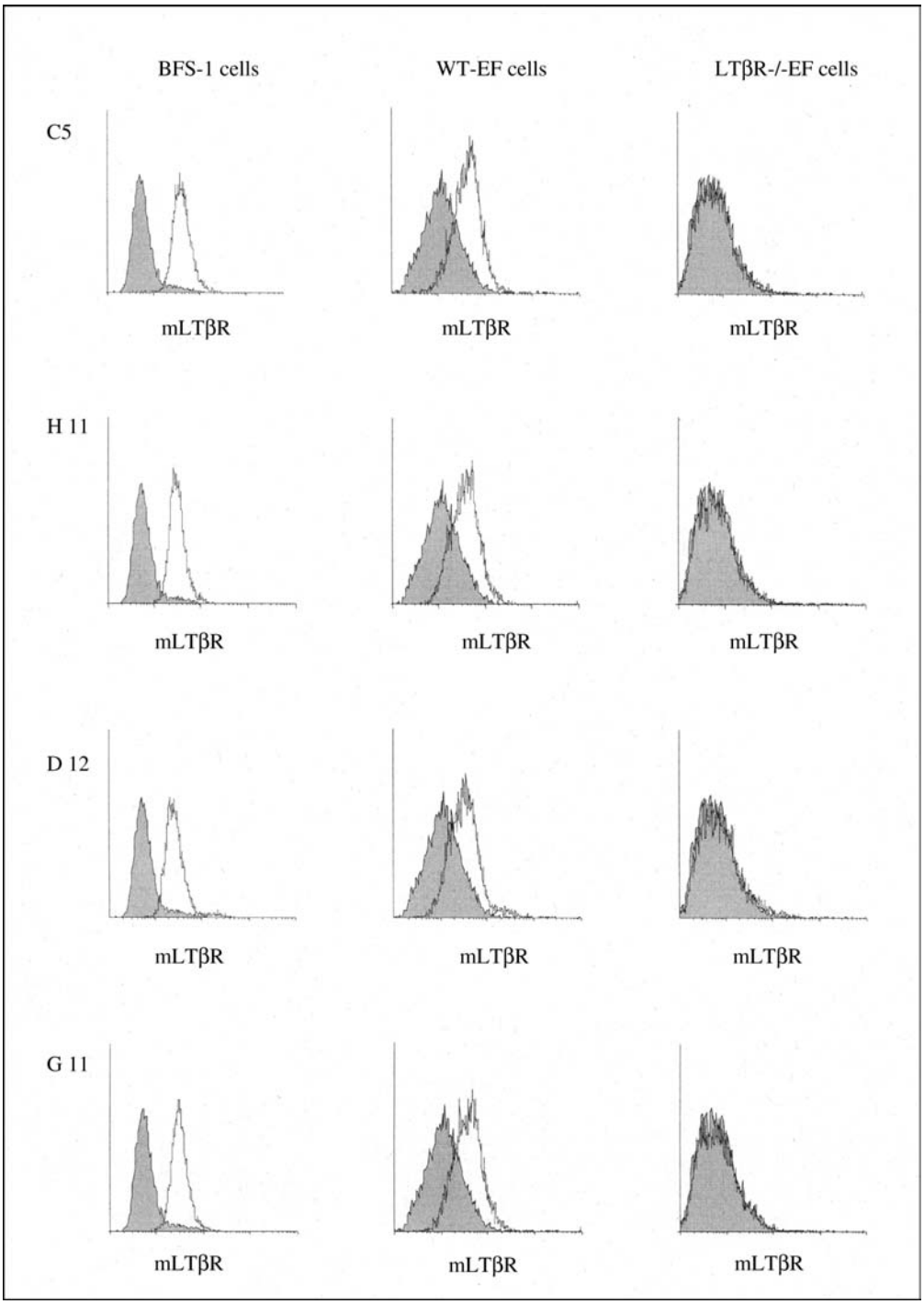

Figure 2
Flow cytometric analysis of endogenous LTβR on mouse fibroblast cells. BFS-1 cells, embryo fibroblasts from either wild type mice or LTβR-deficient mice were incubated with mAbs C5, H11, D12, or G11. FITC-labeled anti-rat Ig was used as secondary antibody. As control for nonspecific binding, cells were incubated in medium without primary antibody (shaded curve).

stained positive for endogenous mLTβR expression with all four mAbs (Figure 2, left panel). Additionally, endogenous LTβR expression was detected on the mouse fibroblast cell lines NIH 3T3 and L929 and no cross reactivity with rat or human LTβR was seen by FACS analysis using rat primary fibroblasts or HeLa, WiDr, or HT29 cells lines (data not shown).

