Differential RelA- and RelB-dependent gene transcription in LTβR-stimulated mouse embryonic fibroblasts

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Abstract

**Background:** Lymphotoxin signaling via the lymphotoxin-β receptor (LTβR) has been implicated in biological processes ranging from development of secondary lymphoid organs, maintenance of spleen architecture, host defense against pathogens, autoimmunity, and lipid homeostasis. The major transcription factor that is activated by LTβR crosslinking is NF-κB. Two signaling pathways have been described, the classical inhibitor of NF-κB α (IkBα)-regulated and the alternative p100-regulated pathway that result in the activation of p50-RelA and p52-RelB NF-κB heterodimers, respectively.

**Results:** Using microarray analysis, we investigated the transcriptional response downstream of the LTβR in mouse embryonic fibroblasts (MEFs) and its regulation by the RelA and RelB subunits of NF-κB. We describe novel LTβR-responsive genes that were regulated by RelA and/or RelB. The majority of LTβR-regulated genes required the presence of both RelA and RelB, revealing significant crosstalk between the two NF-κB activation pathways. Gene Ontology (GO) analysis confirmed that LTβR-NF-κB target genes are predominantly involved in the regulation of immune responses. However, other biological processes, such as apoptosis/cell death, cell cycle, angiogenesis, and taxis were also regulated by LTβR signaling. Moreover, LTβR activation inhibited expression of a key adipogenic transcription factor, peroxisome proliferator activated receptor-γ (pparg), suggesting that LTβR signaling may interfere with adipogenic differentiation.

**Conclusion:** Microarray analysis of LTβR-stimulated fibroblasts provided comprehensive insight into the transcriptional response of LTβR signaling and its regulation by the NF-κB family members RelA and RelB.
Background

NF-κB transcription factors are essential for innate and adaptive immunity, cell survival, cellular stress responses, development and maintenance of lymphoid organ structures, and other biological functions [1-3]. The vertebrate NF-κB family includes five structurally related members, the Rel proteins RelA (p65), RelB, cRel, and the NF-κB proteins p50 and p52. Among the Rel/NF-κB family, only RelA, RelB, and cRel contain C-terminal transcriptional activation domains enabling them to directly regulate transcription. The other two members, p50 and p52, are synthesized as p105 and p100 precursors, respectively. The Rel and NF-κB proteins can form different homo- and heterodimers (for example p50-RelA or p52-RelB) that bind to DNA target sites, so-called κB sites. In resting cells, Rel/NF-κB proteins associate with inhibitory κB molecules (IkBα) and are retained in the cytoplasm as inactive forms [4].

Two major NF-κB signaling pathways can be distinguished, the classical or canonical and the alternative or non-canonical pathway. In response to stimulation of transmembrane receptors like tumor necrosis factor receptor (TNFR)-1 or Toll-like receptor (TLR)-4, signaling cascades are initiated that lead to the liberation of Rel/NF-κB complexes from their IkB molecules. As a result, they translocate to the nucleus and regulate transcription of numerous target genes. This classical pathway involves phosphorylation of IkBα by the NEMO (NF-κB essential modulator)/IKKγ- and IKKB-containing IkB kinase (IKK) complex followed by its ubiquitin-dependent proteasomal degradation. Regulation of gene transcription is predominantly mediated through p50-RelA and p50-cRel heterodimers and target genes are mainly involved in innate immunity, cell survival, and inflammation. A few inducers of NF-κB, like LTβR, are able to trigger an additional, so-called alternative or non-canonical pathway through the activation of NF-κB-inducing kinase (NIK) and IKKα. The alternative pathway governs gene regulation mainly through p52-RelB heterodimers that are generated from the inactive cytoplasmic p100-RelB complex via signal-dependent processing of the p100 inhibitor to p52. This pathway controls genes that are predominantly involved in adaptive immunity and lymphoid organ development [5-11]. Recent findings by Hoffmann and colleagues extend this scenario. They could show that not only RelB- but also RelA-containing complexes can be released from the p100 inhibitor after LTβR stimulation [12-14].

This report focuses on the transcriptional response downstream of the LTβR and its regulation by RelA and RelB. The role of LTβR signaling in development and organization of secondary lymphoid structures is well documented (reviewed in [8,15-17]). We are interested in similarities and differences in RelA and RelB function in lymphoid organ development. However, a major problem is that RelA-deficient (relA−/−) mice are embryonic lethal due to tumor necrosis factor (TNF)-induced hepatocyte apoptosis [18]. Moreover, RelB-deficient (relB−/−) mice display impaired secondary lymphoid organ development and suffer from an autoinflammatory syndrome that also affects organization and function of lymphoid tissues [19,20]. Thus, stromal compartments that display LTβR signaling and thereby have an organizational role in the development of lymphoid organs cannot be used for in vivo gene expression studies from the above animals. Therefore, we applied MEFs established from wild-type (wt), relA−/−, and relB−/− mice as an in vitro model system. Also, there is increasing evidence that LTβR functions beyond lymphoid organs, as it is involved in liver regeneration, hepatitis [21], and hepatic lipid metabolism [22]. We therefore hypothesized that LTβR signaling, via RelA and/or RelB, may participate in physiological processes other than lymphorganogenesis. MEFs with different genotypes (wt, relA−/−, and relB−/−) allowed us to dissect specific RelA and RelB activities in the regulation of gene transcription after LTβR stimulation. In wt MEFs, LTβR signals were predominantly transduced by RelA- and/or RelB-containing dimers. Upon LTβR signaling in relA−/− cells, gene regulatory events were mediated by RelB and vice versa in relB−/− cells, changes in gene expression were mediated by RelA. Using this system, we describe novel LTβR-responsive genes that were regulated solely by RelA or RelB or by both RelA and RelB.

Results and discussion

LTβR stimulation of MEFs

For LTβR stimulation, MEFs of each genotype were either left untreated or were treated with agonistic anti-LTβR monoclonal antibody (mAb) for 2.5 or 10 h. For each treatment group, cells from four experiments were pooled. Nuclear protein extracts were used in electrophoretic mobility shift assays (EMSAs) to verify proper LTβR signaling (Figure 1). In wt cells, LTβR signaling resulted in modest induction of κB-binding complexes at the early time point (2.5 h) but strong induction after 10 h of stimulation. Dissection of these complexes with supershifting antibodies revealed that the faster migrating complex contained RelB and the slower migrating complex contained RelA. As expected, in wt cells both RelA and RelB complexes were activated in response to LTβR signaling, whereas in relA−/− cells only RelB- and in relB−/− cells only RelA-containing κB-binding complexes were induced (Figure 1). Recently, slow and relatively weak DNA-binding of NF-κB complexes in response to LTβR ligation was reported [12]. The plateau was reached between 10 and 15 h of LTβR stimulation corresponding to a 2- to 3-fold induction of NF-κB DNA binding. Our results are in agreement with these observations: for each
genotype the strongest induction of κB-binding complexes was observed at 10 h. For gene expression profiling we therefore used total RNA isolated from untreated (0 h) and 10 h agonistic anti-LTβR mAb treated wt, relA⁻/⁻, and relB⁻/⁻ MEFs, assuming that stronger DNA-binding activity reflects stronger gene expression changes controlled by NF-κB transcription factor complexes.

