Balance between NF-κB p100 and p52 Regulates T Cell Costimulation Dependence

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c-IAP1 and c-IAP2 are ubiquitin protein ligases (E3s) that repress noncanonical NF-κB activation. We have created mice that bear a mutation in c-IAP2 that inactivates its E3 activity and interferes, in a dominant-negative fashion, with c-IAP1 E3 activity (c-IAP2H570A). The immune response of these animals was explored by infecting them with the Th1-inducing parasite Toxoplasma gondii. Surprisingly, c-IAP2H570A mice succumbed because of T cell production of high levels of proinflammatory cytokines. Unlike naïve wild-type (WT) cells, which require signals generated by the TCR and costimulatory receptors to become fully activated, naïve c-IAP2H570A T cells proliferated and produced high levels of IL-2 and IFN-γ to stimulation via TCR alone. c-IAP2H570A T cells had constitutive noncanonical NF-κB activation, and IκB kinase inhibition reduced their proliferation to anti-TCR alone to WT levels but had no effect when costimulation via CD28 was provided. Notably, T cells from nfkbia−/− mice, which cannot generate the p52 component of noncanonical NF-κB, were also costimulation independent, consistent with the negative role of this unprocessed protein in canonical NF-κB activation. Whereas T cells from nfkbia−/− mice behaved like WT, coexpression of a single copy of c-IAP2H570A resulted in cleavage of p100, upregulation of p52, and T cell costimulation independence. Thus, p100 represses and p52 promotes costimulation, and the ratio regulates T cell dependence on costimulatory signals. The Journal of Immunology, 2013, 190: 549–555.

The NF-κB family of transcription factors plays critical roles in a variety of biological processes (1, 2). Two mechanisms activate NF-κB. The majority of stimuli that activate NF-κB, such as TNF-α, IL-1β, and LPS, do so via the so-called canonical pathway (1). In resting cells, IκBα constitutively binds NF-κB dimers and retains them in the cytosol. Upon stimulation, the IκB kinase (IKK) β subunit is activated, leading to phosphorylation and subsequent ubiquitination and proteasome-mediated degradation of IκBα, freeing NF-κB to migrate to the nucleus and activate transcription. Activation of the canonical NF-κB pathway depends exclusively on IκBα degradation, does not require protein synthesis, and is rapid and transient (2, 3). Noncanonical NF-κB signaling is initiated by TNFR-associated factor 2-mediated, lysin (K) 63–linked ubiquitination of c-IAP1 and c-IAP2. This modification diverts their activity from NF-κB–inducing kinase (NIK) to K48–linked ubiquitination of TNFR-associated factor 3, inducing its proteasomal-mediated degradation and release of NIK from the complex. As a result of both protein stabilization and de novo synthesis, NIK accumulates in the cytosol, where it phosphorylates and activates IKKα, which, in turn, phosphorylates the NF-κB family member p100, inducing its K48 polyubiquitination and partial proteolysis to p52. The resulting RelB/p52 heterodimers migrate to the nucleus where they activate transcription of target genes (4–8). Such a regulatory mechanism ensures the absence of receptor-unrelated signals by suppressing the level of NIK in unstimulated cells, and is characterized by relatively slow kinetics (on the order of hours) (3).

c-IAP1 and c-IAP2 are highly homologous members of the IAP family, characterized by the presence of one or more baculovirus IAP repeat domains (an ~70-aa region that mediates protein–protein interactions) and a RING domain conferring E3 activity (9). The Journal of Immunology, 2013, 190: 549–555.
Institute of Allergy and Infectious Diseases, National Institutes of Health). All mice were bred and maintained in a National Cancer Institute pathogen-free animal facility. For adoptive transfer experiment, RAG1−/− mice were injected i.v. with 107 T cells from age-matched wild-type (WT) or c-IAP2H570A mice. Study protocols were approved by the Institutional Animal Care and Use Committee of the National Cancer Institute.

