

ORIGINAL RESEARCH



## Deubiquitinases A20 and CYLD modulate costimulatory signaling via CD137 (4–1BB)

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### ABSTRACT

TRAF2 dependent K63-polyubiquitinations have been recently shown to connect CD137 (4–1BB) stimulation to NF- $\kappa$ B activation. In a search of deubiquitinase enzymes (DUBs) that could regulate such a signaling route, A20 and CYLD were found to coimmunoprecipitate with CD137 and TRAF2 complexes. Indeed, overexpression of A20 or CYLD downregulated CD137-elicited ubiquitination of TRAF2 and TAK1 upon stimulation with agonist monoclonal antibodies. Moreover, overexpression of A20 or CYLD downregulated CD137-induced NF- $\kappa$ B activation in cultured cells and in gene-transferred hepatocytes *in vivo*, while silencing these deubiquitinases enhanced CD137 costimulation of primary human CD8 T cells. Therefore A20 and CYLD directly downregulate the signaling from a T and NK-cell costimulatory receptor under exploitation for cancer immunotherapy in clinical trials.

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### KEYWORDS

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## Introduction

The family of the TNF receptor lacks cytoplasmic enzymatic domains and relies on partnering with adaptors, such as members of the TRAF family, for signal transduction.<sup>1–3</sup> While some members of the TNFR family recruit a caspase activating machinery, others lack death domains and primarily activate inflammatory functions.<sup>4</sup> This is the case, for instance, of CD137, CD27, OX40, GITR that are well known T-cell costimulators.<sup>5</sup> In fact, upon ligation these surface receptors enhanced antitumor immune responses and agonist mAb are currently being tested across multiple clinical trials.<sup>6</sup>


TNFRSF9 (CD137 or 4–1BB) is a TNFR family member that is able to costimulate T and NK lymphocytes upon ligation with its natural ligand (4–1BBL) or with agonist monoclonal antibodies (mAbs).<sup>7</sup> Its intracytoplasmic domain has been found to physically interact with the adaptors TRAF1 and TRAF2.<sup>8</sup> While TRAF1 biochemical activity largely remains a mystery,<sup>9</sup> TRAF2 encompasses a RING domain predicted to act as an E3 ubiquitin ligase generating K63 polyubiquitin chains. K63 polyubiquitination of protein substrates or free K63 polyubiquitin chains<sup>10</sup> act as docking sites to sustain downstream signaling to the NF- $\kappa$ B pathway ubiquitinating several substrates such as TAK1, NEMO, IKK $\alpha$  and IKK $\beta$ .<sup>10</sup> In the case of CD137, we have recently reported that receptor stimulation leads to K63 polyubiquitination of TRAF2 itself and that this pathway is necessary for canonical NF- $\kappa$ B

signaling and for receptor internalization.<sup>11</sup> Since the catalytic domain of TRAF2 is structurally doubtful to accommodate the E2 enzyme Ubc13,<sup>12</sup> it is possible that this activity could be mediated by cIAPs, other E3 enzymes that are known to physically associate with TRAF2.<sup>13,14</sup>

E3 K63 polyubiquitin reactions are controlled by proteases that degrade these complexes and conform the so called Deubiquitinase (DUB) family.<sup>15–17</sup> Among the DUBs able to degrade K63 polyubiquitins 2 enzymes stand out for their known regulatory activity on the NF- $\kappa$ B pathway and on immune and inflammatory responses, namely A20 and CYLD (Cylindromatosis Turban Tumor Syndrome).<sup>10,15,17</sup>

A20<sup>18</sup> is encoded by the TNF $\alpha$ -inducible gene 3 (*TNFAIP3*) and is a complex enzyme since it encompasses an N-terminal OTU domain with K63 deubiquitinase activity and its C-terminal region a Zinc finger domain (ZF4 domain) with E3 K48-ubiquitin ligase activity that targets proteins for proteasomal degradation.<sup>18–20</sup> A20 is considered a tumor suppressor gene in lymphoma.<sup>21,22</sup> Gene-targeting experiments conclusively demonstrate an anti-inflammatory role for A20 that prevents autoimmunity and excessive inflammation.<sup>23–26</sup> Selective gene deletion in the skin and immune cells including T and B cells<sup>23,26</sup> strongly supports this critical regulatory role. In humans, A20 polymorphisms have been statistically linked to autoimmune conditions.<sup>27</sup>

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CYLD<sup>28</sup> is another DUB that removes and degrades K63-linked polyubiquitin chains from substrates,<sup>29,30</sup> including those attached to TRAF2<sup>17,31</sup> and other molecular targets in the activation of the canonical NF- $\kappa$ B pathway.<sup>17,32</sup> Its ability to downregulate the NF- $\kappa$ B is favored by its strong physical association with NEMO (IKK $\gamma$  subunit). Deficient function of CYLD was first ascribed to cause predisposition to cylindromatosis, a condition causing multiple benign skin tumors.<sup>28</sup> Knock-out mouse models have shown involvement of CYLD not only in immune cell function,<sup>33</sup> but also in osteoclast function<sup>34</sup> and spermatogenesis.<sup>35</sup> Deubiquitination of mediators in NF- $\kappa$ B MAPK and Wnt signaling routes have been found to be involved in such processes.

Suspecting that E3 K63 polyubiquitin-removing enzymes could downregulate CD137-TRAF2 signals, we have found that both A20 and CYLD coimmunoprecipitate with this multiprotein complex. Furthermore, a marked inhibition of CD137-dependent NF- $\kappa$ B activation was observed when A20 or CYLD were overexpressed, whereas silencing of such genes upregulates the intensity of CD137 signaling. These results are consistent with a role of A20 and CYLD in downregulating CD137 costimulation.

## Results

### **A20 and CYLD coimmunoprecipitate with CD137-TRAF2 complexes regulating ubiquitination reactions**

In a previous report, we showed that CD137 ligation results in K63-polyubiquitination of TRAF2.<sup>11</sup> Indeed TRAF2 is well known to physically interact with the cytoplasmic domain of CD137 as a signaling mediator and we mechanistically proposed that TRAF2, under CD137 stimulation, K63-polyubiquitinates sister TRAF2 moieties.<sup>7,16</sup> Fig. 1A shows coimmunoprecipitation experiments confirming CD137-TRAF2 interaction.<sup>8</sup> In these immunoprecipitates, we searched for the presence of deubiquitinases that would be candidates to downregulate such CD137 signaling pathway. As can be seen in Fig. 1A, when 293 T cells were cotransfected with CD137 and either Flag-tagged A20 and/or CYLD, both DUBs were found to associate with the CD137 complex. In these experiments TRAF2 was not transfected since 293 T constitutively express this adaptor protein. Furthermore, as shown in Fig. S1, an anti-CD137 mAb pulled down both TRAF2 and 2 different deletion mutants of A20: an N-terminal deletion missing the OTU deubiquitinase domain and a C-terminal deletion mutant lacking the ZF4 domain which mediates K48 E3 ligase activity.<sup>36</sup> These data are interpreted in the sense that binding of A20 to the complex is independent of these enzymatic functions.<sup>17,37</sup>

