Activation-induced Tumor Necrosis Factor Receptor-associated Factor 3 (Traf3) Alternative Splicing Controls the Noncanonical Nuclear Factor κB Pathway and Chemokine Expression in Human T Cells*

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Background: Many pre-mRNAs are alternatively spliced upon T cell activation, but functional implications remain largely unexplored.

Results: Alternative splicing of the signaling adaptor Traf3 controls expression of effector proteins in activated T cells.

Conclusion: Cell type-specific and activation-dependent alternative splicing regulates signaling and gene expression in T cells.

Significance: Alternative splicing plays a fundamental role in regulating functionality upon T cell activation.

Alternative splicing is widely recognized as a mechanism to increase the coding capacity of the genome and to have a large regulatory potential (1). Recent deep sequencing analyses have produced an enormous amount of data revealing widespread cell type-, activation-, differentiation-, and species-specific alternative splicing patterns (2–4). One of the model systems to investigate dynamic changes of alternative splicing is activation of T cells (5). Upon T cell receptor engagement or treatment with pharmacological mimics profound alterations in gene expression result in well defined changes in cell division, viability, and production and secretion of effector molecules among others (6). Several studies have used genome-wide approaches such as splicing sensitive microarrays or RNA-Seq to identify signal-induced changes in alternative splicing patterns during T cell activation, and many exons have been identified that are differentially spliced in resting versus activated conditions (5). However, a role of such splicing events in regulating functional changes has been investigated in only very few cases leaving the question to which extent alternative splicing contributes to T cell biology largely unanswered. This is also true for other model systems, where, despite the growing evidence pointing to alternative splicing as a substantial source of proteome diversity, functional implications are only beginning to be addressed. Such analyses have shown isoform-specific functions of some genes and, as a result, an important regulatory role of alternative splicing (7–10), but the vast majority of alternative splicing events remains unexplored with respect to functionality. The notion that alternative splicing plays a fundamental role in regulating cellular functionality on a genome-wide scale is further supported by the finding that alternative exons are enriched in motifs participating in protein-protein interactions thus potentially controlling signaling pathways and protein interaction networks in a cell type-dependent manner (11, 12).

Members of the NFκB family of proteins play fundamental roles in cellular differentiation, viability, and proliferation (13). Two NFκB pathways exist, the canonical and the noncanonical, that regulate distinct target genes (14). The noncanonical (nc) pathway results in intramolecular processing of the p100 pro-

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4 The abbreviations used are: nc, noncanonical; cIAP, cellular inhibitor of apoptosis; IP, immunoprecipitation; MO, morpholino; NIK, NFκB-inducing kinase; PBMC, peripheral blood mononuclear cell; PMA, phorbol 12-myristate 13-acetate; qPCR, quantitative PCR; Traf2, TNF receptor-associated factor 2; Traf3, TNF receptor-associated factor 3; WCE, whole cell extract.
tein to form active p52, which is capable of binding a dimerization partner, mainly RelB, and entering the nucleus (15). Although little is known about the functional role and regulation of ncNFκB signaling in T cells, the pathway has been well described in B cells and stromal cells. For example, it is required for secondary lymphoid organ formation as it induces essential chemokines such as CxCL13 in stromal cells (14, 16, 17). Inducible CxCL13 expression in a subset of human CD4+ T cells may contribute to B cell activation (18—20), but the signaling pathway leading to chemokine expression in T cells remains unknown. Activity of the ncNFκB pathway critically depends on the presence of the upstream kinase NIK. NIK expression is kept at a low basal level by an interaction with Traf3 (TNF receptor-associated factor 3), which targets NIK for ubiquitination by Traf3-associated Traf2-cIAP (cellular inhibitor of apoptosis), leading to its degradation (21—25). Degradation of Traf3 itself, e.g., upon stimulation of CD40 or BAFF receptors in B cells, or 4-1BB in T cells, separates NIK from Traf2-cIAP thus allowing accumulation of NIK to initiate ncNFκB signaling (26). A further regulatory layer is added through the control of receptor-induced Traf3 degradation by the deubiquitinating enzyme OTUD7B, underlining the necessity of tightly controlled Traf3 expression and ncNFκB signaling for proper immune function (27). Together, these studies unequivocally identified Traf3 as a negative regulator of ncNFκB signaling. Furthermore, T cell-specific deletion of Traf3 in mice leads to a defective T cell-dependent antibody response, suggesting an involvement of Traf3 in T helper cell function (28). Whereas several splicing isoforms of Traf3 have been described, regulated isoform expression and isoform-specific functions in an endogenous setting remain unexplored (29).