T. gondii infection and parasite burden evaluation

T. gondii cysts from the avirulent strain ME-49 were prepared from the brains of infected C3HBL/6 mice. For experimental infections, mice were inoculated i.p. with an average of 20 cysts/animal and monitored daily for survival. To measure parasite burden, we harvested peritoneal cells on day 7 postinfection, and the number of infected cells was assessed evaluating Diff-Quik–stained Cytospin smears of exudates.

Cell preparation and purification

T cells were purified from lymph nodes using EasySep T cell enrichment kit (Stem Cell Technologies) following the manufacturer’s protocol, and the number of live cells was assessed by trypan blue exclusion. Purity was determined by flow cytometry, and for all experiments was >90%. T cells were cultured in RPMI 1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, and 50 μM 2-ME.

Reagents and Abs

Hoechst 3342 and Pyronin Y were purchased from Molecular Probes and Polysciences, respectively. iK inhibitor XII (12) was from Calbiochem. Anti-mouse CD3 (145-2C11), anti-mouse CD28 (37.51), and anti-mouse CD40 (HM40-3), and all Abs for flow cytometry were purchased from BD Biosciences. PMA, ionomycin (Iono), and anti-β-actin were purchased from Sigma-Aldrich; CFSE from Invitrogen; Abs to p100/p52, phospho-STAT3, IκBα, c-IAP2, and c-IAP1 from Santa Cruz. Anti-IκBα, cyclin D3, RelA, RelB, and Lamin A were from Cell Signaling; and Abs to IκBα, cyclin D3, RelA, RelB, and Lamin A were from Santa Cruz.

Flow cytometry and sorting

All stainings were performed in PBS plus 1% FCS and 0.01% NaN3 in the presence of 1:500 Fc-blocking Abs (2.4G2). CFSE was used at a concentration of 500 nM, and cells were stained following the manufacturer’s instructions. For DNA/RNA staining, cells were incubated with Hoechst 33342 and Pyronin Y, respectively, as described previously (13). Sorting experiments were performed on a BD FACS Aria. All data were analyzed with FlowJo software (Tree Star).

Proliferation assay

Assays were performed in 96-well flat-bottom plates in a final volume of 200 μl. Wells were coated with anti-CD3 alone or in combination with anti-CD28 at the indicated concentrations for 1 h at 37°C or overnight at 4°C in PBS. Cells were cultured for 48 h, pulsed with 1 μCi [3H]thymidine, and harvested 18 h later. [3H]thymidine uptake was determined by a Wallac 1450 MicroBeta Liquid Scintillation Counter. All experimental points were performed in triplicate, and the error bars represent the SEM.

Immunoblotting

T cells were normalized to cell number and lysed in sample buffer (50 mM Tris pH 6.8, 10% glycerol, 2% SDS, 2% 2-ME, and 0.04% bromophenol blue), resolved by SDS-PAGE, and immunoblotted with the indicated Abs. For DNA/RNA staining, cells were incubated with Hoechst 33342 and Pyronin Y, respectively, as described previously (13). Sorting experiments were performed on a BD FACS Aria. All data were analyzed with FlowJo software (Tree Star).

Cytokine and aspartate aminotransferase quantification

Supernatants from T cells cultured with plate-bound anti-CD3 or anti-CD3 plus anti-CD28 were collected at the indicated time points and the level of IL-2 and IFN-γ measured using the IL-2 or IFN-γ ELISA Ready-SET-Go! kit (eBioscience). For T. gondii infection experiments, mice were bled at day 7 or 8 postinfection, and levels of aspartate aminotransferase (AST) were determined using a commercial kit (Boehringer) in an automatic analyzer (model 917; Hitachi). Levels of IL-12, IFN-γ, and IL-10 were determined by ELISA.

Real-time PCR

Total RNA was extracted from purified T cells using RNeasy Mini kits (Qiagen) and reverse transcribed using the Superscript II Reverse Transcriptase kit (Invitrogen) following the manufacturers’ protocols. IL-2 and hypoxanthine phosphoribosyltransferase mRNA were quantified using the respective primers, SYBR Green PCR Master Mix (Applied Biosystems) and the 7500 Real Time PCR System (Applied Biosystems). Values were normalized to hypoxanthine phosphoribosyltransferase, and the percentage increase relative to WT was calculated by dividing c-IAP2H570A values by WT values.