To ascertain if these proteins were actually involved in the regulation of the ubiquitination reactions, HEK 293 T cells expressing CD137 were engaged with anti-CD137 mAb during 5 minutes. Then, ubiquitinated proteins were immunoprecipitated with antibodies against ubiquitin. The total amount of ubiquitinated proteins, TAK1 and TRAF2 (Fig. 1B) contained in these immunoprecipitates was analyzed by SDS-PAGE and Western blotting. These experiments, and their respective quantitative densitometries (Fig. 1C), indicate that both A20 and CYLD downregulated the level of

ubiquitination of the analyzed proteins induced by CD137 stimulation (Fig. 1C). However, such an effect was clearer for both DUBs in the case of TAK1 as a substrate, while in the case of TRAF2, CYLD but not A20 overexpression downregulated the conjugated polyubiquitin content, a finding consistent with previous reports.<sup>17</sup>

### **A20 and CYLD inhibit NF- $\kappa$ B activation elicited by CD137 stimulation**

We next assessed whether overexpression of A20 and CYLD down-regulates the NF- $\kappa$ B transcriptional activity triggered by the stimulation of CD137 with agonist mAb. In a previous study,<sup>11</sup> we showed that CD137-mediated NF- $\kappa$ B activation was dependent on TRAF2 and required K63 ubiquitination, since overexpression of a dominant negative mutant form of ubiquitin (K63R) abrogated NF- $\kappa$ B activation.

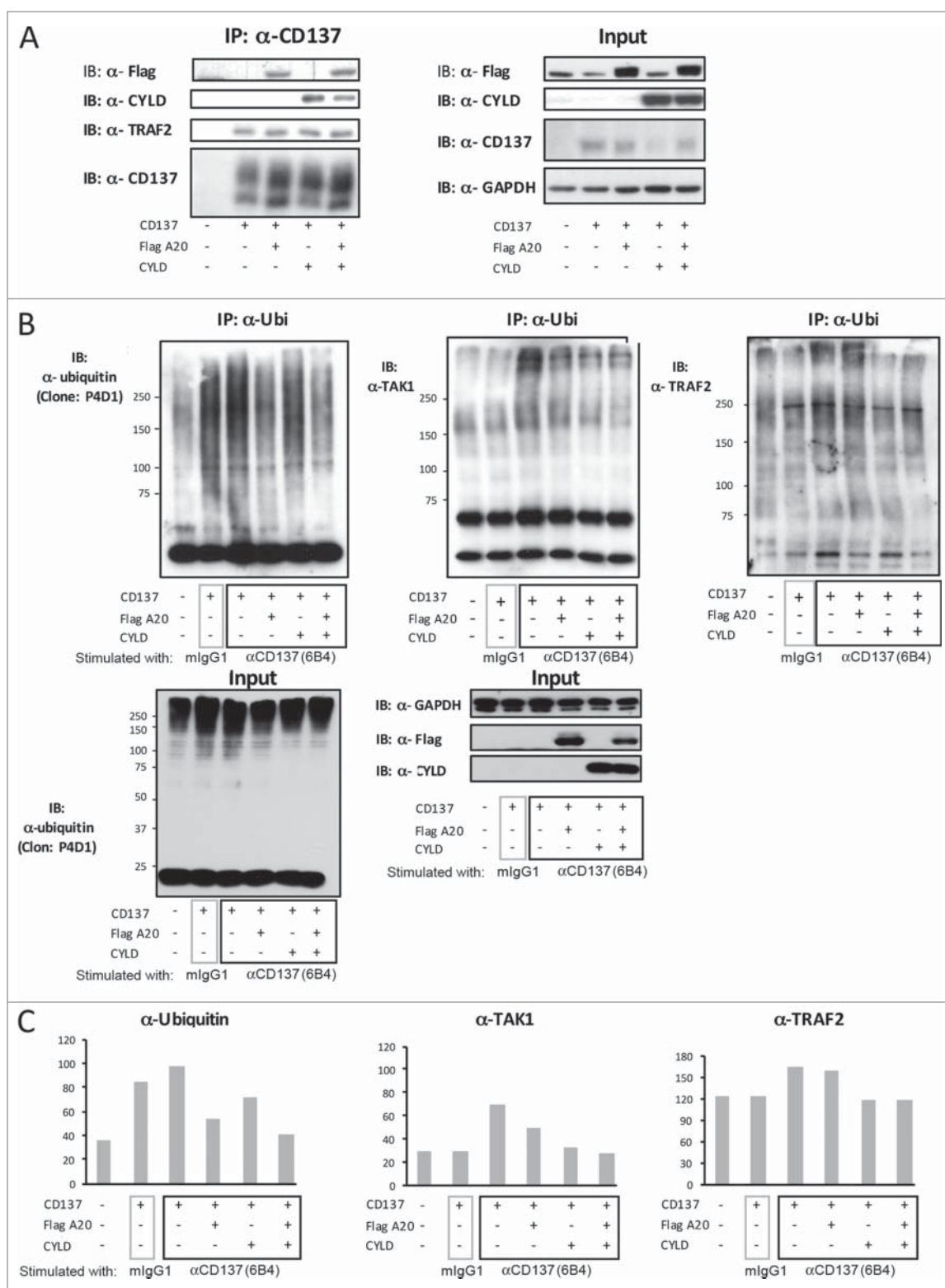
Experiments using a NF- $\kappa$ B luciferase reporter construction indicated that enforced expression of either A20 or CYLD caused the suppression of NF- $\kappa$ B activation in response to the agonist anti-CD137 mAb 6B4 in transiently transfected 293 T cells (Fig. 2A).<sup>11</sup> The extent of the inhibition was comparable to that exerted by mutant ubiquitin K63R through a negative dominant effect. Consistent with these findings attenuation in I $\kappa$ B degradation was observed upon overexpression of A20 and CYLD under similar experimental conditions (Fig. 2B). In addition, the enforced expression of A20 N-terminal and C-terminal deletion mutants showed that both truncated proteins retained the inhibitory activity on NF- $\kappa$ B activation (Fig. S2), even if, as described in the literature, the C-terminal part of the protein has a stronger effect on NF- $\kappa$ B inhibition.<sup>37,38</sup>

The inhibition of CD137-mediated NF- $\kappa$ B activation by A20 and CYLD was also observed in transient transfections of transformed T lymphocytes of the MOLT4 and CEM cell lines (Fig. 2C), thus indicating that A20 and CYLD could regulate CD137 activity in the cell type where CD137 exerts its physiologic costimulatory functions.