Characterization of the anti-mouse LTβR mAbs

Receptor specificity was further demonstrated by using LTβR-deficient embryonic fibrobast (EF) cells. Although wild type EF cells expressed LTβR to a lesser extend (Figure 2, middle panel), compared to the fibrosarcoma cell line BFS-1 (Figure 2, left panel) these cells clearly stained positive compared to EF cells derived from LTβR-deficient mice. This result clearly demonstrates the receptor specificity of the anti-mLTβR mAbs.

To characterize the epitopes recognized by the mAbs, competitive binding to the recombinant extracellular domain of the LTβR was tested by ELISA. The mAbs H11 and C5 competed for antigen binding, indicating that the mAbs H11 and C5 recognized the same epitope.

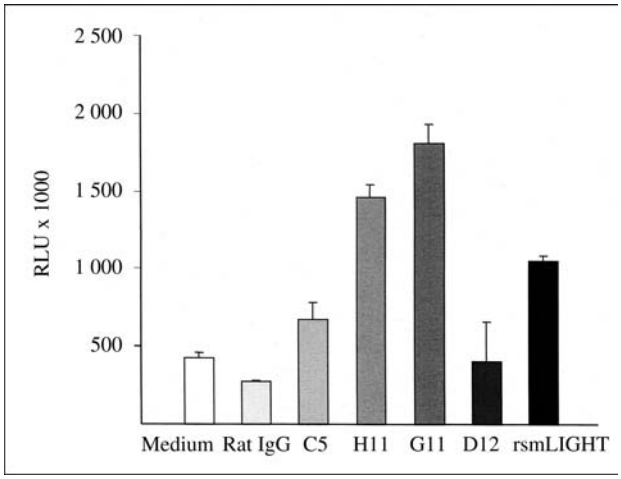

Figure 3
Activation of NFκB in L929 cells.
L929 cells transiently transfected with a luciferase reporter plasmid containing an NFκB-driven promoter were incubated for 16 h with either medium alone, rat IgG, mAbs C5, H11, G11, or D12,or rsmLIGHT (each 10 μg/ml), cells were harvested and the lysate tested for luciferase activity. Results are given as mean + SD.

However, the mAbs D12 and G11 did not interfere with each other nor with H11 or C5 in antigen binding indicating that D12 and G11 recognized two different epitopes on the LTβR extracellular domain (data not shown).

Activation of the LTβR induces secretion of MIP-2 and IL6 in an NFκB-dependent manner

Signaling through the LTβR has been reported to induce NFκB activation and chemokine production in a cell type-restricted manner [12, 13, 15, 16]. We examined the functional consequences of LTβR activation on L929 cells that endogenously express the LTβR, by stimulating the cells with either recombinant soluble mouse LIGHT (rsmLIGHT) or the anti-mLTβR mAbs. As shown in Figure 3, the mAbs C5, H11, and G11 induced significant NFκB activation, comparable to rsmLIGHT, when incubated with L 929 cells. Interestingly, no further cross – linking of the mAbs seemed to be necessary to induce NFκB activation in these cells, indicating that these mAbs activated the LTβR in an agonistic way.

It has been reported earlier that engagement of the human LTβR with a soluble form of $LT\alpha_1\beta_2$ or recombinant soluble human LIGHT, induced IL-8 production in human A375 melanoma cells [12,14]. We therefore examined the response of L929 cells to the anti-mLTβR mAbs (Figure 4). Incubation of wild type L929 cells with the anti-mLTβR mAbs C5, H11, and G11 induced the release of MIP-2, the mouse equivalent the human IL-8, comparably to the induction by rsmLIGHT (Figure 4, black bars). The mAb D12 was not able to induce MIP-2 release when incubated with L929 cells, which is in accordance with the negative result obtained with D12 in activation of NFκB.