Results

c-IAP2H570A mice succumb to T. gondii infection

To explore the biological consequences of c-IAP2H570A mutation in T cells, we infected WT, c-IAP2H570A, and c-IAP2H570A mice with an avirulent T. gondii strain. This model was chosen because protective immunity is B cell independent, relying on vigorous Th1-mediated immunity mediated primarily by dendritic cell–derived IL-12 and T cell–derived IFN-γ and IL-10 (14). Whereas the majority of WT mice survived, c-IAP2H570A mice succumbed in the acute phase of the infection with a median of day 9.5 (Fig. 1A). To distinguish between a requisite role for c-IAP2 and a role for c-IAP2 E3 activity, we also examined mice deficient in c-IAP2, and they were found to survive infection. The death of c-IAP2H570A mice was not due to uncontrolled parasite replication, because the parasite burden was similar in WT and c-IAP2H570A mice (Fig. 1B). In contrast, there was a high serum level of AST, indicating acute liver damage (Fig. 1C). This excessive response to T. gondii was also reflected in high amounts of IFN-γ and IL-10 in the sera of c-IAP2H570A compared with WT and c-IAP2−/− mice (Fig. 1D). The amount of IL-12 and IL-6 was increased as well, although to a lesser extent. Each of these cytokines was found at low to undetectable levels in the sera of uninfected mice of all genotypes. To determine whether the exaggerated response to T. gondii was T cell intrinsic, RAG1−/− mice were reconstituted with WT or c-IAP2H570A T cells and infected. Whereas WT T cells protected four of five RAG1−/− mice for up to 25 d, mice reconstituted with c-IAP2H570A T cells died within 20 d postinfection (Fig. 1E), demonstrating that the death of c-IAP2H570A mice was due to the T cell response.

c-IAP2H570A T cells are costimulation independent

The functional responses of WT versus c-IAP2H570A T cells were compared in vitro. As expected, TCR-driven WT T cell proliferation was largely dependent on the presence of costimulation. Strikingly, c-IAP2H570A T cells proliferated after stimulation with anti-TCR alone, as shown by [3H]thymidine incorporation and CFSE dilution (Fig. 2A, 2B). This was not due to differences in their intrinsic proliferative capacity, because WT and c-IAP2H570A T cells proliferated similarly in the presence of Abs to the TCR and CD28, or PMA and ionomycin, a mitogenic stimulus that bypasses the TCR. Upregulation of the activation markers CD44 and CD25, but not CD69, was much greater in WT T cells stimulated with anti-CD3/CD28 compared with anti-CD3 alone (Fig. 2C). In contrast, CD44 and CD25 upregulation on c-IAP2H570A T cells was the same whether costimulation was present. The expression of these markers at rest was similar between WT and c-IAP2H570A T cells (10). If c-IAP2H570A T cells have a costimulation advantage, WT T cells should proliferate to the same extent as the mutant T cells regardless of whether they are exposed to a sufficient amount of costimulation. Anti-CD28 was titrated in the presence of fixed amounts of anti-CD3 to test this (Fig. 2D). Whereas c-IAP2H570A T cell proliferation did not change as a function of anti-CD3 at any anti-CD3 concentration, the dependence of WT T cell proliferation on anti-CD28 increased as the amount of anti-CD3 decreased. Whether this costimulation advantage was cell intrinsic was addressed by activating cocultured WT and c-IAP2H570A T cells with anti-CD3 alone (Fig. 2E). Proliferation of a 1:1 ratio of WT and c-IAP2H570A T cells in re-
response to anti-CD3 alone was intermediate between the two unmixed populations, indicating that the c-IAP2H570A T cells did not produce a factor that made WT T cells less dependent on costimulation. The dominant-negative effect of E3-defective c-IAP2 on c-IAP1 was needed for this phenotype, because c-IAP2−/− and WT T cells proliferated equally poorly to anti-CD3 alone (Fig. 2F). Hence it is not the loss of c-IAP2 E3 activity per se, but the resulting upregulation of noncanonical NF-κB that is responsible for costimulation independence.