Over the past years, the Jurkat-derived Jsl1 T cell line has become a prime model system to investigate activation induced alternative splicing (30, 31). A recent RNA-Seq approach in Jsl1 cells suggested an inducible switch in Traf3 isoform expression (3). Here we show that activation- and cell type-specific Traf3 exon 8 alternative splicing generates an isoform, Traf3DE8, that in contrast to the full-length protein, activates ncNFκB signaling. Traf3DE8 disturbs the NIK-Traf3-Traf2 complex to allow accumulation of NIK, initiation of ncNFκB signaling, and chemokine expression. Traf3 exon 8 skipping and the ncNFκB pathway are also activated upon anti-CD3 stimulation of primary human CD4+ T cells, leading to induction of B lymphocyte chemoattractant (CxCL13). Together, we provide evidence for a new functionally important splicing switch during activation of a model T cell line as well as primary human T cells. Our data suggest a model in which Traf3 alternative splicing contributes to the regulation of the T cell-dependent immune response by activating ncNFκB signaling and chemokine expression to participate in B cell chemoattraction and activation.

MATERIALS AND METHODS

Cell Culture and Transfections—Jsl1 cells and culture conditions have been described previously (30). Ramos and Raji cells were cultured under the same conditions. HeLa cells were maintained in DMEM +10% FBS + penicillin/streptomycin. Media and supplements were from Invitrogen. Transfection of HeLa cells using Lipofectamine 2000 was done according to the manufacturer’s instructions. PMA (Sigma) stimulation of Jsl1, Raji, and Ramos cells was performed as described (30). Unless otherwise stated RNA and proteins were prepared 2 days after stimulation. For actinomycin D experiments, ActD (Sigma, 5 μg/ml) was added 40 h after stimulation for another 24 h. Morpholino (MO) transfection has been described previously (30). In brief, 10 × 10⁶ Jsl1 cells at a density of 1 × 10⁶ cells/ml were spun down, washed twice in RPMI 1640 medium, resuspended in 400 μl of RPMI 1640 medium, mixed with MOs (10 nmol), and then electroporated at 250 mV and 960 microfarads. Cells were placed in fresh growth medium overnight and then treated as indicated. The following MOs were obtained from Gene Tools: Traf3_E8 (CTCAACTGCAACGGGAAAGAGAGCT and standard negative control (CCTCTACCTTCAGTACACTTTATA).

For siRNA transfection, 20 pmol of a Traf3DE8 targeting siRNA (spanning the junction of exons 7—9, GGAGC-GAGGGGAACAAAACA) or a nonsilencing control was transfected using HiPerFect according to the manufacturer’s protocol. One day after transfection cells were treated with PMA or solvent control, and RNA was prepared and analyzed 48 h later. Generation of stable cell lines was done as described (32). PBMCs were obtained from heparin-treated whole blood from healthy volunteers according to University policies. PBMCs were purified using Ficoll centrifugation and cultured in RPMI +10% FBS + penicillin/streptomycin. For stimulation, 1 μg of anti-CD3 (UCHT1; BD Biosciences) was precoated in 250 μl of PBS for 2 h at 37 °C per well of a 24-well plate, the well was washed once with PBS, and 1.5 × 10⁶ cells in 1 ml of medium were added for 2 days. CD4+ T cells were negatively purified using Dynabeads (Invitrogen), and purity was assessed using CD4-FITC staining (BD Biosciences) and was routinely above 80%. Stimulation was performed as with PBMCs.

Constructs—For Traf3 expression constructs, ORFs were PCR-amplified using primers introducing restriction sites with cDNA from resting or stimulated Jsl1 cells. PCR products were digested and ligated directly into a pCMV-N3-FLAG expression vector. All constructs were verified by sequencing.

