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The chemical biology of IL-12 production *via* the non-canonical NFκB pathway

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Interleukin-12 (IL-12) has emerged as an attractive cytokine for cancer therapy because it has direct anti-cancer effects and additionally plays a critical role in enhancing checkpoint inhibitors. Given these multiple modes of actions, identifying means to pharmacologically induce IL-12 production in the tumor microenvironment has become important. In this review, we highlight therapeutics that promote IL-12 induction in tumor-associated myeloid cells through the non-canonical NFκB pathway. We discuss existing clinical trials and briefly examine the additional pathway targets that warrant further exploration for drug discovery.

Introduction

Cancer immunotherapy has fundamentally changed the landscape of oncologic treatment options. Substantial excitement has been generated by the successes of checkpoint inhibitors, which promote the cytotoxic activity of anti-tumorigenic T cells.^{1,2} While such therapies have induced durable remissions in cancers refractory to other treatments, they are not without limitations, most notably in that they work only in a fraction of patients.³ As such, numerous avenues are being explored to increase the fraction of responders while minimizing the development of resistance and systemic side effects.

Similar to targeted therapeutics, a promising approach being pursued is combination therapy.^{4–8} For example, it has been shown in multiple models that activation of innate immune cells can improve the efficacy of checkpoint inhibitors.^{9,10} In particular, several groups have shown a critical role of interleukin-12 (IL-12) in sensitizing tumors to anti-PD-1 therapy.^{11–13} Specifically, production of IL-12 in a subset of tumor-associated dendritic cells, termed DC3, is essential for response to anti-PD-1 treatment.^{11,14} Treatment of mice with anti-PD-1 antibodies leads to an increase in levels of interferon-gamma (IFNγ) in T-cells, which turns on IL-12 production in dendritic cells (Fig. 1a). The full-fledged activation of antitumor immunity triggered by immunotherapy is thus not direct, but rather involves T-cell:dendritic cell crosstalk and is licensed in part by IL-12.¹¹

Beyond its emerging role in immunotherapy, IL-12 also has numerous other anti-tumorigenic effects and has thus been of interest therapeutically for some time.¹⁵ In fact, it is worth

noting that one of the oldest cancer immunotherapies, Coley's toxins,¹⁶ a mixture of dead bacteria, likely induced IL-12 production in patients, as components of bacterial membrane components are known to do so. Today, we know that IL-12 (i) elicits broad anti-tumor effects in multiple cancer models, (ii) acts on various immune cell types including NK-, B-, and T-cells, and (iii) turns on signaling pathways aiding in the activation of T cells.^{11,15,17} Despite these numerous anti-tumorigenic effects, recombinant IL-12 delivered intravenously as a therapeutic has also been shown to have considerable toxicity in humans, thereby limiting its systemic use.^{15,17}

Given these findings, it will be important to identify pharmacological interventions that more selectively turn on IL-12 production in tumor-associated immune cells and thus locally broaden the therapeutic window. Preclinical work has demonstrated that IL-12 is produced by multiple immune cell types.^{18–21} However, in tumor microenvironments, the highest IL-12 producing cells have been shown to be dendritic cells in the DC3 cluster.¹¹

The non-canonical NFκB pathway

IL-12 production appears to be tightly regulated by the non-canonical NFκB pathway, as summarized in Fig. 1b.¹¹ The central, key regulator of this pathway is NFκB-inducing-kinase (NIK, also known as MAP3K14).^{22,23} In unstimulated cells, NIK levels are very low, as a complex of E3 ligases ubiquitinates the protein. The added chain of ubiquitin has a lysine 48 (K48) linkage, which marks NIK for degradation by the proteasome. This ubiquitin ligase complex that modifies NIK typically consists of the proteins TRAF3, TRAF2, and either cellular Inhibitor of Apoptosis 1 or 2 (cIAP1 or 2). Other proteins may also be included, again depending on contexts such as cellular type. TRAF3 is unable to ubiquitinate directly, so it binds to either