**Global gene expression in response to LTβR stimulation in MEFs**

To identify RelA- and RelB-regulated genes after LTβR stimulation, we carried out microarray analysis using total RNA from the experiment described above hybridized to CodeLink UniSet Mouse 20K I bioarrays. For statistical analysis, different genotypes were analyzed separately and significantly differentially expressed genes between time points 0 h and 10 h were identified (p < 0.05). The fold change (FC) threshold was determined from the minimal detectable fold change (MDFC) calculated by the Code-Link Expression Analysis v4.1 software (wt: 1.48; relA⁻/⁻: 1.54; relB⁻/⁻: 1.36). In response to LTβR stimulation, a total of 528 genes were regulated in wt cells. In line with the moderate NF-κB activation seen in the EMSAs the observed gene regulation was also modest: gene expression changes were in the range of +5-fold (induction) and -5-fold (repression). We assigned the 528 LTβR-responsive genes to 4 categories: genes that were significantly regulated (i) only in wt cells (category I, n = 366), (ii) in wt and relA⁻/⁻ cells (category II, n = 30), (iii) in wt and relB⁻/⁻ cells (category III, n = 102), and (iv) genes that were significantly regulated in all 3 genotypes (category IV, n = 30) (Figure 2A; for the list of LTβR-responsive genes in wt cells see Additional file 1).

The genes in these four categories could be segregated into further subcategories, which helped us to assign regulatory mechanisms underlying the expression patterns of individual genes (see schematic depiction of gene expression behavior in Figure 2B and lists of genes belonging to different subcategories in Additional files 2, 3, 4, 5).

Category (cat) I genes were significantly regulated only in wt cells in response to LTβR stimulation. This group of genes required both RelA and RelB for their LTβR-dependent activation (cat I/1, n = 161) or repression (cat I/2, n = 205). Therefore, expression of these genes did not signifi-
LTβR-responsive genes can be allocated into distinct categories

**Figure 2**

**LTβR-responsive genes can be allocated into distinct categories.** (A) Venn-diagram of significantly ($p < 0.05$) regulated genes. (B) Schematic depiction of gene expression patterns. The four main categories in (A) can be segregated into further subcategories, depending on whether their genes were upregulated or downregulated. The arrows in the plots show the direction of gene expression changes from non-induced (0 h) to the 10 h induced state in response to LTβR stimulation. The first arrow describes gene expression behavior in wild-type, the second in relA−/−, and the third in relB−/− cells. Horizontal arrows indicate lack of change or statistically insignificant change in gene expression. Arrows pointing upwards or downwards indicate significant positive or negative regulation, respectively.
cantly change in either of the mutant cell lines in response to agonistic anti-LTβR mAb treatment (Figure 2B, Additional file 2).

Category II genes were significantly regulated in wt and relA⁻/⁻ cells upon LTβR ligation. Genes upregulated (cat II/1, n = 13) or downregulated (cat II/2, n = 17) in both wt and relA⁻/⁻ cells, but not significantly regulated in relB⁻/⁻ cells, were considered to be RelB target genes in response to LTβR signaling. Other theoretical patterns could also be appointed to category II, but we did not find any example in our analysis for these subcategories (cat II/3, n = 0 and cat II/4, n = 0) (Figure 2B, Additional file 3).

Genes belonging to category III were significantly regulated in wt and relB⁻/⁻ cells in response to LTβR stimulation. Genes upregulated (cat III/1, n = 54) or downregulated (cat III/2, n = 43) in both wt and relB⁻/⁻ cells, but not significantly regulated in relA⁻/⁻ cells, were considered to be RelA target genes in response to LTβR signaling. Negligible numbers of genes in category III could also be allocated to cat III/3 and III/4 (n = 3 and n = 2, respectively) (Figure 2B, Additional file 4). However, these genes were not further analyzed. The significantly larger number of RelA- (cat III) compared to RelB-regulated genes (cat II; Figure 2A) is likely to be a consequence of the stronger LTβR-induced DNA binding of RelA compared to RelB complexes (Figure 1).

Category IV genes were significantly regulated in each of the genotypes in response to LTβR ligation. Although eight theoretically possible gene expression behaviors exist, we only found genes that belonged to two easily explainable scenarios: genes were either upregulated (cat IV/1, n = 20), or downregulated (cat IV/2, n = 10) in each genotype upon LTβR signaling (Figure 2B, Additional file 5). Most likely, both RelA and RelB contributed redundantly to their regulation or alternatively, a third factor/pathway controlled these genes in response to LTβR stimulation. JNK (c-Jun N-terminal kinase) is a possible candidate for such a third pathway, as there are indications that LTβR stimulation leads to activation of JNK. However, the experimental setup in those studies was different from ours as LTβR-overexpressing HEK293 cells [23] or treatment of MEFs with the LTβR agonist LIGHT (lymphotoxin-related inducible ligand that competes for glycoprotein D binding to herpesvirus entry mediator on T cells) [24] were studied.

FC values observed in the three cell lines at 10 h compared to 0 h are displayed in a heatmap that also reflects the four categories and their subcategories (Figure 3, for a zoomable/enlarged version of FC heatmaps supplied with gene symbols and GenBank Accession Numbers see Additional file 6).

Interestingly, in the two subcategories with the largest number of genes both RelA and RelB together were required for LTβR-induced gene regulation (161 cat I/1 genes for their activation and 205 cat I/2 genes for their repression). In case one of the transcription factors was
missing the other one was not able to ensure regulation alone, suggesting significant crosstalk between the two NF-κB activation pathways. In response to LTβR stimulation, sequential engagement of the classical and alternative pathway was suggested, resulting in initial DNA binding by RelA followed by RelB complexes [7,9]. These findings may suggest a scenario where RelA binds first to the DNA in the promoter of category I genes, loosens up chromatin, thereby enabling subsequent DNA binding and gene regulatory action by RelB [25]. Alternatively, since relB is an NF-κB target gene [26] RelA may ensure sufficiently high expression of RelB and in the absence of RelA the reduced RelB levels cannot mediate proper regulation of certain LTβR target genes. This possibility is supported by the observation that in the absence of RelA both RelB protein levels and binding of RelB to κB sites were reduced (Figure 1 and data not shown) [13].

**Meta analysis of LTβR-dependent transcriptomes**

LTβR signaling is best known in the context of secondary lymphoid organ development and a recent expression profiling study described LTβR-dependent transcriptomes in lymph nodes and follicular dendritic cells (FDCs) [27]. However, increasing evidence suggests that LTβR also plays a role in non-lymphoid organs such as epithelial tissues during embryonic development [28] and adult liver [21,22].

To interpret our results in the light of other studies investigating LTβR signaling, we compared our LTβR-responsive genes with two recently published LTβR-dependent transcriptomes. Huber et al. identified transcripts in murine mesenteric lymph nodes affected in vivo by the administration of a soluble LTβR-Ig decoy receptor which blocks LTβR signaling [27]. A gene cluster of 80 unique transcripts that showed decreased expression after LTβR blockade was further analyzed. Twelve genes in this cluster were also associated with germinal centers (GCs)/FDC. A few common genes were found between our analysis and the LTβR-dependent transcriptomes described by Huber et al. Delk1 and enpp2 (doublecortin-like kinase 1; GenBank Accession Number: NM_019978 and ectonucleotide pyrophosphatase/phosphodiesterase 2 or autotaxin; GenBank Accession Number: NM_015744) expression was moderately decreased 3 d after LTβR blockade (FC: 0.70× and 0.66×, respectively) [27]. In our hands, both genes were upregulated in response to LTβR stimulation in a RelA-dependent manner (cat III/1, for enpp2 see also Table 12). Enpp2 was also found to be associated with GC/FDC in mesenteric lymph nodes [27]. Moreover, Enpp2 (also called autotaxin) has been recently described as a new molecule in lymphocyte homing through high endothelial venules (HEVs) [29]. Collectively, these findings suggest that LTβR, in addition to its well-described effect on the HEV differentiation program [30], further contributes via RelA-dependent upregulation of enpp2 to lymphocyte homing through HEVs. Unfortunately, we could not detect further genes with a similar regulation pattern in our and Huber and colleagues’ studies. This lack of overlap could be the consequence of several reasons: (i) different modes of function and kinetics of antagonistic LTβR-Ig vs agonistic anti-LTβR mAb application, (ii) incubation time (3 d treatment with LTβR-Ig vs 10 h treatment with agonistic anti-LTβR mAb), or (iii) in vivo collection of different cell types influenced by the treatment vs in vitro cell culture system using MEFs.