**Increased IL-2 and IFN-γ production by c-IAP2H570A T cells**

Costimulatory signals are required for the production of effector cytokines such as IL-2 and IFN-γ (15). Purified T cells were stimulated with anti-CD3 in the presence or absence of anti-CD28 and cytokine production measured (Fig. 3A). Whereas WT T cells produced little IL-2 unless CD28 was engaged, anti-CD3 alone was sufficient to induce c-IAP2H570A T cells to produce as much IL-2 as WT cells in the presence of both anti-CD3 and anti-CD28, which was increased another 2-fold by anti-CD28. IL-2 mRNA was also increased in c-IAP2H570A T cells (Fig. 3B). As with IL-2, WT T cells secreted IFN-γ only in the presence of a costimulatory signal, whereas a comparable amount of IFN-γ was detected in supernatants of c-IAP2H570A T cells stimulated with anti-CD3 alone (Fig. 3A). Thus, lack of c-IAP2 E3 activity allows TCR signals alone to induce cytokine expression, the high levels of IL-2 likely being responsible for proliferation in the absence of costimulation.

**Costimulation of naive versus memory c-IAP2H570A T cells**

The requirement for costimulation in TCR-mediated proliferation is more pronounced in naive than in memory cells (reviewed in Ref. 15). To determine whether the proportion of memory cells in c-IAP2H570A mice was altered, we analyzed T cell subpopulations. There was no difference in the CD44, CD62L, CD25, and CD69 expression between WT and c-IAP2H570A T cells (Fig. 4A). Two important characteristics of memory cells are elevated expression of cyclin D3 and a higher content of total RNA compared with naive T cells (13, 16). Although cyclin D3 expression was not increased in naive c-IAP2H570A T cells (Fig. 4B), they did have higher total RNA levels than their WT counterparts (Fig. 4C), consistent with their being in a state of readiness similar to memory cells. Accordingly, TCR stimulation alone was sufficient to induce proliferation of naive (CD44hi) c-IAP2H570A, but not naive WT T cells (Fig. 4D). In contrast, memory T cells (CD44hi) of both genotypes responded similarly, demonstrating that it was predominantly naive T cells that were affected by activation of noncanonical NF-κB.

**Constitutive activation of noncanonical NF-κB in c-IAP2H570A T cells**

Lack of c-IAP2 E3 activity caused constitutive activation of noncanonical NF-κB in resting B cells, an effect that was difficult to see in T cells because of the much higher levels of p52 in WT B cells compared with WT T cells (10). Therefore, we addressed the state of NF-κB activation specifically in T cells. Freshly isolated c-IAP2H570A T cells expressed higher levels of NIK and p52, and lower levels of the unprocessed p52 precursor p100 than WT T cells (Fig. 5A). The p100/p52 ratio was 16-fold lower relative to WT, as determined by densitometry. As expected, c-IAP2−/− T cells did not display any changes in the state of noncanonical NF-κB activation (Fig. 5B) and had a p100/p52 ratio of 0.8 relative to WT T cells, confirming that c-IAP1 compensates for the lack of c-IAP2 in the T cell inhibitory complex. To question whether the c-IAP2H570A mutation affects other pathways that are known to regulate costimulation, we analyzed Akt activation. At rest, phospho-Akt was undetectable in both WT and c-IAP2H570A T cells, and it increased in both in a similar fashion after activation through TCR or TCR/CD28 (Fig. 5C). Other potential candidates, including mammalian target of rapamycin, which prevents anergy, and p27, which is required for induction of T cell anergy (17), were analyzed, and no differences were found between c-IAP2H570A and WT T cells (data not shown). Similarly, canonical NF-κB activation as measured by IkBα degradation was comparable between WT and c-IAP2H570A T cells as well (data not shown). These results suggest that the dysregulation of noncanonical NF-κB accounts for c-IAP2H570A T cell costimulation independency.