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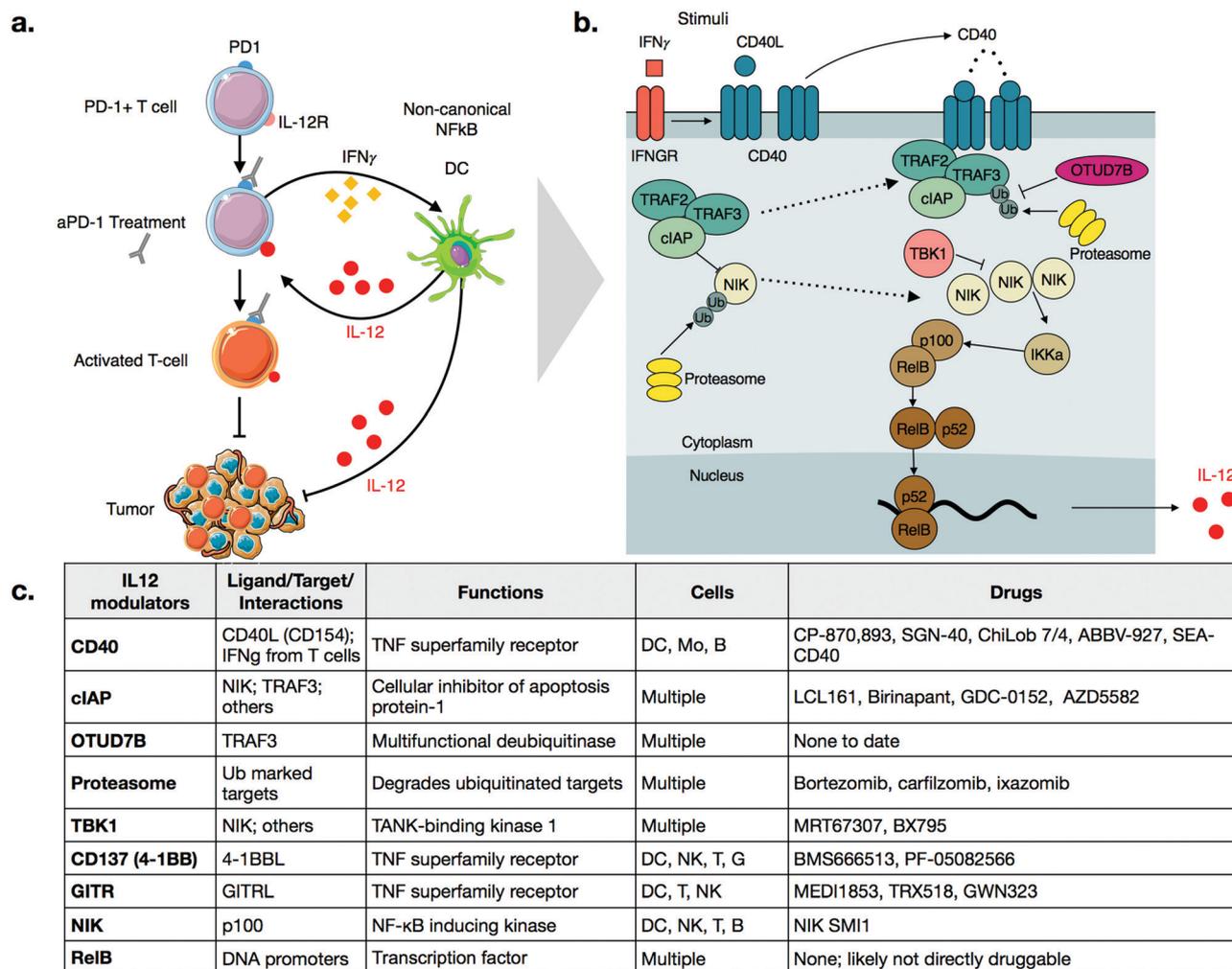


Fig. 1 (a) Schematic of IL-12 modulation of checkpoint therapy through T-cell:dendritic cell cross talk. aPD-1 treatment induces IFN γ in T-cells, which promotes production of IL-12 in dendritic cells. IL-12 then further activates anti-tumor T-cells. IL-12 activates other cell types as well, which leads to additional anti. (b) Schematic of the non-canonical NF κ B pathway. Activation of CD40, either through CD40L binding or through IFN γ signaling, disrupts the TRAF2-TRAF3-cIAP complex, allowing NIK levels to rise. NIK then promotes p100 processing via IKKa, which leads to an active RelB-p52 complex that can translocate to the nucleus and cause transcription of IL-12. (c) Description of various signaling nodes in the non-canonical NF κ B pathway.

cIAP1 or cIAP2 (hereinafter referred to as cIAP), which are responsible for the ubiquitination of NIK.²² The bridging of cIAP to TRAF3 is mediated by TRAF2.²⁴