Lo et al. described a hepatic gene expression profile of wt vs lck-LIGHT transgenic mice (overexpressing the LTβR ligand LIGHT on the surface of T lymphocytes) [22]. A group of significantly regulated genes (n = 19) involved in lipid and cholesterol metabolism was identified. The gene that displayed the highest level of regulation (23-fold repression in transgenic vs wt mice) encodes for hepatic lipase, a key enzyme in lipid metabolism. We did not observe repression of hepatic lipase in our experiments, most probably due to its restricted expression on the surface of hepatocytes. However, we found another gene belonging to the lipid/cholesterol metabolism-related group described by Lo and colleagues. Ralgds (ral guanine nucleotide dissociation stimulator, GenBank Accession Number: NM_009058) expression was increased in the liver of transgenic mice and also upregulated in our LTβR stimulation experiments, belonging to the RelA-responsive genes (cat III/1, Table 12).

**Gene Ontology (GO) enrichment analysis**

Our goal was not only to define the LTβR-dependent transcriptome in MEFs, but also to assign regulatory mechanisms to LTβR signaling, i.e. to examine which part of the LTβR transcriptome is regulated by RelA, RelB, or both. We started out with GO enrichment analysis of significantly regulated genes to identify biological processes, molecular functions, and cellular components putatively regulated in the categories described above. Compared to molecular functions and cellular components, GO analysis of biological processes yielded the most conclusive results.

First, GO analysis was performed on the total LTβR transcriptome in wt cells to see how LTβR signaling influences biological processes in these fibroblasts, regardless whether these genes were also regulated in relA−/− or relB−/− cells (Category: Total wild-type, Table 1). For interpretation of our data we chose GO terms with p < 0.01. As lower limit, we did not consider GO terms with less than 3 annotated genes in the list of differentially regulated genes since they are too specific. As upper limit we did not use GO terms represented by more than 600 genes on the microarray since they are too general. Among the consid-
| GO number   | GO term                         | Type of biological process | p value    | n sel. | n tot. |
|-------------|---------------------------------|----------------------------|------------|--------|--------|
| GO:0007049  | Cell cycle                      | CCY                        | 1.80E-05   | 39     | 559    |
| GO:0006915  | Apoptosis                       | A/CD                       | 1.00E-04   | 34     | 499    |
| GO:0008219  | Cell death                      | A/CD                       | 0.00011    | 35     | 523    |
| GO:0016265  | Death                           | A/CD                       | 0.00011    | 35     | 523    |
| GO:0012501  | Programmed cell death           | A/CD                       | 0.00012    | 34     | 503    |
| GO:0006259  | DNA metabolic process           | CCY                        | 0.00016    | 32     | 469    |
| GO:0022402  | Cell cycle process              | CCY                        | 0.00034    | 30     | 447    |
| GO:0042981  | Regulation of apoptosis         | A/CD                       | 0.00063    | 23     | 319    |
| GO:0043067  | Regulation of programmed cell death | A/CD                   | 0.00068    | 23     | 321    |
| GO:0009607  | Response to biotic stimulus     | IR                         | 0.0035     | 11     | 124    |
| GO:0006260  | DNA replication                 | CCY                        | 0.0037     | 10     | 107    |
| GO:0043066  | Negative regulation of apoptosis| A/CD                       | 0.0045     | 11     | 128    |
| GO:0000074  | Regulation of progression through cell cycle | CCY                    | 0.0047     | 19     | 287    |
| GO:0043069  | Negative regulation of programmed cell death | A/CD                  | 0.0048     | 11     | 129    |
| GO:0051726  | Regulation of cell cycle        | CCY                        | 0.0051     | 19     | 289    |
| GO:0002376  | Immune system process           | IR                         | 0.0053     | 30     | 534    |
| GO:0030968  | Unfolded protein response       |                            | 0.0054     | 3      | 11     |
| GO:0007610  | Behavior                        |                            | 0.0054     | 17     | 249    |
| GO:0009953  | Dorsal/ventral pattern formation|                            | 0.0057     | 5      | 37     |
| GO:0016567  | Protein ubiquitination          |                            | 0.0064     | 5      | 35     |
| GO:0006730  | One-carbon compound metabolic process |                  | 0.0067     | 7      | 65     |
| GO:0048514  | Blood vessel morphogenesis      | BR                         | 0.0078     | 12     | 157    |
| GO:0040029  | Regulation of gene expression, epigenetic |                  | 0.0082     | 5      | 37     |
| GO:0007631  | Feeding behavior                |                            | 0.0084     | 4      | 24     |
| GO:0001525  | Angiogenesis                    | BR                         | 0.0087     | 10     | 121    |
In our study, we analyzed the Gene Ontology (GO) terms associated with the LTβR transcriptome in wild-type cells. We found that apoptosis/cell death (A/CD) and cell cycle (CCY)-related processes were overrepresented. We also identified genes annotated with "response to biotic stimulus", "immune system process" (immune related (IR) features) as well as "blood vessel morphogenesis" and "angiogenesis" (blood vessel development related (BR) features) that were enriched. Collectively, these data indicate that LTβR signaling largely influences cell survival/cell proliferation features. Moreover, it has an impact on immune responses and blood vessel development/angiogenesis related processes. Since these GO terms were found in LTβR-stimulated non-immune fibroblasts, it is likely that LTβR signaling regulates similar biological processes in stromal cells of secondary lymphoid tissues governing lymphorganogenesis and maintaining lymphoid tissue architecture.

Next, we carried out GO analysis for the four main categories and for all subcategories with at least 20 genes. Interpretation of the data was performed applying the same criteria as above. GO analysis of category I genes revealed those biological processes that were overrepresented only in LTβR-stimulated wt cells, i.e. in the presence of both RelA and RelB (Table 2). Amongst these processes, CCY-related terms dominated. Subsequently, we analyzed cat I/1 (containing genes that were upregulated exclusively in

| GO number   | GO term                              | Type of biological process | p value   | n sel. | n tot. |
|-------------|--------------------------------------|----------------------------|-----------|--------|--------|
| GO:0006259  | DNA metabolic process                | CCY                       | 1.40E-05  | 27     | 469    |
| GO:0007049  | Cell cycle                           | CCY                       | 1.80E-05  | 30     | 559    |
| GO:0022402  | Cell cycle process                   | CCY                       | 0.00033   | 23     | 447    |
| GO:0040029  | Regulation of gene expression, epigenetic |                             | 0.0016   | 5      | 37     |
| GO:0006260  | DNA replication                      | CCY                       | 0.0036    | 8      | 107    |
| GO:0022403  | Cell cycle phase                     | CCY                       | 0.0041    | 12     | 211    |
| GO:006730   | One-carbon compound metabolic process |                             | 0.0041    | 6      | 65     |
| GO:0051301  | Cell division                        | CCY                       | 0.0045    | 11     | 187    |
| GO:0031497  | Chromatin assembly                   |                            | 0.0047    | 5      | 47     |
| GO:0016458  | Gene silencing                       |                            | 0.0068    | 3      | 17     |
| GO:009953   | Dorsal/ventral pattern formation     |                            | 0.0079    | 4      | 34     |
| GO:0043543  | Protein amino acid acylation         |                            | 0.008     | 3      | 18     |
| GO:000278   | Mitotic cell cycle                   | CCY                       | 0.0081    | 10     | 175    |
| GO:0016567  | Protein ubiquitination               |                            | 0.0087    | 4      | 35     |

GO analysis was performed the same way as for category "total wild-type" described in Table 1 legend. CCY, cell cycle.
wt cells) and found enrichment of IR- and cell/biological adhesion (important events in immune cell migration)-related terms on the list of biological processes (Table 3). This finding indicates that in the absence of RelA or RelB a considerable portion of LTβR-stimulated immune response-related events cannot be carried out; fibroblasts need both molecules to execute these processes. In cat I/2 (containing genes that are downregulated exclusively in wt cells) we found enrichment of CCY-related terms on the list of overrepresented biological processes (Table 4). This finding indicates that in wt cells an important action of RelA and RelB is to downregulate numerous genes that are implicated in cell cycle regulation in response to LTβR signaling.