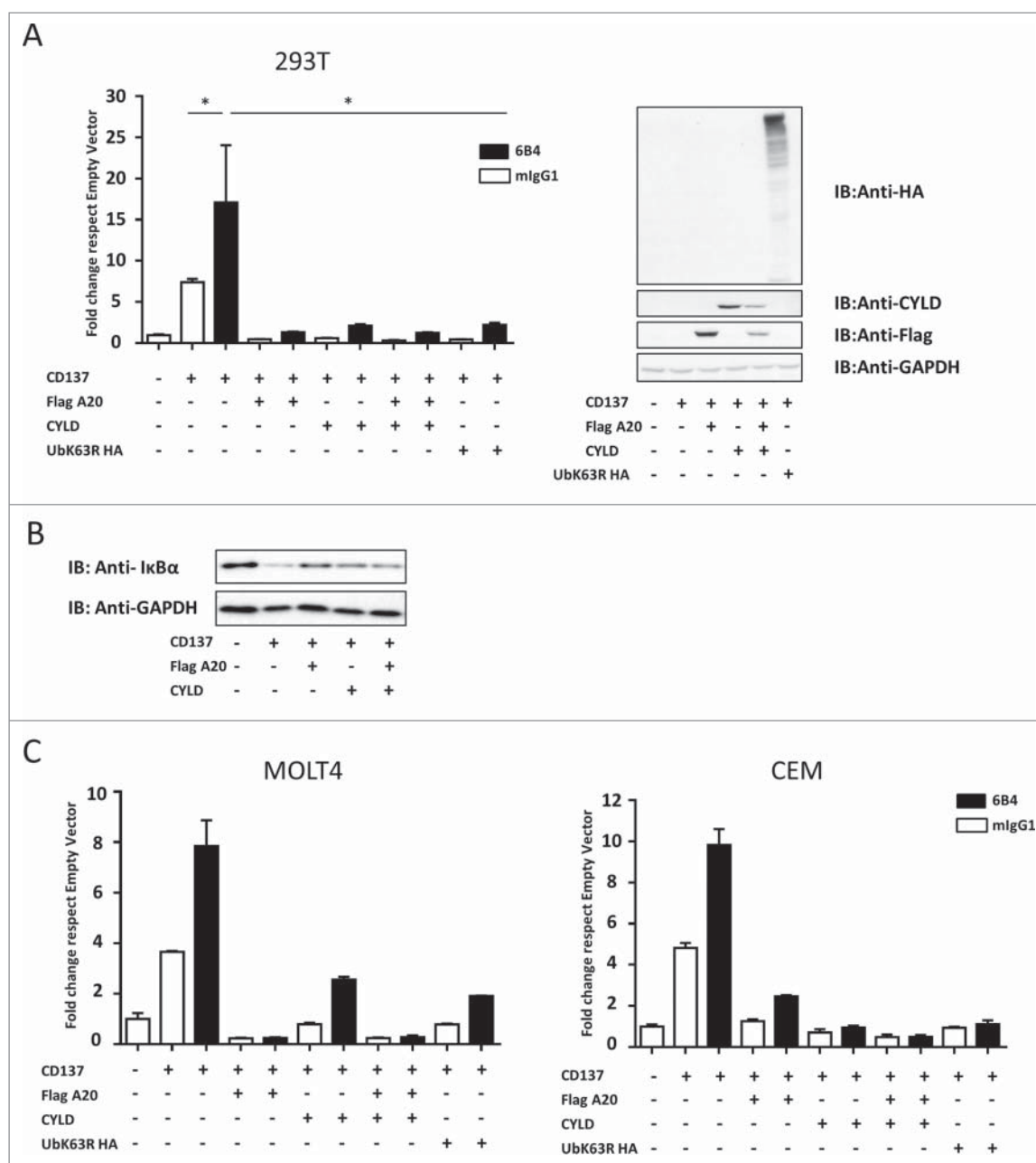
### **Silencing A20 and/or CYLD up-regulates NF- $\kappa$ B activation following CD137 stimulation**

We hypothesize that if A20 and CYLD immunoprecipitate with the CD137 proximal signaling complex and regulate the intensity of the signal, their reduced expression should result in NF- $\kappa$ B enhanced transcriptional activity.

To explore this point, we used magnetically sorted human CD8 T lymphocytes that were stimulated for 24 hours with anti-CD3/CD28 coated beads. Lymphocytes were then transduced with lentivirus encoding EGFP with or without 2 distinct shRNAs targeting the A20 transcript. Lymphocytes were then cultured on plates coated with anti-CD3 + anti-CD137 (6B4) mAbs or anti-CD3 mAb + control mIgG1 antibody (Fig. 3A). Three days later, GFP<sup>+</sup> (transduced) and GFP<sup>-</sup> (non-transduced) lymphocytes (Fig. 3B) were analyzed for Bcl-xL expression by intracellular immunofluorescence as a parameter to measure CD137 costimulatory activity.<sup>7</sup> Fig. 3 C (top) and D show an increase in Bcl-xL in CD8 T cells blasts transduced with the shRNAs targeting A20



**Figure 1.** A20 and CYLD coprecipitate with CD137-TRAF2 and downregulate ubiquitination of TAK1 and TRAF2. (A) Lysates from 293 T cells transiently co-transfected with expression plasmids containing the indicated expression cassettes encoding human CD137, human A20-flag and CYLD were immunoprecipitated with Protein G sepharose-coupled anti-CD137 mAb (6B4). SDS-PAGE blots were probed with antibodies to detect flag, CYLD, TRAF2 and CD137. GAPDH was used as a loading control. (B, C and D) 293 T cells were cotransfected with plasmids encoding the indicated expression cassettes and 24 h following transfection, cells were incubated with the agonist anti-CD137 mAb 6B4 or control mouse IgG1. Lysates were immunoprecipitated with the anti-ubiquitin antibody FK2 coupled to protein G sepharose beads and blots were probed with the indicated detection antibodies anti-Ub, anti-TAK1 and anti-TRAF2. Content of expressed proteins in the input cell lysates are shown in each panel. (C) Maximal intensity band regions were quantified by densitometry and relative values presented in the bar diagrams. A representative case out of at least 4 experiments similarly performed is shown.



**Figure 2.** Overexpression of A20 and/or CYLD downregulates NF- $\kappa$ B activation elicited by CD137 ligation. (A) 293 T cells transiently transfected with the indicated expression plasmids for 18 hours were stimulated for 5 hours with anti-CD137 (6B4) or control mlgG1. Experiments were read out by means of cotransfection with the NF- $\kappa$ B luciferase reporter construction and subsequent incubation with luciferin. Results are shown as relative luminescence units between firefly luciferase activity and renilla luciferase as an internal control (mean  $\pm$  SEM) given as fold change with respect to the empty vector (left panel). Immunoblots in lysates of the indicated transfectants confirm expression of the corresponding proteins (right panel). (B) Immunoblots for I $\kappa$ B $\alpha$  and anti-GAPDH (loading control) in lysates from 293 T cells transfected as in A with the indicated constructs. (C and D) Experiments performed as in A in MOLT4 and CEM T cells electroporated with the plasmids encoding the indicated expression cassettes. A representative set of experiments out of at least 6 performed is shown. In this case, cells were electroporated 18 hours before the experiment and cells were exposed to antibodies for 24 hours before NF- $\kappa$ B activity measurement in lysates. Quantitative RT-PCRs confirming mRNA expression of the plasmid were performed and CD137 surface expression was checked by flow cytometry (not shown). Shown experiments were consistent with at least 5 similarly performed.