The finding that in the mouse system rsmLIGHT is capable of inducing MIP-2 secretion from fibroblast cells parallels the observation of IL-8 secretion in human A375 melanoma cells after stimulation with rshLIGHT. Since IL-8 gene expression after LTβR activation has been reported to depend on NFκB and AP-1 activation

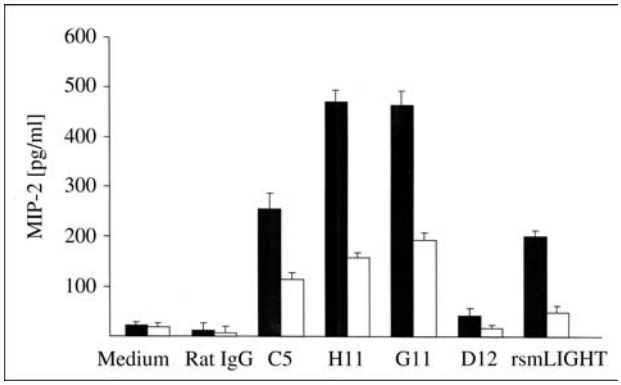

Figure 4
MIP-2 production by L929 cells.
MIP-2 concentrations were determined by ELISA in supernatants of L929 cells (black bars) or L929 stably expressing IκB (white bars) treated for 24h with medium alone, rat IgG, mAbs C5, H11, G11, or D12,or rsmLIGHT (each 10 μg/ml). Results are given as mean ± SD.

[19], we tested MIP-2 release from L929 cells transfected with IκB. Also, MIP-2 secretion as a consequence of LTβR activation seemed to depend on NFκB activation because the L929 cells, over-expressing IκB, exhibited strongly reduced MIP-2 secretion (Figure 4, white bars).

Activation of TNFR family members, which belong to the "non-death domain" receptors, such as the p75TNFR or CD40, can lead to secretion of the inflammatory cytokine IL-6 [20]. Therefore, we tested whether LTβR activation also induces IL-6 release from L929 cells. As shown in Figure 5, the anti-mLTβR mAb C5, H11, and G11 induced the release of IL-6 comparably to rsmLIGHT (Figure 5, black bars). Again, the mAb D12 did not induce IL-6 production when incubated with L929 cells. Also, the IL-6 secretion resulting from LTβR activation seemed to depend on NFκB activation, because L929 cells, over-expressing IκB, exhibited strongly reduced Il-6 secretion (Figure 5, white bars).

Taken together, these results demonstrate that the anti-mLTβR mAbs C5, H11, and G11 are agonistic with respect to NFκB activation, MIP-2 induction, and IL-6 release. The mAb D12 specifically binds to the mLTβR

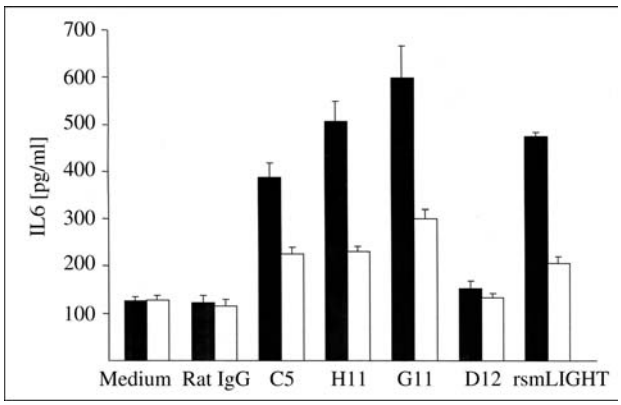

Figure 5
IL-6 production by L929 cells.
IL-6 concentrations were determined by ELISA in supernatants of L929 cells (black bars) or L929 stably expressing IκB (white bars) treated for 24h with medium alone, rat IgG, mAbs C5, H11, G11, or D12,or rsmLIGHT (each 10 μg/ml). Results are given as mean ± SD.

but does not seem to induce signaling as far as we have tested. Also, the mAb D12 did not compete with rsm-LIGHT for LTβR activation as measured by NFκB activation, MIP-2 release, or IL-6 release (data not shown). Therefore, the mAb D12, even though it binds specifically to the LTβR, seems to lack both agonistic and inhibitory activity.

None of the four anti-LTβR mAbs recognizing the mLTβR specifically in ELISA, Western blotting and binding to endogenously LTβR-expressing mouse cell lines and primary cells, could be used so far for staining the LTβR in tissue sections.

ACKNOWLEDGEMENTS. This work was supported by a grant of the Deutsche Forschungsgemeinschaft SFB 585-02 to T. Hehlgans and D.N. Männel.

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