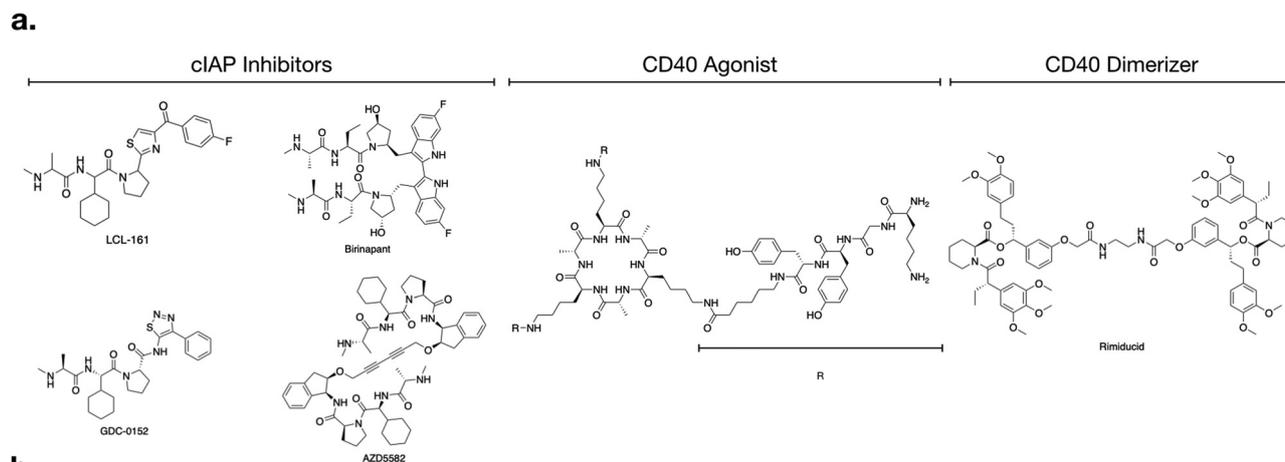
Precise triggers of the non-canonical NF κ B pathway in dendritic cells are still incompletely known, but dendritic cell sensing of IFN γ may play a role, since dendritic cells in IFN γ receptor-deficient mice show decreased expression of non-canonical NF κ B pathway genes.²⁵ Other activators of the non-canonical NF κ B pathway include ligands of Tumor Necrosis Factor Superfamily Receptors (TNFSFR),²² which can be expressed by dendritic cells as well as many other cell types including macrophages, T cells, B cells, NK cells, granulocytes as well as some non-immune cells.²⁶ In dendritic cells, examples of TNFSFRs include CD40, CD137 (4-1BB, TNFRSF9), and the lymphotoxin beta receptor (LTBR).²⁷ While the details of signaling vary among the receptors, a common mechanism is that agonization of the receptor by its ligand disrupts cytosolic TRAF3-TRAF2-cIAP complex. Most receptors have a domain that can bind to TRAF3,

thereby drawing it away from NIK. With some receptors, cIAP subsequently begins to ubiquitinate TRAF3, marking its degradation.^{22,28} Disruption of the complex frees NIK from ubiquitination, allowing its levels to accumulate slowly, as the gene must be transcribed and then translated. This step is notable because it explains why activation of the non-canonical NF κ B pathway is relatively slow, especially in comparison to the canonical NF κ B pathway, which elicits a more transient response to stimuli.²⁹

As NIK levels increase, it phosphorylates the kinase IKKa, a key kinase relevant in the canonical NF κ B pathway as well. IKKa phosphorylates p100, the precursor of NF κ B2. Phosphorylation of p100 leads to its ubiquitination and subsequent degradation, forming the active form of NF κ B2, p52. NF κ B2 p52, in complex with RelB, then translocate to the nucleus, turning on production of cytokines such as IL-12.^{11,30}

As with all innate immune pathways, mechanisms are also in place to prevent inappropriate hyperactivation. The kinase,





b.