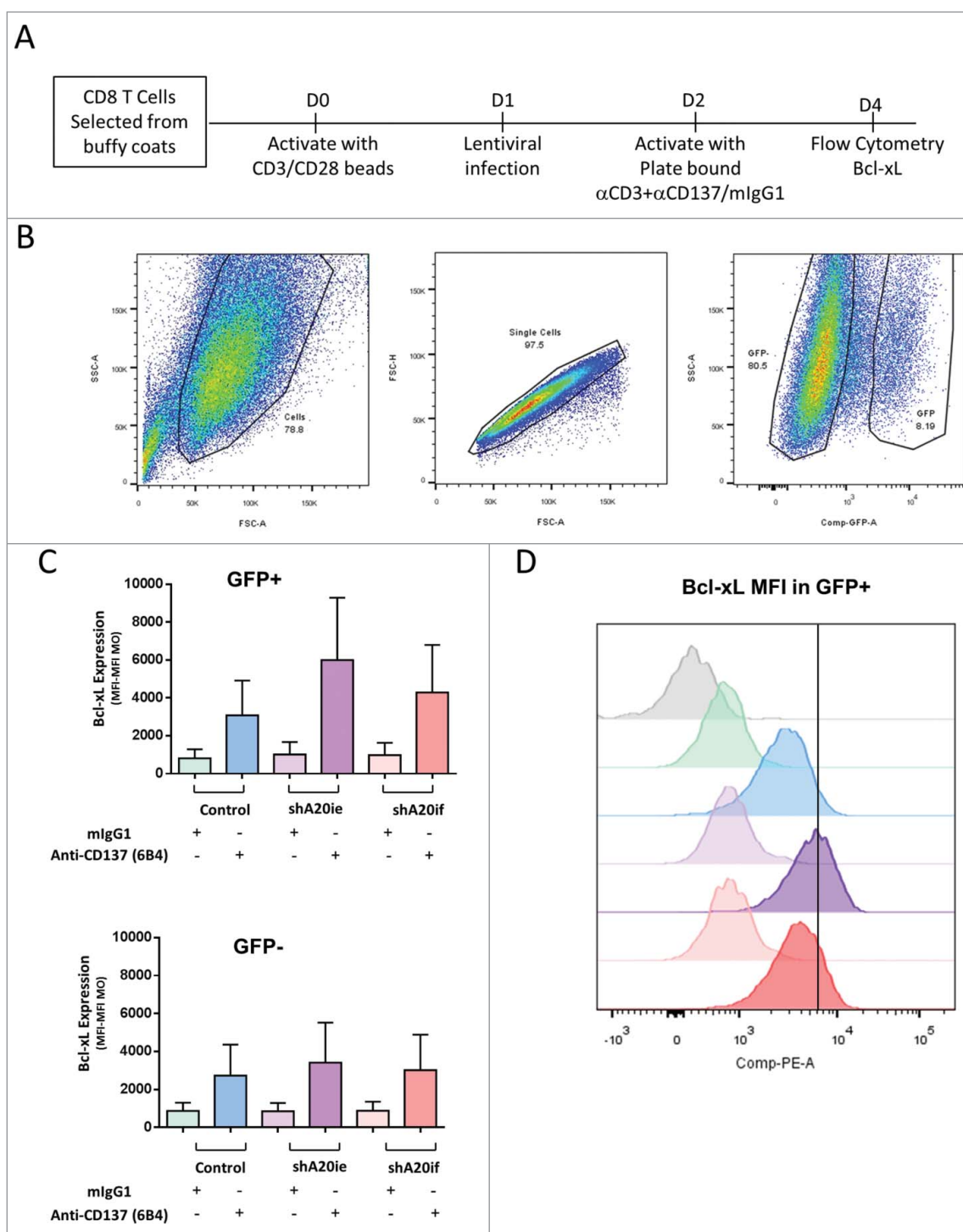
compared with the non-transduced cells and control virus transduced cells (Fig. 3C and D).

Furthermore, we electroporated siRNA oligonucleotides into human primary CD8 T cells to assess the effect of A20 and CYLD on the costimulating effects of CD137 (Fig. 4A). Fig. 4B shows a representative experiment indicating that upon A20 or CYLD transient silencing, CD137 costimulation triggered higher expression of CD25 and Bcl-xL, which are well known effects of CD137 costimulation.<sup>7</sup>

### **A20 and CYLD increased in vivo the NF- $\kappa$ B activation triggered by treatment with agonist anti CD137 mAb**

Using hydrodynamic gene transfer technology, cDNA-encoding vectors can be forced to enter into hepatocytes allowing the expression of the encoded genes *in vivo*.<sup>39</sup> Using this approach, we have previously shown that hydrodynamic gene transfer of plasmids encoding CD137 and a NF- $\kappa$ B luciferase reporter system were efficiently expressed in hepatocytes *in*