**IKK inhibition prevents costimulation-independent proliferation**

To determine whether the c-IAP2H570A costimulation advantage was a consequence of NF-κB activation, we used a synthetic inhibitor of IKKα and IKKβ, IKK XII. Inhibition of IKK completely blocked the costimulation independence of c-IAP2H570A T cells (Fig. 6A, 6B). This effect was highly specific, because at the concentrations used, the IKK inhibitor had little effect when

**FIGURE 1.** c-IAP2H570A mice succumb to *T. gondii* infection. (A) WT (*n* = 8), c-IAP2−/− (*n* = 5), and c-IAP2H570A (*n* = 8) mice were infected i.p. with 20 cysts of the ME49 strain of *T. gondii* and animal survival was monitored daily. (B) Number of tachyzoite-infected PEC in three WT and three c-IAP2H570A mice. (C) Level of infected WT (*n* = 4), c-IAP2−/− (*n* = 5), and c-IAP2H570A (*n* = 3) mice. (D) Serum levels of IFN-γ, IL-10, IL-12–p40, and IL-6 produced by c-IAP2H570A T cells stimulated with anti-CD3 and anti-CD28. IL-2 mRNA was increased another 2-fold by anti-CD28. IL-2 mRNA and IL-2 protein was detected in supernatants of c-IAP2H570A T cells stimulated with anti-CD3 and anti-CD28. IL-2 mRNA was also increased in c-IAP2H570A T cells (Fig. 3B). As with IL-2, WT T cells secreted IFN-γ only in the presence of a costimulatory signal, whereas a comparable amount of IFN-γ was detected in supernatants of c-IAP2H570A T cells stimulated with anti-CD3 alone (Fig. 3A). Thus, lack of c-IAP2 E3 activity allows TCR signals alone to induce cytokine expression, the high levels of IL-2 likely being responsible for proliferation in the absence of costimulation.

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costimulation was mediated by CD28, which mediates costimulation by activation of Akt and is independent of noncanonical NF-κB. Higher concentrations of IKK XII blunted both CD28-dependent and -independent proliferation, without affecting cell viability, consistent with the known role of canonical NF-κB activation via IKKβ (17) (data not shown). To characterize the effect of IKK XII on IKKα, we incubated WT splenocytes with anti-CD40 to activate noncanonical NF-κB. CD40-induced p100 phosphorylation, a hallmark of IKKα activity, was decreased by IKK XII, confirming its negative effect on IKKα, as was, to a lesser extent, constitutive p-100 phosphorylation in c-IAP2H570A T cells (Supplemental Fig. 1A, 1B). RelA (p65) and RelB nuclear translocation in anti-CD40–stimulated splenocytes was also inhibited (Supplemental Fig. 1C, 1D). These results indicate that costimulation provided by c-IAP2H570A, but not CD28, relies on NF-κB activation.

The balance between p100 and p52 regulates costimulation dependence

Noncanonical NF-κB activation is the result of processing p100 to p52, which was reflected in c-IAP2H570A T cells by a change in the ratio of these two proteins. p100 has been shown to have an inhibitory role on naive T cell proliferation, presumably via its binding to, and inhibition of, RelA (18, 19). To evaluate the possible contribution of decreased p100 to costimulation independence, we analyzed T cells from mice in which the gene encoding p100 (nfkb2) was disrupted. nfkb2−/− T cells proliferated in response to TCR stimulation alone, and its lack results in T cell costimulation independence.