RNA, RT-PCR, Quantitative PCR—RT-PCRs were done as described (30). Briefly, RNA was extracted using PevGold RNApure, and 1 μg of RNA was used in a gene-specific RT-PCR reaction. PCR with a [32P]-labeled forward primer was performed using different cycle numbers, and quantification was performed using a low cycle number where the amplification was in linear range with regard to input RNA. Quantification was done after denaturing PAGE using a PhosphorImager (Fuji FLA3000) and ImageQuant TL software. Quantifications are given as mean values of at least three independent experiments (exact number in figure legends); error bars represent S.D. The following primer pairs were used to detect Traf3 exon 8 and Traf3 exon 7-8-9 splicing: 8for, CACGAAGACACCGACTCTGCTTG; 8rev, CTGATTGTGCAAACCTTGGATGGCTTG. For qPCR the same RT protocol was used as above combining up to four gene-specific primers in one RT-reaction. qPCR was then performed in 96-well format using the ABSolute QPCR SYBR Green mix (Thermo Fisher) on Stratagene Mx3000P instru-
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FIGURE 1. Traf3 exon 8 is alternatively spliced upon stimulation of Jsl1 T cells. A, domain structure of Traf3 and regions encoded by exons 7–9. The location of primer pairs used in B and D is indicated. Protein size in amino acids is for the human protein, R, RING; Z, zinc finger. B, Jsl1 T cells or Raji and Ramos B cells treated with vehicle control or PMA for 2 days. Traf3 exon 8 alternative splicing was analyzed by radioactive RT-PCR with primer pair 1 (left) and quantified by Phosphorimage analysis (right). Multiple experiments showed the same result. Error bars, S.D. C, Jsl1 cells treated with PMA or dimethyl sulfoxide (−) for 40 h. Actinomycin D (ActD) was added for an additional 24 h. Shown is the mean-fold change of Traf3fl by actinomycin D treatment under resting or stimulated conditions (n = 3). D, radioactive RT-PCR and Phosphorimage quantification (right) as in B using primer pair 2 to detect alternative splicing of Traf3 exons 7, 8, and 9 in Jsl1 T cells. The quantification shows -fold induction relative to unstimulated cells; graphs represent mean values ± S.D. (n = 9). E, WCEs prepared from cells treated as in B and analyzed by Western blotting (top). The Traf3 isoform induced by PMA has the same electrophoretic mobility as the isoform induced by MO-mediated skipping of exon 8 (bottom, see Fig. 3 for details of the MO experiment).

Western Blotting, Immunoprecipitation (IP), and Antibodies—Whole cell extracts (WCEs) and nuclear extracts were generated as described (30). SDS-PAGE and Western blotting were performed using standard procedures. For IPs 100 μg of WCEs of HeLa cells expressing the indicated FLAG proteins was incubated in 500 μl of IP buffer (60 mM Tris-HCl, pH 7.5, 30 mM NaCl, 1 mM EDTA, 1% Triton X-100, 3% BSA with protease inhibitors). After a 1-h rotation at 4 °C anti-FLAG beads (Sigma) that were prewashed in the same IP buffer were added, and incubation was continued for 1 h at 4 °C. Beads were washed five times in IP buffer, and after the last wash SDS-sample buffer was added. Samples were boiled and analyzed by SDS-PAGE and Western blotting. Endogenous IPs were performed with 100 μg of Jsl1 WCE in IP buffer as above. Lysates were pre-cleared with protein A/G beads (Santa Cruz Biotechnology) for 1 h at 4 °C; afterward beads that were prebound to Traf2 antibody were added, and incubation was continued overnight. Beads were then washed and analyzed as above. The following antibodies were used: Traf2 (C-20), Traf3 (H-122), NIK (H-248), HDAC1 (H-51), RelB (C-19), p65 (C-20) from Santa Cruz; hnRNP L (4D11) from Abcam; p100/p52 (4882), IκBα (4814), and FLAG (2368) from Cell Signaling; and GAPDH from Genetex (GTX100118).