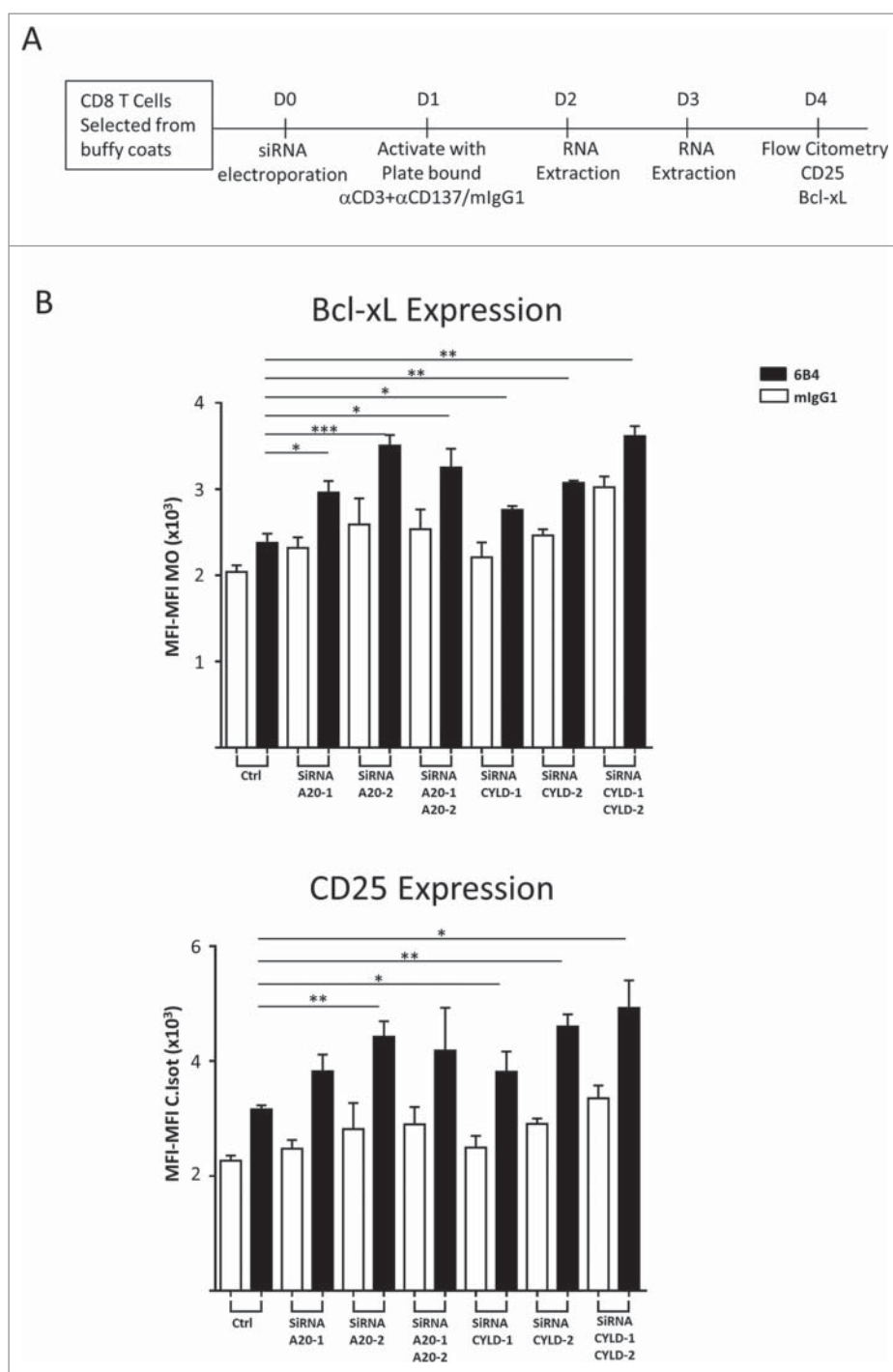


**Figure 3.** Silencing of A20 in primary human T cells with a lentivirus encoding a specific shA20 enhances Bcl-xL expression following CD137 stimulation. CD3-CD28 activated human CD8 T cells were transfected with pLVTHM/A20ie, pLVTHM/A20if viruses or a control pLVTHM lentivirus. A scheme of the experiments is provided in (A). EGFP expression permits FACS gating of the successfully transfected cell population as shown in (B). In (C) Bcl-xL intracellular immunostaining intensity (MFI) is shown on gated GFP-positive and GFP-negative cells following stimulation with plate bound anti-CD137 mAb or control antibody during 72 hours in culture. In (D) a representative case is presented with the intensity of fluorescence of the corresponding color-coded histograms for each experimental condition. A representative experiment out of 2 independently performed is shown.

*in vivo* and could respond to systemic administration of agonist anti-CD137 mAb with increased liver luciferase biosynthesis.<sup>11</sup>

In the experiments shown in Fig. 5, using hydrodynamic gene transfer, we assessed the effect of enforced expression of A20 or/and CYLD on CD137-mediated NF- $\kappa$ B activity (Fig. 5A). Both the anti-CD137 mAb 6B4 and the clinical

grade anti-CD137 mAb Urelumab enhanced the luciferase-dependent light emission in the liver area upon CD137 engagement by agonist antibodies (Fig. 5B and C). However, enforced coexpression of either A20 or CYLD hindered luciferase expression. Fig. 5D shows the light emission pattern in representative mice. These data indicate that both A20 and



**Figure 4.** siRNA-silencing of A20 and CYLD augments costimulation triggered by CD137 stimulation on human primary CD8 T cells. (A) Schematic representation of the experiments. (B) Human CD8 T cells were electroporated with the indicated siRNAs and underwent stimulation with anti-CD3 plus anti-CD137 mAb. Four days later CD25 and Bcl-xL expression were monitored by immunofluorescence and flow cytometry. Data are shown as mean intensity of fluorescence (mean  $\pm$  SEM). Downregulation of the corresponding mRNAs was confirmed by qRT-PCR (not shown). A representative experiment out of at least 3 independently performed is shown. Statistical comparisons were performed using students t tests (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

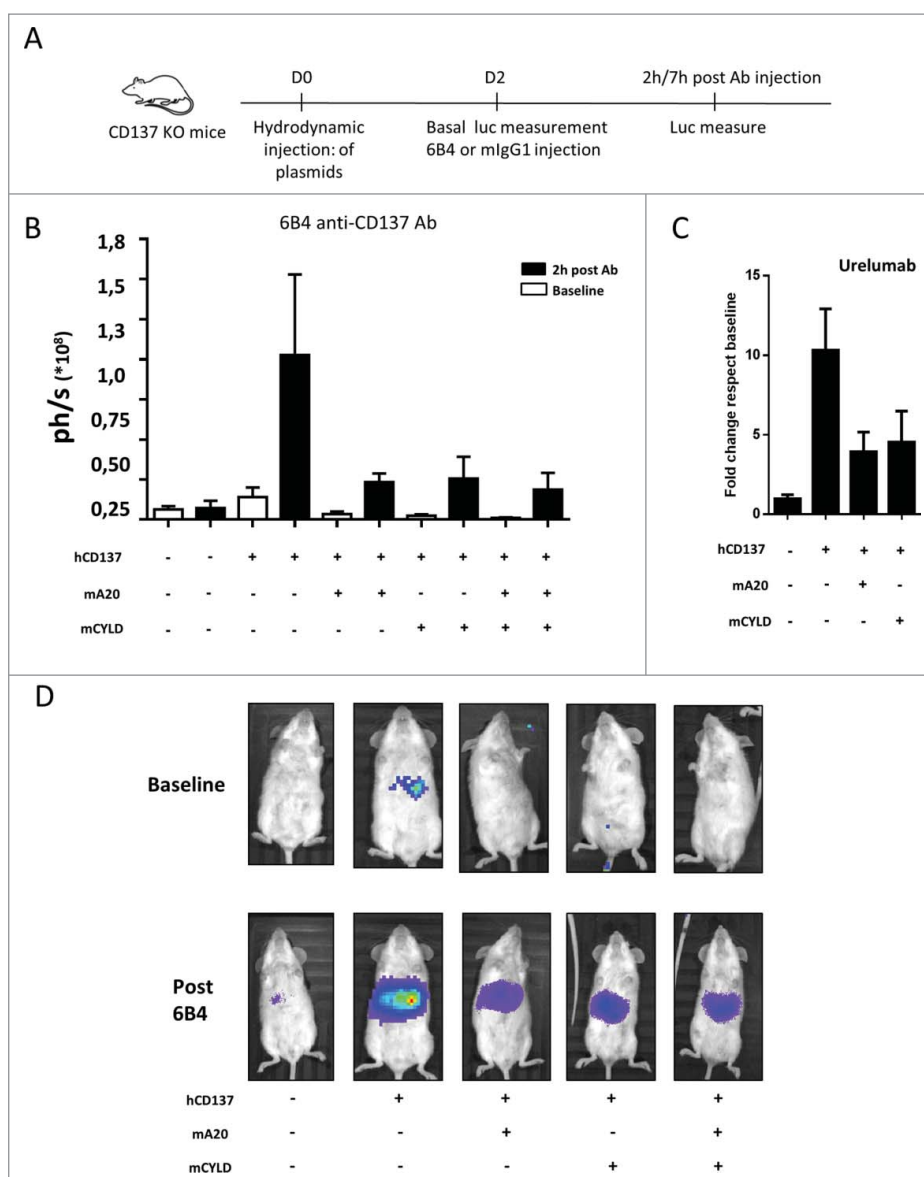
CYLD can efficiently repress ligand-activated CD137-signaling, an outcome that could be exploited to improve the T-cell costimulatory activity of CD137 agonists.