Since cat II/1 and II/2 had only few genes (n = 13 and n = 17, respectively), investigation of GO terms for these groups of genes was not meaningful. GO analysis of the main category II (containing genes that were regulated – either up or down – in wt and relA−/− cells, n = 30) revealed only one enriched GO term, the cell cycle (Table 5). Thus, in response to LTβR signaling a characteristic feature of RelB was to influence cell cycle-related events.

Category III contains genes that were regulated – either up or down – in each of the cell types in response to LTβR stimulation (Table 9). IR processes were overrepresented, but the terms related to hematopoietic or lymphoid organ development (LY) and taxis (T) were also present on the list of enriched biological processes. Unfortunately, we could not analyze cat IV/2, as it comprises too few genes (n = 10). Cat IV/1 contains 20 genes that were upregulated, irrespective of the genotype (Table 10). These genes primarily belong to IR and T. Possibly, RelA and RelB redundantly regulate these events or alternatively a RelA- and RelB-independent third factor/pathway (e.g. JNK) controls these biological processes following LTβR ligation. Table 11 shows a summary of our GO analysis.

**Verification of microarray results by qRT-PCR**

The changes in mRNA levels of several known as well as novel LTβR-responsive genes on the microarray were confirmed by quantitative real-time reverse-transcription-PCR (qRT-PCR), using RNA from three independent LTβR stimulation experiments (Table 12). In agreement with previous reports, we also found induction of nfkβ2 [5,6], ccl2/mcp1 [6], and ikbα expression [31] in LTβR-stimulated wt fibroblasts. In addition, our data indicate that both RelA and RelB redundantly contributed to the proper regulation of these genes in response to LTβR stimulation. However, we did not observe LTβR-dependent upregulation of lymphorganogenic chemokines as described by others. Ccl21, ccl19, ccl13, and ccl12 were shown to be LTβR-induced genes in spleen 8 h after peritoneal injection of an agonistic anti-LTβR mAb [5]. Possibly, cell context-specific signaling accounts for the difference observed between splenocytes and established 3T3 fibroblasts used in our experiments. Basak et al. observed modest upregulation of ccl13 and ccl21 in established wt 3T3 fibroblasts after 24 h treatment with agonistic anti-LTβR mAb [13]. To reduce indirect gene regulatory effects due to rather long stimulation we activated LTβR signaling only for 10

| GO number | GO term | Type of biological process | p value | n sel | n tot |
|-----------|---------|---------------------------|---------|-------|-------|
| GO:00045087 | Innate immune response | IR | 0.0027 | 4 | 58 |
| GO:0002526 | Acute inflammatory response | IR | 0.0037 | 4 | 63 |
| GO:0007155 | Cell adhesion | IR | 0.0054 | 11 | 447 |
| GO:0022610 | Biological adhesion | IR | 0.0054 | 11 | 447 |

GO analysis was performed the same way as for category “total wild-type” described in Table 1 legend. IR, immune related.
Table 4: Gene Ontology analysis of category I/2

| GO number    | GO term                                      | Type of biological process | p value    | n sel. | n tot. |
|--------------|----------------------------------------------|----------------------------|------------|--------|--------|
| GO:0007049   | Cell cycle                                   | CCY                        | 3.10E-07   | 24     | 559    |
| GO:0006259   | DNA metabolic process                         | CCY                        | 9.50E-07   | 21     | 469    |
| GO:0022402   | Cell cycle process                            | CCY                        | 7.00E-06   | 19     | 447    |
| GO:0022403   | Cell cycle phase                              | CCY                        | 2.40E-05   | 12     | 211    |
| GO:0051301   | Cell division                                 | CCY                        | 3.90E-05   | 11     | 187    |
| GO:0000278   | Mitotic cell cycle                            | CCY                        | 0.00011    | 10     | 175    |
| GO:0006730   | One-carbon compound metabolic process          |                            | 0.00022    | 6      | 65     |
| GO:0006468   | Protein amino acid phosphorylation            |                            | 0.00025    | 17     | 487    |
| GO:0006260   | DNA replication                               | CCY                        | 0.00055    | 7      | 107    |
| GO:0000279   | M phase                                       | CCY                        | 0.00057    | 9      | 176    |
| GO:0016310   | Phosphorylation                               |                            | 0.00076    | 17     | 536    |
| GO:0009953   | Dorsal/ventral pattern formation              |                            | 0.001      | 4      | 34     |
| GO:0040029   | Regulation of gene expression, epigenetic     |                            | 0.0014     | 4      | 37     |
| GO:0007067   | Mitosis                                       | CCY                        | 0.0015     | 7      | 126    |
| GO:0000087   | M phase of mitotic cell cycle                 | CCY                        | 0.0015     | 7      | 127    |
| GO:0043543   | Protein amino acid acylation                  |                            | 0.0016     | 3      | 18     |
| GO:0007224   | Smoothened signaling pathway                  |                            | 0.0038     | 3      | 24     |
| GO:0006913   | Nucleocytoplasmic transport                   |                            | 0.004      | 5      | 79     |
| GO:0051169   | Nuclear transport                             |                            | 0.004      | 5      | 79     |
| GO:0007178   | Transmembrane receptor protein serine/threonine kinase signaling pathway | | 0.0083 | 4 | 60 |
| GO:0022613   | Ribonucleoprotein complex biogenesis and assembly | | 0.0093 | 6 | 135 |
| GO:0035295   | Tube development                              |                            | 0.0096     | 6      | 136    |

GO analysis was performed the same way as for category "total wild-type" described in Table 1 legend. CCY, cell cycle.

Table 5: Gene Ontology analysis of category II

| GO number    | GO term                                      | Type of biological process | p value | n sel. | n tot. |
|--------------|----------------------------------------------|----------------------------|---------|--------|--------|
| GO:0007049   | Cell cycle                                   | CCY                        | 0.0059  | 5      | 559    |

GO analysis was performed the same way as for category "total wild-type" described in Table 1 legend. CCY, cell cycle.
where modulation of these chemokines was not observed.

Importantly, we verified novel LTβR-responsive genes and appointed regulatory molecules to them. For a complete list of verified genes see Table 12. Here, some of those verified genes are discussed in more detail.

GO analysis revealed that LTβR stimulation resulted in the regulation of IR processes (Table 11). Except category “Total wild-type”, where we could not assign regulatory molecules, in all categories where IR processes were enriched, RelA alone or together with RelB acted as a positive factor. Cx3cl1 (chemokine C-X3-C motif ligand 1/fractalkine) is one of the IR genes in cat I/1. Several studies document that NF-kB upregulates cx3cl1, e.g. in rat aortic endothelial cells upon interleukin-1β (IL-1β), TNF, and lipopolysaccharide treatment [32] or in human coronary artery smooth muscle cells [33]. The latter work shows that atherogenic lipids induce adhesion of artery smooth muscle cells to macrophages via the upregulation of cx3cl1 in a TNF/NF-kB-dependent manner. In our experiments this gene was upregulated in response to LTβR stimulation dependent on RelA and RelB. This data suggests that LTβR, via employing RelA and RelB together, may act as a proatherogenic factor.