Therapeutic	Target	Sample Clinical Trial	Indication	Status	Sponsor
LCL161	cIAP	NCT01240655	Advanced solid tumor with paclitaxel	Ph 1 completed	Novartis
Birinapant	cIAP	NCT02587962	Solid tumors in combination with pembrolizumab	Recruiting for Ph1/2	Medivir (Merck)
GDC-0152	cIAP	NCT00977067	Melanoma	Terminated (not due to safety)	Genentech
CP-870,893	CD40	NCT01456585	Pancreatic cancer, combination with gemcitabine	Ph1 completed	Pfizer
ABBV-927	CD40	NCT02988960	Advanced solid tumors; combination with ABBV-181 (aPD-1)	Ph 1 Recruiting	AbbVie
SEA-CD40	CD40	NCT02376699	Multiple cancers; combination with pembrolizumab, gemcitabine, and nab-paclitaxel	Ph1 Recruiting	Seattle Genetics
BMS-663513	CD137 (4-1BB)	NCT00309023	Metastatic and locally advanced solid tumor	Terminated	Bristol-Myers Squibb
MEDI1873	GITR	NCT02583165	Advanced solid tumor	Ph1 completed	MedImmune
TRX518	GITR	NCT01239134	Metastatic Melanoma and other solid malignancies	Ph1 completed	Leap Therapeutics

Fig. 2 (a) Structure of various cIAP inhibitors (left), CD40 agonist (middle), and rimiducid (right), a tool compound used in cellular therapy. (b) Table of various clinical trials highlighting agents that modulate non-canonical NF κ B signaling.

any effect on non-canonical NF κ B signaling.⁵² The pathways underlying apoptosis have been reviewed elsewhere,^{51,53,54} but it is important to note that development of various cIAP inhibitors were motivated with this pro-apoptotic mechanism in mind.⁵⁵ IAP members are also often amplified in cancer, furthering the rationale for clinical use of IAP antagonists as a pro-apoptotic agents acting directly on the tumor.⁵²

Several preclinical studies have focused on the apoptotic effects of cIAP inhibitors, in both solid tumors and hematological malignancies, often in combination with other cytotoxic drugs.^{56–58} With the realization that cIAP inhibitors also activate non-canonical NF κ B signaling, attention has been directed to the immunostimulatory capabilities as well. Most preclinical studies at the *in vivo* level have found broad immunostimulatory effects in multiple cell types.⁵⁹ However, further work has clarified that cIAP inhibitors indeed potently induce IL-12 directly in dendritic cells.^{11,60}

As with most small molecules, cIAP inhibitors exhibit polypharmacology, and different drugs have varying degrees of selectivity against cIAP and xIAP. In Table 1, we highlight the affinities of various cIAP inhibitors against selected members of the IAP family. LCL161 and birinapant, two cIAP inhibitors in

Table 1 IC₅₀ (in nM) of small molecule inhibitors against cIAP1/2 and xIAP. Data taken from multiple ref. 55, 115–117

	cIAP1	cIAP2	xIAP
LCL161	0.4	N/A	35
Birinapant	45	N/A	< 1
AZD5582	15	21	15
GDC-0152	17	43	28

clinical trials, exhibit preferential activity against cIAP compared to xIAP, but nonetheless are still reasonably potent inhibitors of xIAP. Other inhibitors, such as AZD5582 and GDC-0152, exhibit less preferential activity against cIAP. Whether and how this polypharmacology manifests in a clinical setting remains to be determined.

It remains to be determined whether the therapeutic mechanism in man of cIAP inhibitors is due to anti-tumor immunity, pro-apoptotic effects, or both. We expect both mechanisms to be relevant. In fact, it is reasonable that they may be synergistic, since promoting apoptosis would lead to dying tumor cells that could be a source of antigens for the immune system.⁶¹ Conceptually, such a dual mechanism is similar to how



increasing numbers of other cytotoxic drugs, such as PARP inhibitors, both kill tumor cells and prime immune cells for activation.⁷ Nonetheless, it is likely that the mechanism is highly dependent on the context of the model system. One study, using the cIAP1 and xIAP inhibitor, LCL161, found that the drug was curative in mouse models of multiple myeloma.⁶² Response was associated with an innate immune signature, and in fact was independent of direct cytotoxic effects of the drug on the tumor.

Numerous clinical trials using cIAP inhibitors have investigated both effects of the drug.^{63,64} Phase 1 studies have established the safety and pharmacokinetic properties of LCL161, developed by Novartis. In one study, a common toxicity was cytokine release syndrome, consistent with immunomodulation.⁶⁴ GDC-0152 is a structurally similar inhibitor,⁵⁵ but its phase 1 trial was terminated for reasons not due to safety or efficacy.