## Discussion

Our study shows that CD137 signaling toward canonical NF- $\kappa$ B activation is regulated by A20 and CYLD. This study

herein extends our previous results showing that CD137 stimulation induced TRAF2-dependent K63 polyubiquitination events resulting in NF- $\kappa$ B activation,<sup>11</sup> further supporting the key importance of K63-ubiquitination in CD137 signaling.

The signaling events downstream of CD137 are an important issue due to the critical roles of this TNFR-family member in cancer immunotherapy.<sup>40</sup> On the one hand, agonist anti-CD137 mAb potently induce CD8-mediated anti-tumor



**Figure 5.** A20 and CYLD decrease CD137-elicited NF- $\kappa$ B activation in vivo. (A) Mice (6 per group) were hydrodynamically gene transferred to the liver with expression plasmids encoding the NF- $\kappa$ B luciferase reporter construct and the indicated genes (A). 48 hours later mice were dosed intraperitoneally with the anti-CD137 mAb 6B4 (B) or urelumab (C) and bioluminescence was monitored by light emission from the upper abdomen (photons/sec in B and fold change over baseline in C). (D) Shows images of representative mice in B. Baseline was measured immediately before intraperitoneal mAb injection. Results represent mean  $\pm$  SEM of an experiment out of at least 4 performed under similar conditions and rendering similar results.

immunity in mice.<sup>41</sup> On the other hand, the CD137 cytoplasmic tail is critical for the functions of chimeric antigen receptors targeting CD19 with successful application in patients against B-cell lymphomas and leukemias.<sup>42-44</sup> Therefore elucidating the mechanisms and regulation of the CD137 signaling pathway is relevant for understanding its immunotherapeutic functions and to gain insights into how to modulate those to improve its therapeutic efficacy.

There is evidence for both TRAF2 and TRAF1 functioning as adaptors for CD137 signaling.<sup>45-47</sup> There is ample published evidence for TRAF2 involvement in ubiquitination.<sup>10</sup> A recent report has also linked TRAF1 to the function of the linear ubiquitin binding complex (LUBAC) although it is unknown if this applies to TNFR members in addition to TLR members.<sup>48</sup>

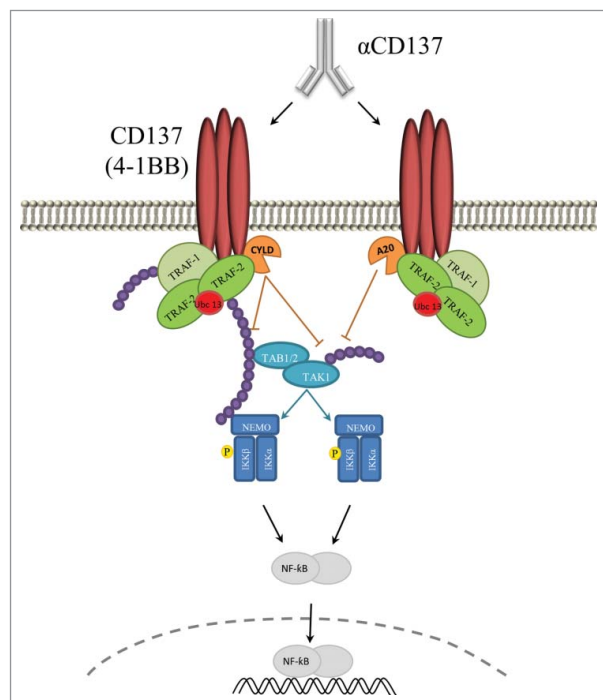
TRAF2 is a RING domain containing protein reportedly endowed with E3 ubiquitin ligase activity, generating K63

polyubiquitin chains in conjunction with Ubc13 as the necessary E2 enzyme.<sup>49</sup> A number of reports have shown a K63 ubiquitin ligase reaction attributed to TRAF2,<sup>49,50</sup> although questions have been raised on the ability of TRAF2 to actually accommodate the transubiquitination reaction based on the structural characteristics of the TRAF2 E3 domain.<sup>12</sup> Therefore, it cannot be ruled out that another E3 enzyme associated with the complex might be responsible for the K63 ubiquitination reactions. Interestingly, TRAF2 itself is an important substrate of the polyubiquitination reaction and we propose that CD137 crosslinking causes TRAF2-mediated polyubiquitination of neighboring sister TRAF2 molecules in the CD137 activation complex, which could be one of the earliest signaling events,<sup>7</sup> in addition to TRAF2 phosphorylation<sup>51</sup> and a cofactor requirement for sphingosine 1 phosphate.<sup>52</sup>

The function of K63 ubiquitination is to create docking sites for protein-protein interactions allowing the signaling cascade to proceed downstream toward NF- $\kappa$ B activation.<sup>53</sup> Several proteins, such as TAB1/2, TAK1, NEMO and IKK $\beta$  are polyubiquitinated.<sup>53</sup> Lymphocyte activation pathways need elements to regulate the function by feed-back quenching signals. Following the example of kinases and phosphatases, K63 polyubiquitin reactions do not go unchecked, since a broad family of K63 deubiquitinases control the extent of K63-ubiquitination<sup>10,17</sup>

Indeed, we have shown that A20 and CYLD bind to the CD137-TRAF2 complex thereby permitting coimmunoprecipitation of these proteins. TRAF2 has been shown to interact with other members of the TNFR family such as OX40, CD40, GITR and CD27 and hence binding to these DUB enzymes is unlikely to be exclusive of CD137. A20 is well known to regulate signaling toward NF- $\kappa$ B activation through TNFR1 and CD40.<sup>54,55</sup> In turn, CYLD has been recently discovered to regulate ubiquitination elicited by TNFR1 by means of the SPATA2 adaptor that links CYLD to the signaling receptor machinery.<sup>56</sup>

Consistent with the presence of CYLD and A20 in the complex, polyubiquitination of proteins in the CD137 activation complex was reduced and, more importantly, overexpression of A20 or/and CYLD decreased NF- $\kappa$ B activation. The effects were prominent on TAK1 which is a hub kinase in the pathway.<sup>57</sup> We cannot rule out that A20 and CYLD might also be exerting an additional role downstream in the NF- $\kappa$ B pathway, but overexpression and silencing data speak of a direct regulation on proximal CD137 signaling activity. Figure 6 shows a sketch of the proposed functional and protein-to-protein interaction toward NF- $\kappa$ B activation and its regulation by CYLD and A20.