### Table 6: Gene Ontology analysis of category III

| GO number   | GO term                                      | Type of biological process | p value | n sel | n tot |
|-------------|----------------------------------------------|----------------------------|---------|-------|-------|
| GO:0006939  | Smooth muscle contraction                    | T                          | 0.00018 | 3     | 16    |
| GO:0048675  | Axon extension                               | T                          | 0.00027 | 3     | 18    |
| GO:0006935  | Chemotaxis                                   | T                          | 0.00058 | 5     | 95    |
| GO:0042330  | Taxis                                        | T                          | 0.00058 | 5     | 95    |
| GO:0009605  | Response to external stimulus                | T                          | 0.0011  | 9     | 364   |
| GO:0006936  | Muscle contraction                            |                            | 0.0011  | 4     | 64    |
| GO:0007610  | Behavior                                     | T                          | 0.002   | 7     | 249   |
| GO:0048858  | Cell projection morphogenesis                 |                            | 0.003   | 6     | 200   |
| GO:0032990  | Cell part morphogenesis                       |                            | 0.003   | 6     | 200   |
| GO:0030030  | Cell projection organization and biogenesis   |                            | 0.003   | 6     | 200   |
| GO:0007626  | Locomotory behavior                          | T                          | 0.0072  | 5     | 169   |
| GO:0042981  | Regulation of apoptosis                       | A/CD                       | 0.0077  | 7     | 319   |
| GO:0043067  | Regulation of programmed cell death           | A/CD                       | 0.0079  | 7     | 321   |
| GO:0042221  | Response to chemical stimulus                 | T                          | 0.0082  | 7     | 323   |
| GO:0006915  | Apoptosis                                     | A/CD                       | 0.009   | 9     | 499   |
| GO:0012501  | Programmed cell death                         | A/CD                       | 0.0094  | 9     | 503   |
| GO:0048522  | Positive regulation of cellular process       | IR                         | 0.0096  | 10    | 596   |
| GO:0069555  | Immune response                               | IR                         | 0.0097  | 7     | 334   |

GO analysis was performed the same way as for category “total wild-type” described in Table 1 legend. T, taxis, response to external/chemical stimulus; A/CD, apoptosis/cell death; IR, immune related.
IR- and T-related processes were also enriched in cat III and cat III/1 according to the GO analysis. \textit{Cd74/ii} (invariant polypeptide of major histocompatibility complex, class II antigen-associated) and \textit{cxcl10/ip10} (chemokine C-X-C motif ligand 10/interferon-inducible protein-10) are two genes in cat III/1 and assigned to IR and T. CD74/Ii is involved in antigen processing and presentation and CXCL10 is chemotactic for monocytes and T cells. Moreover, expression of CXCL10, along with two other CXCR3-binding chemokines CXCL9 and CXCL11, can be induced in carcinoma cells by LT\textit{β}R agonists. These chemokines function as potent chemoattractants for activated T, NK, and dendritic cells, which may contribute to antitumor immune responses [34]. In our experiments, expression of \textit{cd74/ii} and \textit{cxcl10/ip10} was upregulated by LT\textit{β}R signaling in wt and \textit{relB}-/- cells. Thus, LT\textit{β}R signaling via RelA may (i) attract T lymphocytes and promote antigen presentation by dendritic cells in the context of MHC class II and (ii) facilitate antitumor responses against cancer cells.

As indicated by GO analysis, IR- and T-related biological processes were significantly regulated in cat IV and cat IV/1. Amongst others, genes encoding proteins that participate in innate immune responses, like \textit{ccl7/mcp3}, are also represented in these groups. \textit{Ccl7/mcp3} encodes the proinflammatory chemokine C-C motif ligand 7/monocyte chemotactic protein-3. Expression of \textit{ccl7/mcp3} was upregulated by LT\textit{β}R signaling in each of the genotypes, indicating redundant positive regulation by RelA and RelB or upregulation via another RelA- and RelB-independent pathway.

Collectively, positive regulation of the expression of proinflammatory chemokines like \textit{cx3cl1}, \textit{cxcl10}, and \textit{ccl7} (but also others, see Table 12) by LT\textit{β}R suggests that LT\textit{β}R signaling, besides regulating development and organization of secondary lymphoid structures, also participates in innate/inflammatory immune responses and for that primarily RelA action seems to be necessary.

Moreover, we found that LT\textit{β}R signaling functions beyond the regulation of immune responses and organization of lymphoid structures. PPAR\textgamma (peroxisome proliferator activated receptor \gamma) is a key-regulatory transcription factor in the process of adipocyte differentiation and activation of PPAR\textgamma promotes the storage of fat [35]. The work of Fu and colleagues suggests that LT\textit{β}R affects lipid homeostasis by downregulating hepatic lipase expression [22]. Hepatic lipase is expressed on the surface of hepatocytes in the liver. It promotes receptor-mediated uptake of plasma lipoproteins that harbor triglycerides and cholesterol and specifically catalyzes hydrolysis of triglycerides, actions that are suppressed when LT\textit{β}R signaling is switched on. Expression of \textit{pparg} was negatively affected by LT\textit{β}R signaling in wt and \textit{relA}+/- but not in \textit{relB}+/- cells (belonging to cat II/2 genes), indicating

| GO number   | GO term               | Type of biological process | p value  | n sel. | n tot. |
|-------------|-----------------------|----------------------------|----------|--------|--------|
| GO:0006955 | Immune response       | IR                         | 2.00E-04 | 7      | 334    |
| GO:0009605 | Response to external stimulus | T           | 0.00034 | 7      | 364    |
| GO:0006935 | Chemotaxis            | T                          | 0.00041  | 4      | 95     |
| GO:0042330 | Taxis                 | T                          | 0.00041  | 4      | 95     |
| GO:0002376 | Immune system process | IR                         | 0.00065  | 8      | 534    |
| GO:0007610 | Behavior              | T                          | 0.0022   | 5      | 249    |
| GO:0007626 | Locomotory behavior   | T                          | 0.0035   | 4      | 169    |
| GO:0006954 | Inflammatory response | IR                         | 0.0036   | 4      | 171    |
| GO:0006952 | Defense response      | IR                         | 0.0052   | 5      | 305    |
| GO:0002252 | Immune effector process | IR                  | 0.0064   | 3      | 102    |
| GO:0042221 | Response to chemical stimulus | T          | 0.0066   | 5      | 323    |

GO analysis was performed the same way as for category “total wild-type” described in Table 1 legend. IR, immune related; T, taxis, response to external/chemical stimulus.
that this gene was downregulated by RelB in response to LTβR stimulation. Our finding is a further indication that LTβR signaling represses lipogenesis and it may do so via RelB. It has been shown that ligand-induced transactivation by PPARγ is suppressed by IL-1 and TNF and that this suppression is mediated through NF-κB (p50-RelA) [36]. However, unlike suppression of PPARγ by p50-RelA, where this heterodimer blocks PPARγ binding to DNA by forming a complex with PPARγ and its co-activator PGC-2, LTβR-mediated suppression of pparg occurred via transcriptional repression executed by RelB. Further experiments are required to find out whether RelB directly or indirectly mediates repression of pparg transcription in response to LTβR signaling. The repressive effect of LTβR signaling on adipogenesis has been confirmed in MEFs that were induced for adipogenic differentiation. LTβR stimulation resulted in attenuated lipid droplet accumulation as well as in reduced pparg and adipogenic marker gene (fabp4/ap2) expression under conditions that promote differentiation into adipocytes (unpublished results).