EMSA—1 μg of nuclear extract (30) was used in binding reactions containing 3 μg of poly(dI-dC) in 20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM MgCl2, 3 mM DTT, 10% glycerol for 15 min at 30 °C. Where indicated, antibodies were added for an additional 15 min at 30 °C. 1 ng of 32P-end-labeled NFκB oligonucleotide (33) was added, and incubation was continued for 15 min at 30 °C. The reaction mixture was then separated by a 4% native PAGE, the gel was dried, and complexes were visualized by autoradiography.

RESULTS

Traf3 Exon 8 Is Alternatively Spliced during Activation of Jsl1 T Cells—In a recent RNA-Seq approach we found Traf3 to undergo a profound change in isoform expression upon PMA-activation of Jsl1 T cells (Fig. 1 and Ref. 3). The exon predicted to be differentially included, exon 8, encodes part of the zinc finger domain (Fig. 1A), and its exclusion results in a protein lacking one zinc finger (Traf3DE8). We used an established radioactive RT-PCR protocol to confirm an increase of TraflDE8 from 5% to >40% upon PMA treatment of Jsl1 T cells (Fig. 1B and Ref. 3). When we PMA-treated two human B cell lines, Raji and Ramos, or HeLa cells, we did not observe increased Traf3 exon 8 skipping, suggesting a T cell-specific
regulation (Fig. 1B and data not shown). The changed Traf3 isoform expression was not due to changed mRNA stability, as we did not observe a selective stabilization of either one of the two isoforms in resting or activated cells (Fig. 1C). As Traf3 exons 7 and 9 have also been reported to be alternatively included (29), we tested whether the observed regulation was specific for exon 8. We detected five Traf3 isoforms, but only exon 8 was regulated upon PMA stimulation of Jsl1 cells whereas none of these five isoforms changed in PMA-treated Raji or Ramos B cells (Fig. 1D and data not shown). As a further specificity control, we analyzed alternative splicing of the corresponding exons in Traf5, the closest Traf3 homolog. We did detect some exclusion of Traf5 exon 10 (corresponding to exon 9 in Traf3), which, however, did not change upon PMA stimulation (data not shown). At the protein level, a Traf3 isoform with the expected size of Traf3DE8 was only detected in PMA-treated Jsl1 cells (Fig. 1E, top). The electrophoretic mobility of this PMA-induced Traf3 isoform corresponds exactly to that of the Traf3 isoform induced by MO-mediated exon 8 skipping (Fig. 1E, bottom, and see below), strongly arguing for the additional Traf3 band in PMA-treated Jsl1 cells being Traf3DE8.

From the above experiments we conclude that Traf3 exon 8 is alternatively spliced in an activation- and cell type-specific manner, leading to a corresponding change in Traf3 protein expression.

**Two-day PMA Treatment Selectively Activates the Noncanonical NFκB Pathway in Jsl1 T Cells**—To identify a functional role of Traf3 alternative splicing, we analyzed the activity of both NFκB pathways in activated T cells. Upon 2-day PMA treatment of Jsl1 cells p52 protein (noncanonical) was increased whereas the amount of IκBα (canonical) was unchanged (Fig. 2A). Consistently, p52 protein and its dimerization partner RelB were detected in nuclear extract only upon PMA stimulation (Fig. 2B). Using EMSAs we observed increased binding to an NFκB-specific probe (33) in nuclear extracts from PMA-treated cells (Fig. 2C, lanes 2 and 3), whereas an unrelated probe showed no such difference (Fig. 2C, lanes 8 and 9) and confirmed equal loading. The PMA-induced shift could be partially blocked by addition of p52 and RelB antibodies (Fig. 2C, lanes 4 and 5), whereas a p65 antibody (lane 6) had no such effect. Furthermore, an unlabeled NFκB oligonucleotide was much more efficient in a competition assay than an unrelated control oligonucleotide (data not shown). These experiments together confirm the presence of the ncNFκB heterodimer in the nucleus of 2-day PMA-treated T cells.