**Figure 6.** Schematic representation of the proposed regulation of CD137 signaling by A20 and CYLD. Graphical abstract of the reported physical and functional signaling interactions of CD137 shown to be regulated by A20 and CYLD in such a way that attenuate NF- $\kappa$ B activation. Purple beads indicate K63 polyubiquitin chains that can be degraded by A20 and CYLD.

A20 is a double-edge enzyme with K63 deubiquitylation activity in its N-terminal OTU domain and a K48 ubiquitin ligase E3 function on the ZF4 domain. Both functions are important for TNFR-family signaling, deactivating proteins and eliminating docking sites in the case of K63 deubiquitinase reactions, and targeting inhibitors for degradation by means of K48-ubiquitylation.<sup>54, 58</sup> Our data with deletion variants do not provide a definitive answer, since both mutants retain the ability to coprecipitate with the CD137-TRAF2 complex and inhibit CD137-mediated NF- $\kappa$ B activation. A possible interpretation is that both K63 deubiquitination and K48 polyubiquitination give rise to similar functional results but this intriguing finding warrants further investigation. In this line, A20 knock-in mice with loss of function mutations either in the OTU domain or in the ZF4 domain do not recapitulate the severe inflammatory phenotype of A20 knockout mice.<sup>59</sup> Indeed our results are consistent with recent findings in A20-deficient MEFs which show that transfection of A20 mutants of either domain do not rescue the inhibition effect on NF- $\kappa$ B activation as elicited by TNF $\alpha$ .<sup>58</sup> Further complexity to fully understand A20 regulation on CD137 is expected to come from post-transcriptional modifications of the protein by serine phosphorylations,<sup>60</sup> dimerization<sup>58</sup> and oxidation.<sup>61</sup>

We propose that CD137 signaling below a certain threshold, for instance, due to stochastic receptor associations or in the absence of strong CD137L interactions, would be rapidly quenched by A20 or CYLD deubiquitinase activities. However, in response to a strong stimulus CD137 signaling would be able to proceed once sufficient E3 polyubiquitin ligase activity is in progress. In addition A20 and CYLD may destroy K63-polyubiquitin chains to terminate signaling once the agonist mAb or CD137L are no longer available. At this point, it is unclear if A20 and CYLD are redundant for this function or not, as shown for B-cell function and development.<sup>62</sup> In our experiments TRAF2 itself seems to be mainly a substrate for CYLD but not for A20 in agreement with previous literature.<sup>17</sup>

Moreover, A20 and CYLD relative levels of expression are known to change with cell activation<sup>17</sup> and hence are both DUBs are not likely to be redundant even if in our experiments both attain similar levels of inhibition on CD137-elicited NF- $\kappa$ B activation. It is noteworthy that A20 is the most induced transcriptional target regulated by the canonical NF- $\kappa$ B pathway.<sup>18</sup> This can be interpreted as a negative feedback loop acting to curtail excessive signaling through CD137 and other NF- $\kappa$ B activating receptors. This interpretation, if correct, provides translational relevance to our findings and is consistent with the observation that A20 and CYLD overexpression mitigate CD137 signaling in hepatocytes *in vivo*.

It is of note that TRAF6 and TRAF5 are also endowed with E3 K63-ubiquitin ligase activity<sup>3</sup> and it is not surprising that CYLD, A20 (or perhaps other DUBs) control these signaling functions as well.<sup>63-66</sup> In this way A20 and CYLD would regulate signaling from other T-cell costimulatory members of the TNFR family. By contrast, it is unlikely that A20 or CYLD would affect CD28-mediated co-stimulatory signaling offering another difference between CD137 and CD28 costimulatory signaling, that for instance show different time courses in the induction of Bcl-xL and CD25 expression (data not shown).



Another interesting area of research will be opened up if the LUBAC machinery is connected to TRAF1 signal transduction. How deubiquitinases regulate linear polyubiquitins might become important to understand CD137 regulation.<sup>48</sup> Interestingly A20 and CYLD have recently been shown to regulate this pathway<sup>67</sup> and A20 is known to bind linear M1-polyubiquitins through its C-terminal ZF7 domain.<sup>17,68</sup>

From a translational point of view, A20 and CYLD could be critical modulators of immunotherapy with anti-CD137 mAbs. We can, for instance, assess the activity of the anti-CD137 mAb Urelumab administered to mice in an experimental setting that could recapitulate events that are also taking place in patients undergoing urelumab clinical trials.<sup>69,70</sup> In this context, the interplay of CD137 with TRAF2, A20 and/or CYLD offers possibilities to devise biomarkers, enhance costimulatory activity and might be conducive to strategies supporting the critical activity of the CD137 cytoplasmic domain in clinically successful chimeric antigen receptors for adoptive T-cell therapy (CARs).<sup>42</sup>

## Materials and methods

### Transfections and expression cassettes

HEK293 T cells were obtained from ATCC in 2008. Cells were cultured in complete RPMI medium (RPMI 1640 with Glutamax [Gibco, Invitrogen] containing 10% heat-inactivated FBS [Sigma-Aldrich], 100 IU/ml penicillin and 100  $\mu$ g/ml streptomycin [Gibco]). Cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. For overexpression of CD137, A20, CYLD, we used the following plasmids: pCMV6-hCD137(GeneCopoeia), pCMV6-mA20 (Origen), pCEFL-FLAG-hA20 WT, N-ter (1–390), C-ter (390–790) and pCMV6-mCYLD (Origen).

### Immunoprecipitation and immunoblotting

HEK293 T cells were transfected as described above with plasmids encoding CD137, FLAG-A20 and/or CYLD. Eighteen hours after transfection, stimulation with anti-CD137 mAb for 5 min was accomplished with 5  $\mu$ g/ml anti-CD137 (clone 6B4) or mIgG1 (Biolegend) as a control. Then cells were lysed at 4°C in lysis buffer (50 mM Tris-HCl [pH 8.5], 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, and EDTA free complete protease inhibitor [Roche]). Soluble lysate was immunoprecipitated with anti-ubiquitin mAb (clone FK2, ENZO Life sciences) coupled to protein G-Sepharose 4 Fast Flow beads (GE Healthcare). Proteins were eluted in Laemmli buffer, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Millipore). Membranes were blocked in TBS containing 0.05% Tween-20 and 5% non-fat milk for 1 h. Immunoblots were performed with the following antibodies against ubiquitin (clone P4D1, Mouse monoclonal; Santa Cruz), TAK1 (rabbit polyclonal; Invitrogen), TRAF2 (C-20) (rabbit polyclonal; Santa Cruz), CYLD (rabbit monoclonal; Cell signaling), I $\kappa$ B $\alpha$  (mouse monoclonal; Cell signaling), FLAG (mouse monoclonal; Sigma-Aldrich) and HA (Rabbit polyclonal; Abcam). GAPDH (mouse monoclonal; Sigma-Aldrich) was used as a loading control. Blots were developed with the SuperSignal West Dura

Extended Duration Substrate (Pierce). Bands were quantified by densitometry using ImageJ (NIH) software.