**Conclusion**

This study is the first systematic dissection of the RelA- and RelB-driven transcriptome response downstream of the LTβR. We confirmed previously described LTβR-regulated genes. More importantly, we identified novel LTβR-responsive genes and assigned underlying regulatory mechanisms executed by RelA and/or RelB to them (Table 13). We found that the majority of LTβR-regulated genes

| GO number   | GO term                                      | Type of biological process | p value | n sel. | n tot. |
|-------------|----------------------------------------------|----------------------------|---------|-------|-------|
| GO:0006939  | Smooth muscle contraction                    |                            | 1.40E-05| 3     | 16    |
| GO:0006936  | Muscle contraction                           |                            | 3.90E-05| 4     | 64    |
| GO:001525   | Angiogenesis                                 |                            |         |       |       |
| GO:0048514  | Blood vessel morphogenesis                   |                            |         |       |       |
| GO:0048646  | Anatomical structure formation               |                            |         |       |       |
| GO:0030005  | Cellular di-, tri-valent inorganic cation homeostasis | ION                    | 0.0016  | 3     | 77    |
| GO:0055066  | Di-, tri-valent inorganic cation homeostasis  | ION                        | 0.0017  | 3     | 78    |
| GO:0008015  | Circulation                                  | BR                         | 0.0012  | 4     | 157   |
| GO:0030003  | Cellular cation homeostasis                  | ION                        | 0.0021  | 3     | 84    |
| GO:0001568  | Blood vessel development                     | BR                         | 0.0021  | 4     | 182   |
| GO:0055080  | Cation homeostasis                           | ION                        | 0.0021  | 3     | 85    |
| GO:0006873  | Cellular ion homeostasis                     | ION                        | 0.0022  | 3     | 86    |
| GO:0055082  | Cellular chemical homeostasis                | ION                        | 0.0022  | 3     | 86    |
| GO:0001944  | Vasculature development                      | BR                         | 0.0023  | 4     | 185   |
| GO:0050801  | Ion homeostasis                              | ION                        | 0.003   | 3     | 96    |
| GO:0065008  | Regulation of biological quality             |                            | 0.004   | 5     | 354   |
| GO:0065008  | Chemical homeostasis                         | ION                        | 0.0062  | 3     | 124   |
| GO:0070507  | Heart development                            | BR                         | 0.0088  | 3     | 141   |

GO analysis was performed the same way as for category “total wild-type” described in Table 1 legend. BR, blood vessel development related; ION, ion homeostasis.
Table 9: Gene Ontology analysis of category IV

| GO number | GO term                                      | Type of biological process | p value       | n sel. | n tot. |
|-----------|----------------------------------------------|----------------------------|---------------|--------|--------|
| GO:0002376 | Immune system process                        | IR                         | 4.40E-05      | 7      | 534    |
| GO:0006955 | Immune response                              | IR                         | 0.00038       | 5      | 334    |
| GO:0045595 | Regulation of cell differentiation           |                            | 0.0013        | 3      | 113    |
| GO:0006952 | Defense response                             | IR                         | 0.0026        | 4      | 305    |
| GO:0042221 | Response to chemical stimulus                | T                          | 0.0032        | 4      | 323    |
| GO:0006954 | Inflammatory response                        | IR                         | 0.0043        | 3      | 171    |
| GO:0048534 | Hemopoietic or lymphoid organ development    | LY                         | 0.0064        | 3      | 197    |
| GO:0050793 | Regulation of developmental process          |                            | 0.0067        | 3      | 201    |
| GO:0002520 | Immune system development                    | IR                         | 0.0078        | 3      | 212    |

GO analysis was performed the same way as for category "total wild-type" described in Table 1 legend. IR, immune related; T, taxis, response to external/chemical stimulus; LY, hematopoietic or lymphoid organ developmental processes.

Table 10: Gene Ontology analysis of category IV/1

| GO number | GO term                                      | Type of biological process | p value       | n sel. | n tot. |
|-----------|----------------------------------------------|----------------------------|---------------|--------|--------|
| GO:0002376 | Immune system process                        | IR                         | 1.40E-05      | 6      | 534    |
| GO:0006955 | Immune response                              | IR                         | 2.4E-05       | 5      | 334    |
| GO:0006952 | Defense response                             | IR                         | 0.00032       | 4      | 305    |
| GO:0006954 | Inflammatory response                        | IR                         | 0.00091       | 3      | 171    |
| GO:0009611 | Response to wounding                         | IR                         | 0.0024        | 3      | 240    |
| GO:0015031 | Protein transport                            |                            | 0.0024        | 4      | 523    |
| GO:0045184 | Establishment of protein localization        |                            | 0.0029        | 4      | 546    |
| GO:0008104 | Protein localization                         |                            | 0.0037        | 4      | 586    |
| GO:0042221 | Response to chemical stimulus                | T                          | 0.0056        | 3      | 323    |
| GO:0006886 | Intracellular protein transport              |                            | 0.0057        | 3      | 326    |
| GO:0009605 | Response to external stimulus                | T                          | 0.0078        | 3      | 364    |

GO analysis was performed the same way as for category "total wild-type" described in Table 1 legend. IR, immune related; T, taxis, response to external/chemical stimulus.
required the presence of both RelA and RelB, suggesting significant crosstalk between the two NF-κB activation pathways. Gene Ontology analysis confirmed that LTβR-NF-κB target genes were predominantly involved in the regulation of immune responses. However, other biological processes such as apoptosis/cell death, cell cycle, angiogenesis, and taxis were also regulated by LTβR signaling. Furthermore, we show that LTβR stimulation downregulated expression of the gene encoding PPARγ, suggesting that LTβR signaling may repress adipogenic differentiation by attenuating the levels of this key adipogenic transcription factor. Our findings are significant since they indicate a role for LTβR signaling beyond immune responses and lymphoid organ development and assign underlying gene expression regulatory mechanisms to the LTβR transcriptome.

| Category/Subcategory | Enriched biological processes | Regulatory molecules downstream of LTβR, and their effects on the gene expression |
|----------------------|-------------------------------|--------------------------------------------------------------------------------|
| Total wild-type      | A/CD, CCY, IR, BR            | Molecules not assignable – up and downregulation                                 |
| Cat I                | CCY                           | RelA and RelB together – up and downregulation                                  |
| Cat I/1              | IR                            | RelA and RelB together – upregulation                                           |
| Cat I/2              | CCY                           | RelA and RelB together – downregulation                                          |
| Cat II               | CCY                           | RelB – up and downregulation                                                   |
| Cat II/1             | Not investigated              | RelB – upregulation                                                            |
| Cat II/2             | Not investigated              | RelB – downregulation                                                          |
| Cat III              | T/A/CD, IR                    | RelA – up and downregulation                                                   |
| Cat III/1            | T/IR                          | RelA – upregulation                                                            |
| Cat III/2            | ION, BR                       | RelA – downregulation                                                          |
| Cat IV               | IR, T, LY                     | RelA and RelB via redundant effects – up and downregulation, OR Third pathway – up and downregulation |
| Cat IV/1             | IR, T                         | RelA and RelB via redundant effects – upregulation, OR Third pathway – upregulation |
| Cat IV/2             | Not investigated              | RelA and RelB via redundant effects – downregulation, OR Third pathway – downregulation |

Summary of GO analysis: categories/subcategories with their respective enriched biological processes and the assigned regulatory mechanisms are listed. A/CD, apoptosis/cell death; CCY, cell cycle; IR, immune related; BR, blood vessel development related; T, taxis, response to external/chemical stimulus; ION, ion homeostasis; LY, hematopoietic or lymphoid organ developmental processes. Since cat II/1, II/2 and cat IV/2 had only few genes (n = 13, 17 and 10, respectively) they were not investigated for GO terms.