As the canonical NFκB pathway is known to respond within minutes and is controlled by negative feedback mechanisms we tested IκBα levels in a shorter time frame. After 30 min PMA stimulation a clear loss of IκBα was observed whereas p100/p52 levels remained unchanged (data not shown). These data sug-
gest a biphasic activation with the canonical NFκB pathway following a fast and transient kinetic, whereas activation of the ncNFκB pathway, which requires de novo protein synthesis (17), follows a slower pattern. This time frame is consistent with data from mouse CD4+ T cells, suggesting a switch from predominantly canonical to noncanonical NFκB signaling in later stages of an immune response (33). Interestingly, the 2-day stimulation required to activate the ncNFκB pathway coincides with the kinetics of Traf3 alternative splicing, which reaches its full extent 2–3 days after stimulation and also requires de novo protein synthesis (data not shown).

To confirm increased ncNFκB activity in PMA-treated T cells with an independent method, we used RT-qPCR of four known ncNFκB target genes (CxCL12, CxCL13, RxRa, and IcosL; Refs. 14, 34), chosen based on detectable expression in unstimulated Jsl1 cells. Expression of all four genes showed a significant increase upon stimulation, strongly suggesting higher ncNFκB signaling in PMA-treated cells (Fig. 2D). The different magnitude of the response could indicate varying p52-RelB affinities for the respective promoters; alternatively, PMA could induce additional signaling pathways that enhance or dampen ncNFκB-mediated transcription. Consistent with an unchanged IkBα protein level upon 2-day PMA treatment (Fig. 2A), IkBα mRNA remained unaltered (Fig. 2D).

One of the crucial regulators of the ncNFκB pathway is NIK, which, if present in the cell, initiates signaling. Consistently, NIK expression was increased in PMA-treated Jsl1 T cells (Fig. 2E). Interestingly, the ncNFκB signaling pathway was not activated in PMA-treated Raji or Ramos cells, and neither did we observe an increase in NIK expression in these cells (data not shown). This provided a first link between Traf3 alternative splicing and ncNFκB activation, as both occurred only in Jsl1 T cells. Together these data provide evidence for a selective up-regulation of the ncNFκB pathway upon prolonged stimulation of human T cells leading to increased expression of several chemokines, among them CxCL13.

Expression of Traf3DE8 Mimics PMA Stimulation with Respect to ncNFκB Signaling—Traf3 has been implicated in the regulation of NIK stability in B cells where stimulation of various receptors induces Traf3 degradation to allow NIK accumulation. We reasoned that Traf3 alternative splicing could fulfill a similar role and tested whether overexpressing Traf3DE8 in resting cells would activate ncNFκB signaling. Indeed, we detected increased formation of p52 and higher NIK levels in cells stably expressing Traf3DE8 whereas full-length Traf3 (Traf3fl) had no such effect (Fig. 3A and B). Furthermore, we verified induction of the same ncNFκB target genes that were up-regulated by PMA stimulation in resting Traf3DE8-expressing cells, which was not the case for cells expressing similar amounts of Traf3fl (Fig. 3C). We note that the different targets respond differentially to either PMA stimulation or Traf3DE8 overexpression, which may suggest that PMA induces several pathways that have positive and/or negative effects on the respective promoters. The increase of target gene expression by Traf3DE8 overexpression was again specific for the ncNFκB pathway as IkBα mRNA did not change (Fig. 3C).

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**Traf3 Exon 8 Skipping Is Sufficient to Induce ncNFκB Signaling**—To show a direct correlation between Traf3 exon 8 skipping and ncNFκB activation at endogenous expression levels, we used splice site blocking MO (scheme in Fig. 3D). A MO directed against the upstream splice site of exon 8 induced robust exon skipping detectable at mRNA (Fig. 3D, bottom) and protein level (Fig. 3E). This led to increased formation and nuclear translocation of p52 while IkBα remained at a constant expression level (Fig. 3, E and F). We also observed nuclear translocation of RelB (Fig. 3F) and increased binding to an NFκB-specific probe upon MO treatment, mimicking the results from PMA-treated cells (data not shown). We furthermore detected higher NIK expression in Traf3E8 MO-treated cells (Fig. 3G) and increased expression of all four tested ncNFκB target genes (Fig. 3H), which was slightly less strong than in Traf3DE8-overexpressing cells. The relative magnitude was similar between the two conditions (stronger for CxCL12 and RxRa), suggesting that it is caused by the same pathway with different levels of activation. We also noted a statistically not significant increase in mRNA expression of IkBα in Traf3E8 MO-transfected cells, which could be the result of some cross-talk between the two NFκB branches under certain cellular conditions. However, as we did not detect a change of IkBα protein at different time points after Traf3E8 MO transfection (Fig. 3E and data not shown) a direct activation of the canonical NFκB pathway by the Traf3E8 MO appears unlikely.