### NF- $\kappa$ B luciferase reporter assay

Transient transfection of HEK293 T cells was performed using Lipofectamine 2000 according to the manufacturer's instructions. Luciferase reporter assays were performed with p $\kappa$ B-Luc plasmid which encodes a firefly luciferase reporter gene under the control of a NF- $\kappa$ B-responsive promoter<sup>71</sup> and Renilla luciferase reporter vector pRL-TK (Promega). Briefly, cells were co-transfected with 200 ng p $\kappa$ B-Luc plasmid, 200 ng constitutive expression plasmid Renilla luciferase reporter vector pRL-TK (Promega), and 5 ng pCMV6-hCD137 (GeneCopoeia), 500 ng pcDNA 3.1+HA-ubiquitin K63R (a kind gift from Gijs Versteeg, Mount Sinai School of Medicine), 500 ng pCMV6-mCYLD (Origen) and/or 500 ng pCMV6-mA20 (Origen) as indicated. Five hours after transfection cells were stimulated for 18 hours with 10  $\mu$ g/ml anti-CD137 mAb (clone 6B4) or mAb control (mIgG1, Biolegend) and lysed in dual reporter lysis buffer (Promega). Firefly luciferase signal were normalized using Renilla luciferase values.

For luciferase reporter experiments in lymphoid cell lines, 40  $\times$  10<sup>6</sup> CEM or MOLT4 cells per condition were electroporated using the Gene Pulser MX System (300 V, 950  $\mu$ F Bio-Rad, Hercules, CA) with plasmids encoding human CD137 (40  $\mu$ g), K63R (80  $\mu$ g), A20 (80  $\mu$ g), CYLD (80  $\mu$ g), NF- $\kappa$ B-firefly luciferase reporter (40  $\mu$ g), pRL-TK (40  $\mu$ g), or empty vector (20–30  $\mu$ g) as indicated. After 18 h, live cells were isolated using Ficoll density gradient separation, washed, and stimulated during 24 h with 10  $\mu$ g/ml anti-CD137 mAb (clone 6B4) or control Ab (Mouse IgG1; Biolegend).

### siRNA and shRNA gene silencing

CD8 T cells were isolated by negative immunomagnetic selection (Miltenyi) from a buffy coat (Navarra Blood and Tissue Bank. Navarrabiomed Biobank. Navarra Health Department) and purity verified to be above 90%.

Lentivirus encoding shRNA to silence endogenous A20 expression and EGFP were produced by the Viral Vector Platform at Inbiomed Foundation as described previously.<sup>72,73</sup> Purified human CD8 T cells were preactivated 24 hours with CD3/CD28 magnetic beads prior lentiviral infection, which was performed at a multiplicity of infection of 10 overnight. After, cells were incubated with plated bound anti-CD3 (OKT3) and anti-CD137 (6B4) or mIgG1 mAbs for 48 hours and Bcl-xL expression levels were monitored by flow cytometry. These lentiviruses had been previously validated to silence A20.<sup>73</sup>

Silencing of endogenous A20 or CYLD expression in human CD8 T cells was achieved by RNA interference. The following siRNAs were purchased from Dharmacom Thermo Fisher: human A20 1(GCAAAAGAAUCAAAACAAA), human A20 2 (GCUUUGUAUUUGAGCAAUA), human CYLD 1 (CGCUGAACUCUUUAGCAU), human CYLD 2 (GGAAGAAGGUCGUGUCAAA). Purified human CD8 T cells were electroporated with 200pmol siRNA per 10<sup>7</sup> cells using the Gene Pulser MX System (Bio-Rad, Hercules, CA.) 24 hours following electroporation cells were stimulated with plate bound

anti-CD3 (OKT3) coated at 1  $\mu\text{g/ml}$  and anti-CD137 (6B4) or mIgG1 at 5  $\mu\text{g/ml}$ . 72 hours later Bcl-xL and surface CD25 expression was assessed by flow cytometry.

### Antibodies and flow cytometry

For flow cytometry analysis, the single-cell suspensions were treated with Beriglobine (CSL Behring) in a phosphate buffered saline-based buffer (Gibco/Life Technologies) containing 10% fetal bovine serum (Sigma-Aldrich) to avoid unspecific staining and were stained with the following fluorochrome-conjugated antibodies: anti-CD8- BV510 (SK1 [Biolegend]), anti-Bcl-xL-PE (7 B2.5 [Southern Biotech]), anti-CD25-APC (BC96 [Biolegend]) and Zombie NIR ([Biolegend]). Fluorescence signals were acquired with a BD Biosciences FACSCanto II flow cytometer. Data were analyzed using FlowJo software (Tree Star, Inc.). Stainings were controlled by isotype fluorochrome-matched mAbs and Minus One.

### In vivo hydrodynamic gene transfer and determination of luciferase activity

6–8 week old CD137 KO mice (BALB/c) were injected with a total amount of 42  $\mu\text{g}$  plasmid DNA by hydrodynamic injection, consisting of the administration of 1.8–2.0 ml DNA solution via tail vein in 5 seconds.<sup>39</sup> Animals were injected with plasmids encoding a NF- $\kappa$ B-firefly luciferase reporter (6  $\mu\text{g}$ ), CD137 (12  $\mu\text{g}$ ), A20 (12  $\mu\text{g}$ ), CYLD (12  $\mu\text{g}$ ), or with an empty plasmid (empty vector) as required. 48 h after hydrodynamic injection, mice were i.p. inoculated with 100  $\mu\text{g}$  human anti-CD137 mAb (clone 6B4 or Urelumab) or control mouse IgG1. Bioluminescence imaging was performed before mAb treatment (baseline) and 2–7 hours after mAb treatment.

### Statistics

Descriptive statistics were calculated with PRISMA software and comparisons were performed by U-Mann-whitney tests or student's t tests. Statistical significance ( $p < 0.05$ ) are indicated by \* in the figures.

### Disclosure of potential conflicts of interest

MJ-K is a full time employee in AstraZeneca Medimmune and a former employee of Bristol-Myers Squibb; IM serves as a consultant for Bristol Myers Squibb, AstraZeneca, Lilly, Bayer, Roche-Genentech, Bionotech, Merck-Serono, Incyte, Servier, Alligator and Tusk Therapeutics.

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