**Methods**

**Cell culture**

Mouse embryonic 3T3 fibroblasts (wild-type, relA+/+, and relB+/−; kind gift from A. Hoffmann) were cultured at 37°C in Dulbecco’s modified Eagle’s medium (GIBCO/Invitrogen, Karlsruhe, Germany) supplemented with 10% heat-inactivated bovine calf serum (Perbio Science, Bonn, Ger-
Table 12: Verification of microarray results by qRT-PCR

| Gene Symbol and GenBank Accession Number | CodeLink bioarrays FC and p value (in brackets) for wt/relA^-/-/relB^-/- cells and corresponding subcategory | qRT-PCR FC ± SD for wt/relA^-/-/relB^-/- cells and corresponding subcategory |
|----------------------------------------|-------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------|
| Cx3cl1 NM_009142                        | 1.77 (0.00370)/0.90 (>0.05)/0.96 (>0.05), I/I                                                | 1.66 ± 0.22/0.89 ± 0.10/1.08 ± 0.29, I/I                                        |
| Pparg NM_011146                         | 0.65 (0.00690)/0.55 (0.01800)/1.32 (>0.05), II/2                                           | 0.50 ± 0.02/0.48 ± 0.04/0.81 ± 0.11, II/2                                      |
| Ralgds ^* NM_009058                     | 2.24 (0.00750)/1.48 (>0.05)/1.58 (0.00140), III/1                                          | 2.03 ± 0.42/1.13 ± 0.10/1.17 ± 0.16, I/I – not verified in relB^-/- cells     |
| Enpp2 ^* NM_015744                      | 2.28 (0.00150)/5.10 (>0.05), III/1                                                          | 1.85 ± 0.30/1.35 ± 0.27/3.29 ± 0.91, III/I                                    |
| Birc3 NM_007464                         | 2.77 (0.00090)/2.94 (>0.05), III/1                                                          | 2.86 ± 0.73/1.27 ± 0.11/2.99 ± 0.47, III/I                                    |
| Cxcl10/IP10 NM_021274                   | 1.91 (0.00450)/2.14 (0.03000), III/1                                                        | 2.58 ± 0.21/1.28 ± 0.39/2.67 ± 0.20, III/I                                    |
| Irf1 NM_008390                          | 1.96 (0.00270)/2.90 (0.00075), III/1                                                        | 2.67 ± 0.32/1.77 ± 0.15/2.15 ± 0.19, III/I                                    |
| Cd74 NM_010545                          | 3.11 (0.00300)/3.46 (0.00070), III/1                                                        | 5.01 ± 0.99/1.06 ± 0.18/4.77 ± 0.56, III/I                                    |
| Fosl1 NM_010235                         | 0.49 (0.00290)/0.86 (>0.05)/0.42 (0.00070), III/2                                          | 0.46 ± 0.09/0.90 ± 0.09/0.47 ± 0.10, III/2                                    |
| Nfkbia/IκBα NM_019408                   | 2.18 (0.0029)/1.57 (0.0016)/1.81 (0.0007), IV/1                                            | 2.04 ± 0.37/2.43 ± 0.50/2.74 ± 0.54, IV/1                                      |
| Ccl2/MCP1 NM_011333                     | 2.10 (0.00120)/2.99 (0.00099), IV/1                                                         | 2.29 ± 0.42/3.18 ± 0.13/6.31 ± 1.63, IV/1                                      |
| Nfkb2 NM_013654                         | 2.00 (0.00064)/3.42 (0.00140), IV/1                                                         | 1.77 ± 0.16/2.44 ± 0.34/3.92 ± 0.42, IV/1                                      |
| Ccl7/MCP3 NM_013654                     | 2.22 (0.00041)/4.35 (0.00140), IV/1                                                         | 2.77 ± 0.13/3.15 ± 0.15/5.29 ± 1.68, IV/1                                      |
| Cxcl1/KC NM_008176                      | 2.40 (0.00580)/1.80 (0.00160), IV/1                                                         | 2.40 ± 0.46/3.41 ± 0.61/3.41 ± 0.88, III/1 – not verified in relA^-/- cells   |
| Id2 NM_010496                           | 0.42 (0.00440)/0.39 (0.00075), IV/2                                                         | 0.47 ± 0.11/0.75 ± 0.05/0.57 ± 0.14, IV/2                                      |

qRT-PCR using RNA from 3 independent LTβR stimulation experiments confirmed changes in mRNA levels of several known as well as novel LTβR-responsive genes on the microarray. Gene names (Gene Symbol) and GenBank Accession Numbers are shown in the first column. FC values with corresponding p values in brackets, observed in the 3 cell lines (wt; relA^-/-; relB^-/-) at 10 h with CodeLink bioarrays and corresponding subcategories (in bold) are displayed in the second column. FC values with corresponding standard deviations (SD), observed in the 3 cell lines (wt; relA^-/-; relB^-/-) at 10 h with qRT-PCR using RNA from 3 independent LTβR stimulation experiments and corresponding subcategories (in bold) are displayed in the third column. Genes that are discussed in chapter “Meta analysis of LTβR-dependent transcriptomes” are indicated by an asterisk and genes that are discussed in chapter “Verification of microarray results by qRT-PCR” are listed in bold.
many), penicillin (100 U/ml), streptomycin (100 μg/ml), and Glutamax I (2 mM) (GIBCO/Invitrogen) and treated with agonistic anti-LTβR mAb (1 μg/ml, clone AC.H6; kind gift from J. Browning and P. Rennert).

**Table 13: LTβR responsive qRT-PCR verified genes in literature**

| Gene Symbol and GenBank Accession Number | LTβR responsiveness | In response to LTβR stimulation, transcription is regulated by RelA or RelB, + or - or 0 manner „reference“ if known„this study“ if new |
|-----------------------------------------|----------------------|----------------------------------------------------------------------------------------------------------------------------------|
| Cx3cl1 NM 009142                        | This study           | + regulation by RelA and RelB together, this study                                                                           |
| Pparg NM 011146                         | This study           | 0 RelA, this study                                                                                                             |
| Ralgds * NM 009058                      | Lo et al., 2007 [22] | Mode of regulation uncertain: RelA either alone, or together with RelB enhances Ralgds expression.                             |
| Enpp2 * NM 015744                       | Huber et al., 2005 [27] | + RelA, this study, 0 RelB, this study                                                                                           |
| Birc3 NM 007464                         | This study           | + RelA, this study, 0 RelB, this study                                                                                           |
| Cxcl10/IP10 NM 021274                   | Lukashev et al., 2006 [34] | + RelA, this study, 0 RelB, this study                                                                                           |
| Irf1 NM 008390                          | Kutsch et al., 2008 [41] | + RelA, this study, 0 RelB, this study                                                                                           |
| Cd74 NM 010545                          | This study           | + RelA, this study, 0 RelB, this study                                                                                           |
| Fosl1 NM 010235                         | This study           | - RelA, this study, 0 RelB, this study                                                                                           |
| Nfkbia/IκBα NM 019408.1                 | Dejardin et al., 2002 [5] | + RelA, Dejardin et al., 2002 [5], + RelB, this study                                                                       |
| Ccl2/MCP1 NM 011333                     | Derudder et al., 2003 [6] | + RelA, this study, + RelB, this study                                                                                            |
| Nfkb2 NM 019408.1                       | Dejardin et al., 2002 [5] | + RelA, Dejardin et al., 2002 [5], + RelB, this study                                                                       |
| Cd7/MCP3 NM 013654                      | This study           | + RelA, this study, + RelB, this study                                                                                            |
| Cxcl1/KC NM 008176                      | This study           | + RelA, this study, Positive regulation by RelB is uncertain.                                                                      |
| Id2 NM 010496                           | This study           | - RelA, this study, - RelB, this study                                                                                           |

Genes that are discussed in chapter "Meta analysis of LTβR-dependent transcriptomes" are indicated by an asterisk and genes that are discussed in chapter "Verification of microarray results by qRT-PCR" are listed in bold.

**EMSA**

Preparation of nuclear extracts and EMSAs were essentially performed as previously described [37]. Nuclear and cytoplasmic fractions were prepared according to standard procedures [38].
RNA isolation
Total cellular RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Possible contamination by genomic DNA was removed by DNaseI treatment using the RNaseFree DNase Set (Qiagen). Quality of RNA samples was checked by spectrophotometry and agarose gel electrophoresis. RNAs (2 µg total RNA per sample) were used for cRNA preparation for microarrays only when the ratio A260:A280 was 1.8–2.1 and the RNA was intact.