Given that production of IL-12 in dendritic cells enhances aPD-1 therapy in preclinical models,⁶⁰ it will be interesting to examine cIAP inhibitors in combination with checkpoint inhibitors. LCL161 is currently being tested in combination with PDR001 (aPD-1) for multiple myeloma (NCT02890069). Another active trial is testing birinapant, a bivalent SMAC mimetic, in combination with pembrolizumab (aPD-1) in multiple solid tumor types (NCT02587962). Going forward, we can expect to see more trials using cIAP inhibitors. An additional cIAP inhibitor that has not yet been tested clinically, but has shown promising preclinical activity, is AZD5582.¹¹ All of these SMAC mimetics have varying biochemical affinities towards the different members of the IAP family, and it will become important to evaluate if any specific target is more important than another, and in what context.

Other targets and potential probes for non-canonical NFκB modulation

Additional targets for IL-12 production in dendritic cells include other members of the TNFSFR family, such as LTBR, CD137, and GITR.^{27,65} As signaling through these receptors is similar to that of CD40, we refer the reader to more in-depth publications.^{66–68} Most therapeutics targeting these receptors are biologics and Fig. 2b highlights various clinical trials using them.

There is a need for more small molecule modulators of the non-canonical NFκB pathway. Existing small molecule agonists of other innate immune pathways, such as R848, an imidazoquinoline agonist of TLR7/8, or 2'3' cGAMP, an agonist of STING, enhance IL-12 production but these predominantly signal through canonical NFκB signaling and/or interferon regulatory factor (IRF) pathways.^{69,70} There is some evidence they may also contribute to non-canonical NFκB signaling but more investigation is warranted. Identification of small molecule activators of IL-12 production can be achieved with various screening assays, including using immune cells from an IL-12 YFP reporter mouse⁶⁰ (Fig. 3).

Of the targets shown in Fig. 1, an inhibitor of the deubiquitinase enzyme (DUB), OTUD7B, could specifically activate the non-canonical NFκB pathway. Hu *et al.* showed that depletion of the deubiquitinase enzyme (DUB), OTUD7B, enhanced signaling in dendritic cells.³² DUB inhibitors have been developed for

cancer therapy.⁷¹ While none have been developed specifically for OTUD7B, existing chemoproteomic methods could indicate whether any existing inhibitors have significant off target affinity on OTUD7B.⁷² There are only roughly 100 DUBs, so as more inhibitors are developed, an important goal is to characterize their biochemical affinities across the spectrum of DUBs.

Other pathway targets include TRAF3, TBK1, the proteasome, and NIK. TRAF3 is a logical choice from a biological perspective, as it plays a direct role as a pathway inhibitor, and genetic studies clearly indicate that depletion of TRAF3 activates non-canonical NFκB signaling.^{73–75} Unfortunately, there are no specific small molecule probes for TRAF3, and in general, research into inhibitors of E3 ligases is not a well explored space from a chemical perspective. In contrast, TBK1 is druggable, and there are many available inhibitors, such as MRT67307.⁷⁶ However, MRT67307 failed to induce IL-12 production in murine dendritic cells.⁶⁰ Jin *et al.* indicated TBK1 as a negative regulator, but the work was confined to B-cells, so cell type specificity may be an issue.³¹ There are many inhibitors for the proteasome,⁷⁷ including bortezomib, which is FDA approved for multiple indications. It will be reasonable for future work to examine whether bortezomib can modulate non-canonical NFκB signaling. A challenge here is that the proteasome plays multiple roles in non-canonical NFκB signaling, as it degrades both NIK and TRAF3, which have opposing biological roles. Additionally, from a broader perspective, proteasome inhibitors are toxic, and have wide ranging effects on other pathways; these factors might also complicate their use as IL-12 inducers. Lastly, while NIK is druggable, an inhibitor would block, not induce, IL-12 production, and thus lack translational value in cancer immunotherapy. Nonetheless, NIK inhibitors have been developed for treatment of systemic lupus erythematosus,⁷⁸ and these probes may have value as tools to help further dissect pathway biology.