Additional experiments showed that control MO and Traf3E8 MO-treated cells responded similarly to PMA or TNFα treatment with ERK activation and IkBα degradation, respectively, confirming that the Traf3E8 MO did not cause a general signaling defect (data not shown). Furthermore, the interferon response, which can be regulated by Traf3 (35), was not altered in Traf3E8 MO-treated cells (data not shown). Our combined results are consistent with a model in which Traf3 exon 8 skipping specifically regulates ncNFκB activity to provide a mechanism that controls this signaling pathway in a previously unknown, intrinsic way.

**Traf3DE8 Is Necessary for PMA-mediated Induction of ncNFκB Targets**—Having shown that Traf3 exon 8 skipping is sufficient for ncNFκB activation we investigated whether it is also necessary for ncNFκB activity in stimulated T cells. To this end we designed an siRNA that spans the junction of Traf3 exons 7 to 9 and which should thus specifically target the Traf3DE8 isoform (Fig. 4A). We transfected this siRNA in Jsl1 cells, stimulated with PMA for 2 days and first investigated Traf3 isoform expression. In control samples we observed the expected increase of the Traf3DE8 isoform to 44%. In contrast, the specific siRNA decreased Traf3DE8 expression to 27% (Fig. 4B), and a RT-PCR investigating all five Traf3 isoforms confirmed that Traf3DE8 was specifically reduced (Fig. 4C). Importantly, this partial loss of Traf3DE8 caused reduced induction of the ncNFκB target genes as assessed by RT-qPCR (Fig. 4D). We conclude from this experiment that Traf3DE8 expression is not only sufficient to induce the ncNFκB signaling pathway (Fig. 3) but also necessary to do so in activated T cells (Fig. 4).

**Traf3DE8 Disrupts the NIK-Traf3-Traf2 Complex to Allow NIK Accumulation**—Having established a role for Traf3DE8 in controlling ncNFκB activity and NIK accumulation, we next...
investigated the molecular basis for this regulation. Formation of a NIK-Traf3-Traf2-cIAP complex, in which Traf3 forms a “molecular bridge” to bring NIK in proximity of the Traf2-cIAP ubiquitin ligase, plays a central role in controlling NIK levels (21–25). When performing immunoprecipitations with overexpressed NIK and Traf3fl or Traf3DE8 proteins, we noted a strong reduction in the interaction of NIK with Traf3DE8 compared with full-length Traf3 (Fig. 5A). On the contrary, Traf3DE8 interacted stronger with Traf2 than did Traf3fl (Fig. 5B). A preferred interaction of Traf3DE8 with Traf2 and a reduced interaction with NIK would lead to a dominant negative action, separating NIK from the complex and allowing its accumulation. To confirm this model we coexpressed Traf2-FLAG, Traf3fl-, or Traf3DE8-GFP and NIK, performed FLAG-IP, and assayed for the presence of NIK in the precipitate. Substantial amounts of NIK were only detected in the presence of Traf3fl, whereas NIK-coprecipitation was strongly reduced in the presence of Traf3DE8 (Fig. 5C), again suggesting that Traf3DE8 separates NIK from Traf2. To consolidate this model and to show its relevance during T cell activation we repeated IPs with endogenous proteins in resting and activated T cells. To this end we stimulated Jsl1 cells with PMA, treated them with MG132 to allow NIK accumulation also in resting cells, and then used anti-Traf2 for immunoprecipitation. Consistent with our model that Traf3DE8 separates NIK from Traf2, we found a stronger coprecipitation of NIK in resting T cells (Fig. 5D). These data are in agreement with a model in which the reduced interaction of Traf3DE8 with NIK and the stronger interaction with Traf2 disrupt the NIK-Traf3-Traf2 complex in a dominant negative way, thus separating NIK from the Traf2-cIAP ubiquitin ligase and allowing its accumulation and initiation of ncNFκB signaling in activated T cells.