Microarrays
Microarray analysis was performed using CodeLink UniSet Mouse 20K I bioarrays (GE Healthcare, Munich, Germany), a one-color system where for each of the investigated 19,801 transcripts there is one 30-mer oligo probe spotted per slide. For gene expression profiling, untreated (0 h) and 10 h agonistic anti-IL1βR mAb treated wt, relA−/−, and relB−/− MEFs were used. For every treatment group, cells from 4 experiments were pooled, total RNA isolated, cRNA prepared and hybridized onto the bioarrays in technical triplicates. cRNA target preparation, bioarray hybridization and detection were carried out according to the manufacturer’s protocol provided with the CodeLink Expression Assay Reagent Kit. For scanning microarrays, a GenePix 4000B Array Scanner and GenePix Pro 4.0 software (Axon Instruments Inc./Molecular Devices, Munich, Germany) were employed according to settings suggested by the protocol provided with the CodeLink Expression Assay Reagent Kit. Microarray data have been deposited in NCBI’s GEO http://www.ncbi.nlm.nih.gov/geo/ and are accessible through GEO series accession number GSE11963.

Microarray data preprocessing
Microarray raw data of stimulated and unstimulated MEFs were analyzed using the Codelink™ Expression Analysis v4.1 software (GE Healthcare) and MDFC values were extracted. All subsequent analyses were performed using R and Bioconductor. For the analysis only genes with probe type ‘DISCOVERY’ were considered (19,801 genes) and all genes flagged MSR (Manufactory Slide Report) in any sample were excluded (leaving 19,580 genes). To remove negative expression values (local background > spot intensity) raw intensities with values < 0.01 were set to 0.01. The raw intensities of each array were scaled to the array median. After logarithmizing the expression values quantile normalization was applied across all arrays.

Differentially expressed genes
Array data for the different genotypes were analyzed separately. A gene was included in the analysis if it was flagged ‘G’ (good) or ‘S’ (contains saturated pixels) on at least two arrays in any of the two groups (stimulated or unstimulated). Furthermore, genes selected were required to have a FC higher than or equal to the FC threshold determined from the maximum MDFC in these groups. To identify genes significantly differentially expressed after stimulation, a Student’s t-test was performed for the previously filtered genes. The resulting p values were corrected for multiple testing using the method of Benjamini and Hochberg [39]. Allowing a false discovery rate of 5%, a total of 528 genes were identified that were significantly regulated in wt cells (regardless whether they were regulated somewhere else). From these, 366 genes were regulated exclusively in wt, 30 genes in wt and relA−/−, 102 in wt and relB−/− cells and 30 genes in all 3 genotypes.

Functional analysis with GO
Analysis of functional enrichment was performed employing Fisher’s exact test. The resulting p values (p < 0.01) were used to rank GO terms according to their significance. Terms with more than 600 genes on the array or less than 3 genes on the list of investigated genes were regarded as too general or too specific, respectively, and excluded from the analysis. Expert knowledge was used to assign broader themes to specific GO categories.

qRT-PCR
For qRT-PCR, first strand cDNA was obtained from 2 µg of total RNA for each treatment group using oligo-dT primers and M-MLV Reverse Transcriptase kit (Promega, Mannheim, Germany) according to manufacturer’s protocols. qRT-PCRs were performed in an iCycler Thermal Cycler real-time PCR machine (Bio-Rad Laboratories, Hercules, CA) using SYBR Green I as detector dye and reagents from the Quantace SensiMix DNA Kit (Quantace Ltd., Watford, UK). Primers for qRT-PCRs with Tm of 60°C were designed using Primer3 software (v. 0.4.0; http://frodo.wi.mit.edu) [40]. For individual samples, each gene was tested in triplicates and the mean of the 3 cycle threshold values was used to calculate relative expression levels. For normalization, β-actin was used as an endogenous reference gene to correct for variation in RNA content and variation in the efficiency of the reverse transcription reaction. Statistical analysis of qRT-PCR results from 3 independent LTβR stimulation experiments was performed employing a Welch test. Forward (F) and reverse primers (R) in 5’ to 3’ orientation were: Nfkβ2_F: GCTAATGTTGAATGCCCGGAC, Nfkβ2_R: CCTTGTTGATCCCTCTTGGCTAAGCCTCAGAGCATTG, Ccl2_F: CCCACACCTGCTGCTACT, Ccl2_R: TCTGGACCATTCCCTCTTG, Ikβα_F: TGCACTTGCGCAATCATCCAC, Ikβα_R: TTCTTCGAAAGTCTCGCAAG, Birc3_F: TGTCCTGTCAGAGGATG, Birc3_R: GGCTAAGCTCAGAGGATG, Ralgds_F: CATTCCAGCCCTAAAGAAGA, Ralgds_R: GGGCTTCCTCATGGGTCTCATC, Pparg_F: TCATCGAGGAGGAGTCCT, Pparg_R: GGGCGGTCTCCACTGAGAATA, Enpp2_F: TGCCCCATGTGACATTG, Enpp2_R: GTCGGTTGAGGAGATGAA, Birc3_F: TGACCTGTGACACCAATG, Birc3_R: GGCTAAGCTCAGAGGATG, Ralgds_F: CATTCCAGCCCTAAAGAAGA, Ralgds_R: GGGCTTCCTCATGGGTCTCATC.
Authors' contributions

AL: carried out the molecular genetic studies, analyzed and interpreted data, drafted manuscript. DR: carried out the bioinformatic and statistic analysis, participated in study design, analyzed and interpreted data. DA: participated in the bioinformatic and statistic analysis, analyzed and interpreted data. ZBY: initiated and participated in the molecular genetic studies. UM: participated in the bioinformatic and statistic analysis, analyzed and interpreted data. LT: initiated and participated in the bioinformatic and statistic analysis, participated in the bioinformatic and statistic analysis, drafted manuscript. DR: carried out the molecular genetic studies, analyzed and interpreted data. AJRH: supported bioinformatic analysis. FW: conceived the study, participated in its design and coordination, interpreted data, helped to write the manuscript. All authors read and approved the final manuscript.

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Additional file 1

Total LTβR transcriptome in wt cells. List of the 528 genes that were LTβR responsive in wt cells (10 h), regardless whether they were also regulated in relA−/− or relB−/− cells, or not.

Click here for file

Additional file 2

LTβR-responsive genes in wt cells. List of genes that were significantly regulated in wt cells, but not in relA−/− or relB−/− cells (10 h; upregulation, cat I/1, n = 161; downregulation, cat I/2, n = 205).

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Additional file 3

LTβR-responsive genes in wt and relA−/− cells. List of genes that were significantly regulated in wt and in relA−/− cells (10 h; upregulation, cat II/1, n = 13; downregulation, cat II/2, n = 17).

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Additional file 4

LTβR-responsive genes in wt and relB−/− cells. List of genes that were significantly regulated in wt and in relB−/− cells (10 h; upregulation, cat III/1, n = 54; downregulation, cat III/2, n = 43; cat III/3, n = 3; cat III/4, n = 2).

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Additional file 5

LTβR-responsive genes in wt, relA−/− and relB−/− cells. List of genes that were significantly regulated in each of the genotypes (10 h; upregulation, cat IV/1, n = 20; downregulation, cat IV/2, n = 10).

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Additional file 6

Zoomable/enlarged version of fold change heatmaps. Heatmaps displaying the fold change values observed in the three different cell lines at 10 h compared to 0 h. For figure legend see Figure 3. Gene symbols and GenBank Accession Numbers (in brackets) are also displayed.

Click here for file

Abbreviations

LTβR: lymphotixin-β receptor; IκBα: inhibitor of NF-κB activity; PPARγ/α: peroxisome proliferator activated receptor-γ; TNFR1: tumor necrosis factor receptor 1; TLR4: Toll-like receptor 4; MDMF: minimal detectable fold change; cat I: category; FC: fold change; wt: wild-type; mAb: monoclonal antibody; EMSA: electrophoretic mobility shift assay; FC: fold change; MSR: Manufactory Slide Report; SD: standard deviation.
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