The analysis of nfkb2−/− T cells is complicated by the fact that both p100 and p52, which may have opposing effects, are absent. To address how these proteins might independently regulate T cell responses, we examined T cells expressing no, haploid, or normal levels of p100. nfkb2−/− T cells express ~50% less p100 (Fig. 7C). If c-IAP2H570A T cell costimulation independence relies only on reduced p100-mediated inhibition, nfkb2−/− T cells would be expected to have a similar phenotype. As shown in Fig. 7B, nfkb2−/− T cells were not costimulation independent, whereas nfkb2−/− T cells were. Therefore, a decrease in p100 is not sufficient to deregulate the TCR response. Introduction of one c-IAP2H570A allele into nfkb2−/− T cells was sufficient to induce processing of p100 to
p52, thereby decreasing the p100/p52 ratio by approximately half (Fig. 7B, 7C). This resulted in a more robust TCR-mediated response, although one that was still lower than that of nfkβ2−/− T cells, demonstrating that it is the imbalance of the p100/p52 ratio that affects costimulation dependence. These results indicate that activation of noncanonical NF-κB causes costimulation independence by two related but independent mechanisms: a decrease in

Discussion

T cell activation is one of the fundamental processes determining the outcome of an immune response. Full T cell activation is achieved when the MHC–peptide complex recognized by the TCR is accompanied by a costimulatory signal generated by receptors such as CD28. Lack of costimulation not only prevents full activation but also promotes a state of clonal anergy characterized by lack of cytokine secretion. T cell anergy is one means by which

![FIGURE 3. Increased IL-2 and IFN-γ production by c-IAP2H570A T cells.](http://www.jimmunol.org/)

![FIGURE 4. Costimulation of naive versus memory c-IAP2H570A T cells.](http://www.jimmunol.org/)

![FIGURE 5. Constitutive activation of noncanonical NF-κB in c-IAP2H570A T cells.](http://www.jimmunol.org/)

![FIGURE 6. IKK inhibition prevents costimulation-independent proliferation.](http://www.jimmunol.org/)
potentially self-reactive T cells are rendered unresponsive in the periphery, and also one of the mechanisms that tumor cells use to escape the immune response (20, 21). Occupancy of the most well-studied costimulatory molecule, CD28, activates PI3K, Akt, and canonical NF-\(k\)B, leading to cytokine production and T cell survival (15, 22). Other costimulatory molecules belong to the TNFR family: OX40, 4-1BB, CD27, and TNF-R2 (23, 24). Upon engagement with their respective ligand, these molecules activate canonical NF-\(k\)B, causing enhanced cell survival and effector cytokine production. In addition, they activate noncanonical NF-\(k\)B (25–27), although the contribution of this pathway to biological outcomes is not well understood.

Although the role of noncanonical NF-\(k\)B has been characterized mostly in B cells, a few reports deal with noncanonical NF-\(k\)B activation and T cells. \(aly/aly\) mice bear a mutation in NIK that interferes with the activation of IKK\(\alpha\) and the generation of p52, and T cells from these animals have a slight decrease in TCR-induced proliferation and produce lower amounts of IL-2 than WT cells (28). In line with this, T cells from RelB\(^{-/-}\) mice have a profound defect in IL-2 and IFN-\(\gamma\) production upon activation (29). Conversely, p100\(^{AC/ACT}\) mice, in which the inhibitory ankyrin domain of p100 is deleted resulting in a constitutively active N-terminal fragment, have enlarged lymph nodes, increased T cell proliferation in response to anti-CD3 and anti-CD3/CD28, and enhanced cytokine production (30). More recently, it was suggested that p100 represses canonical NF-\(k\)B by inhibiting RelA nuclear translocation, and that a low level of p100 makes naive T cell costimulation independent (18). Thus, the role of the noncanonical NF-\(k\)B in T cell activation is not well understood. The data in this report show that isolated upregulation of noncanonical NF-\(k\)B in T cells does not result in overt activation or alteration of the distribution of naive and memory T cells, but synergizes with TCR signals to cause naive T cells to proliferate in a memory-like fashion. We refer to this phenomenon as “co-stimulation independence” to mean that c-IAP2\(^{H570A}\) T cells are substantially more responsive to TCR-mediated activation than WT cells (≥10-fold shift in the dose–response curve and a much higher level of proliferation at any concentration of anti-CD3 used). This depended on NF-\(k\)B activation, as demonstrated with the IKK inhibitor, and was very likely contributed to by the corresponding decrease in p100 levels, because T cells completely lacking p100 were costimulation independent as well. In line with this result, the hyperactivation of p100\(^{AC/ACT}\) T cells may also depend on the lack of p100 inhibitory activity, which requires the ankyrin repeats. Consistent with our results with a genetic model, it has recently been reported that treatment of naive T cells with SMAC mimetics, a class of compounds that induces rapid degradation of both c-IAP1 and c-IAP2, activates noncanonical NF-\(k\)B and enables them to proliferate in response to TCR stimulation alone (31). Because the c-IAP2\(^{H570A}\) T cells express c-IAP1 and (E3-dead) c-IAP2, we can conclude that it is the E3 activity of the c-IAPs rather than some other property (e.g., baculovirus IAP repeat–dependent interactions with other proteins such as caspases) that is responsible for the costimulation-independent phenotype. This is supported by the finding that c-IAP2\(^{-/-}\) T cells behave like the WT T cells. In this case, the lack of c-IAP2 is presumably compensated for by c-IAP1, preventing activation of the noncanonical NF-\(k\)B pathway. Our results indicate that T cell requirement for costimulation is regulated by the ratio between p100 and p52. Indeed, the ~50% decrease in p100 levels in \(nkb2^{-/-}\) T cells was not sufficient to deregulate the response to TCR signaling. This was not the case when p52 was increased (with only a small further decrease in p100) by coexpression of c-IAP2\(^{H570A}\). Because the decrease of p100 was not sufficient to induce a costimulation advantage in T cells without a concurrent imbalance of p52, these data argue strongly that it is the relative levels of p100 and p52 that determine whether T cells can proliferate to TCR signals alone.