FIGURE 3. Traf3DE8 expression is sufficient to induce ncNFκB signaling. A and B, WCEs of parental Jsl1 cells (−) or cells stably overexpressing the indicated constructs analyzed by Western blotting with the indicated antibodies. C, RT-qPCR as in Fig. 2D with RNA from three independently generated stable clones overexpressing Traf3fl, Traf3DE8, or parental control (−), each assessed in duplicate and represented as -fold induction relative to control. p values are calculated between control and Traf3DE8. Error bars, S.D. D, scheme of the MO experiment (top) and the effect of Traf3 exon 8 MO (Traf3E8) transfection on Traf3 isoform expression in Jsl1 cells assessed by RT-PCR (bottom). E, Jsl1 cells transfected with a control MO or increasing amounts of a Traf3 exon 8 MO (wedge) for 2 days. WCEs were analyzed by Western blotting. F and G, nuclear extract (F) or WCE (G) from Jsl1 cells transfected as in D and analyzed by Western blotting. H, RT-qPCR performed with RNA from cells as in F. -Fold changes were calculated with cMO-transfected cells set to 1. Values shown are mean values from four (CxCL12, CxCL13) or 5 (IcosL, RxRa, IκBα) independent transfections assessed in duplicate.
Traf3 Exon 8 Skipping and the ncNFκB Pathway Are Activated upon Anti-CD3 Stimulation of Primary Human CD4+ T Cells—To investigate a role of the mechanism described above in a physiologically relevant setting, we characterized Traf3 exon 8 exclusion and ncNFκB activation in primary cells. We focused on human PBMCs as a primary system related to Jsl1 cells and first used prolonged PMA stimulation as in the Jsl1 experiments. Two-day PMA treatment resulted in Traf3 exon 8 exclusion in PBMCs in a magnitude similar to that in Jsl1 cells, confirming the presence of this pathway in primary human cells (Fig. 6A). However, this PMA treatment strongly induced apoptosis in PBMCs (data not shown), preventing further meaningful analyses. We therefore turned to anti-CD3 treatment, as a physiologically relevant, T cell-specific stimulus. Activation of PBMCs with anti-CD3 also increased Traf3 exon 8 skipping (Fig. 6A), but less strong than PMA treatment. The effect was even more pronounced in purified CD4+ T cells than in whole PBMCs, indicating that it mainly occurs in the CD4+ T cell subset (Fig. 6A). The difference in the response to PMA and anti-CD3 stimulation and earlier experiments using phyto-
hemagglutinin stimulation of CD4+ T cells that showed no increase in Traf3 exon 8 skipping (3) suggest a stimulus-dependent effect. In line with our cell culture data, Traf3 alternative splicing and ncNFκB activity correlated, as we detected substantial amounts of p52 and nuclear RelB only in CD3-activated PBMCs (Fig. 6B). Whereas we found several ncNFκB targets to be regulated in our model T cell line, anti-CD3 stimulated primary human PBMCs and CD4+ T cells showed an induction of CxCL13 in particular (Fig. 6C). Anti-CD3 stimulation may activate other transcription factors through different signaling pathways that together control the respective promoters in primary cells; alternatively, the threshold for activation through the ncNFκB pathway could be lower for CxCL13 in primary cells, and the stimulation was not sufficiently strong to activate other targets. Such a lower threshold for ncNFκB signaling could also explain the very strong induction of CxCL13 in activated PBMCs and CD4+ T cells, which may not be fully recapitulated in the Jsl1 T cell line.

DISCUSSION

In our work we provide evidence for a regulatory function of Traf3 exon 8 alternative splicing, namely the activation of the ncNFκB pathway which we propose to impact on the T cell dependent immune response. Our data suggest a model in which Traf3DE8 activates ncNFκB activity by disrupting the NIK-Traf3-Traf2 complex. This results in increased NIK levels and, by activating ncNFκB signaling, induces expression of chemokines such as CxCL13, which is involved in B cell migration and activation. Together, we define a splicing switch that controls chemokine expression upon T cell activation, providing a new example of how alternative splicing contributes to regulate T cell function. An interesting feature of this regulatory pathway is the timing of ncNFκB induction, which happens, in contrast to many signaling events that occur within seconds, only after prolonged stimulation and depend on the kinetics of Traf3 alternative splicing. Other signaling pathways in activated T cells are controlled similarly, by a delayed, splicing-dependent mechanism (e.g. the activity of CD45; Ref. 30), potentially suggesting that alternative splicing is more involved in regulating the later phases of a T cell response rather than being immediately downstream of T cell receptor signaling.