Lastly, it will be interesting to evaluate whether certain small molecule degraders induce IL-12 production. Small molecule degraders are bivalent ligands, composed of an enzyme or receptor inhibitor linked to a E3 ligase binder. The concept behind their mechanism of action is that one part of the molecule binds to its target while another part binds an E3 ligase and brings it near its target; the resulting proximity leads to ubiquitination of the target, thereby promoting its degradation. Numerous studies have indicated that protein degradation has advantages over protein inhibition, and have focused on optimal properties of effective degraders.^{79–81} Interestingly though, a new class of degraders, called SNIPERS,⁸² use SMAC mimetics, such as LCL161, as the E3 ligase binder. Thus, it will be interesting to evaluate whether SNIPERS, by binding cIAP, may also trigger the non-canonical NFκB pathway as an unintended effect. To our knowledge, no study has yet examined whether use of an SMAC mimetic in small molecule degraders might confer immunostimulatory effects. This may give SNIPERS an advantage over other small molecule degraders, such as PROTACs, which use thalidomide analogs instead of SMAC mimetics.



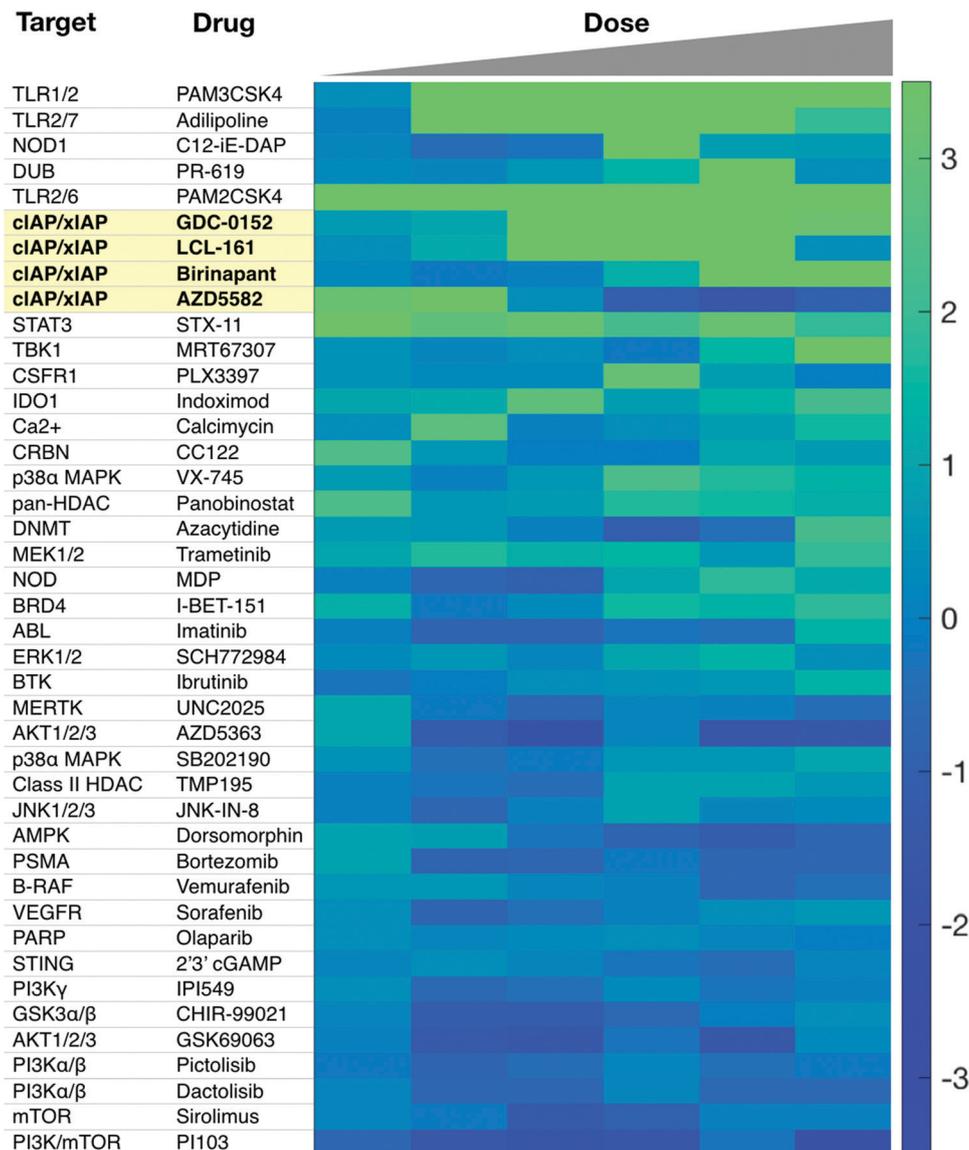


Fig. 3 Sample screen for IL-12 inducers in murine dendritic cells. Several clAP inhibitors scored high in this assay. Reproduced from Koch *et al.* Cell Chem. Biol. (2020).