Naive T cells exist in a state of quiescence (\(G_0\)) with low RNA content and slow activation kinetics. Conversely, memory cells are in \(G_1\) and have higher RNA content (13, 16, 32, 33), which includes mRNA of cytokines (e.g., IFN-\(\gamma\), IL-2) and effector molecules (e.g., perforin, granzyme B) (34), and higher levels of rRNA, which allows for the rapid translation of effector molecule mRNAs (35). Although we found no evidence for constitutive expression of IL-2 mRNA in c-IAP2\(^{H570A}\) T cells, they did express higher levels of RNA than WT T cells. The activation of c-IAP2\(^{H570A}\) T cells by TCR stimulation alone, therefore, may be because noncanonical NF-\(k\)B is always active and the cells are poised to translate upregulated mRNAs rapidly upon stimulation.

There has been a great deal of interest in targeting c-IAP molecules with SMAC mimetics as an anticancer therapy (36). Although there may be initial benefit to activating noncanonical NF-\(k\)B, the long-term sequelae of such an intervention must be considered. There are several lines of evidence that chronic noncanonical NF-\(k\)B activation can contribute to lymphoproliferative disorders. Gene rearrangement of \(nfkb2\) leading to transcription of constitutively active fragments lacking the ankyrin inhibitory domain has been described in some cases of chronic lymphocytic leukemia, multiple myeloma, and cutaneous T cell lymphoma (37–40). In another example, MALT lymphoma, c-IAP2–MALT1 fusion proteins lacking E3 activity induce constitutive p100 to p52 processing (41), and activation of noncanonical NF-\(k\)B in c-IAP2\(^{H570A}\) B cells leads to a MALT lymphoma-like disease (10). Human T cell leukemia virus type 1 Tax protein triggers noncanonical NF-\(k\)B activation in T cells, causing their transformation (8, 42, 43). Although not tested in T cells, in fibroblasts, stable expression of p100 blocked Tax-dependent transformation (44). In
addition to promoting tumors, the observation that c-IAP2<sup>−/−</sup> mice die postinfection with <i>T. gondii</i> because of an exaggerated proinflammatory response adds another potentially deleterious outcome to activating T cell noncanonical NF-κB. Therefore, therapies in which c-IAP expression is targeted may have to contend with broader consequences of noncanonical NF-κB expression that include potentially harmful enhancement of T cell activation.

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