Exclusion of Traf3 exon 8 alters the zinc finger domain within the protein, which connects the N-terminal RING and the C-terminal Traf domains. Earlier studies have shown that both the Traf
and the RING domains are involved in Traf3-mediated ncNFκB inhibition (21, 22). The Traf domain is also required for the Traf3-NIK interaction (22, 25), whereas the Traf3-Traf2 interaction could not be mapped to one domain alone (36), suggesting a more complex interaction surface. This could make the Traf3-Traf2 interaction susceptible for a regulation by an internal deletion as we observe in Traf3DE8. Our data together with previous studies are consistent with a model in which the zinc finger domain of Traf3 is not essential for the interaction with NIK and Traf2 but rather fine tunes these interactions, probably by altering the spatial arrangement of the RING and Traf domains within the Traf3 protein. Our combined data suggest that Traf3DE8 and full-length Traf3 have opposite functions with respect to ncNFκB signaling: whereas Traf3fl leads to formation of the NIK-Traf3-Traf2-clAP complex and NIK degradation, Traf3DE8 disturbs this complex to allow NIK accumulation. As Traf3 exon 8 skipping reduces the amount of Traf3fl and increases the expression of Traf3DE8, this splicing switch serves a dual role in activating ncNFκB signaling upon prolonged T cell activation. Such a dual function increases the impact of Traf3 alternative splicing such that already a modest increase in exon 8 skipping can have a significant influence on NIK levels.

CxCL13 has been well described as an important regulator of B cell recruitment during formation and activation of the adaptive immune system (16, 18), has been suggested to impact B cell activation directly (37), and has been shown to be controlled by the ncNFκB pathway (14). However, expression of CxCL13 (and other organogenic chemokines) has been attributed mainly to stromal cells with an essential role in secondary lymphoid organ formation (17). In this context, signaling through the lymphotixin β receptor is required to induce the ncNFκB pathway leading to chemokine expression. Our data reveal that the ncNFκB pathway is also activated upon stimulation of human T cells. However, the mechanism and the functional outcome in T cells are quite different from stromal cells. In our model, Traf3 alternative splicing activates ncNFκB signaling which is not necessarily dependent on a transmembrane receptor. Traf3 alternative splicing thus provides the cell with the means to regulate ncNFκB signaling in a cell autonomous manner. Functionally, expression of CxCL13 by an activated CD4+ T cell could, by attracting B cells, be an important step in linked recognition and B cell activation (37), which is different from the expression of the same chemokine in stromal cells. This model is in line with and provides a molecular basis for the finding that CD4+ germinal center T cells express CxCL13 in an inducible manner starting 48 h after stimulation (19).

Traf3 exon 8 is excluded specifically upon activation of T cells, whereas no other cell type tested, two B cell lines among others, shows a similar regulation. Establishment of cell type-specific splicing profiles is a matter of intense research, and mechanisms have been proposed in which either one dominant regulator or subtle differences in several splicing regulatory proteins lead to a particular splicing pattern (38). As Traf3 exon 8 skipping requires de novo protein synthesis, this regulation may involve changes in expression levels of RNA-binding proteins (39); however, mechanisms involving stable posttranslational modifications are also conceivable (30). Cell type specificity may be achieved by the T cell–restricted expression of signaling components or proteins that modify expression or posttranslational modification of the respective RNA-binding proteins.

Investigating the functional impact of alternative splicing is one of the challenging questions in postgenome RNA biology (40). With the present work, we provide a new example that connects alternative splicing with T cell function in a model cell line as well as in primary human cells underlining the impact of activation induced alternative splicing on T cell biology.

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Footnote:

5 Michel and F. Heyd, unpublished observation.
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