Canonical NFκB and other signaling pathways

While the primary focus in this review has been on the non-canonical NFκB pathway, it is important to note that IL-12 can also be produced in response to other stimuli as well. Signaling through the canonical NFκB pathway or *via* the interferon-regulatory-factor-3/7 (IRF3/7) pathways can promote IL-12 secretion.^{83–85} Canonical NFκB signaling and IRF3/7 signaling are both activated by upstream pathways. Examples include the Toll-like-receptor (TLR), STING, and RIG-I pathways.^{86–88} These pathways are all similar in that they are triggered by pathogen associated molecular patterns (PAMPs), including bacterial lipopolysaccharide or viral nucleic acid. Each pathway is initiated when a specific receptor binds to a PAMP; activation then funnels into canonical NFκB and/or IRF3/7 signaling, which promote IL-12 secretion.

The canonical NFκB pathway in particular, shares many of the same nodes as the non-canonical pathway. In brief, for the canonical NFκB pathway, an IKK kinase phosphorylates IκB, leading to its degradation by the proteasome. Degradation of the IκB protein allows translocation of a RelA/p50 dimer to the nucleus where it turns on a transcriptional response. In contrast to non-canonical NFκB signaling, canonical pathway responds quickly to stimuli and is transient. For more information on this signaling pathway, we refer the reader elsewhere.^{84,85}

Therapeutics acting on the above pathways include PAMPs themselves or synthetic mimetics. For example, the *Bacillus Calmette-Guerin* (BCG) vaccine, which contains attenuated bacteria, is used in bladder cancer.⁸⁹ Poly-IC, a synthetic dsRNA mimetic that binds RIG-I and TLR3, is being pursued in several cancer immunotherapy strategies,^{90,91} and is also in several clinical trials, including one which it is being used in combination



with a CD40 agonist (NCT01008527). Synthetic small molecule agonists have also been developed, as such therapeutics have improved pharmacokinetic properties compared to PAMPs, which are large, and often charged. Imiquimod and resiquimod are imidazoquinolines that binds TLR7 and TLR8.^{70,92,93} There is substantial interest in developing more improved therapeutics on these pathways, which have potential to potently induce IL-12.

Localized delivery of IL-12 modulators

Ideally, production of IL-12 should be confined to the tumor microenvironment. Systemic administration of recombinant IL-12 has been limited by broad toxicity.^{15,17} A key aspect to improving therapeutic efficacy of IL-12 while minimizing systemic side effects has been to enhance local production in tumor microenvironments. This has and can be achieved by a number of different ways, summarized below.

Intratumoral delivery

In melanoma, a phase II clinical trial (NCT01502293) is ongoing in which an IL-12 tavokinogene telseplasmid is electroporated into tumors. Preliminary results indicate that intratumoral electroporation enhances antitumor immune responses.^{11,17} This is undoubtedly an impressive technological feat with promising results but may require easily accessible tumors and ideally non-metastatic lesions, unless these local treatments can produce systemic antitumor immune responses. For biologics, intratumoral injection of CD40 agonists has shown promising efficacy with fewer side effects compared to systemic treatment.⁹⁴

Targeting myeloid cells with nanotherapeutics

Various nanoformulations have been used to deliver small molecules to the tumor microenvironment. The vast majority of efforts have been centered around the enhanced permeability and retention (EPR) effect and subsequent tumor cell targeting using liposomes, PLGA-PEG, albumin nanoparticles, and graft copolymers, among other formulations.^{95–103} Targeting tumor-associated myeloid cells has also been of interest for diagnostic^{104–106} and therapeutic effects.^{107–109} The nanoformulations most commonly used for myeloid cell targeting include modified dextran and cyclodextrins as well as other carbohydrate-based nanomaterials. Successful examples of myeloid targeting of small molecules include TLR7/8^{70,107,110–112} and cIAP agonists such as LCL161. LCL-161 was complexed to cyclodextrin nanoparticles and shown to (i) increase tumoral IL-12, (ii) decrease tumor volumes, (iii) outperform the free drug control, and (iv) have minimal toxicity.⁶⁰

Further work is needed on clarifying whether certain types of nanoparticles are taken up preferentially by specific types of myeloid or other cells. Advances in intravital imaging and single-cell RNA sequencing will allow for profiling of drug action in various cell types, at a very refined level.¹¹³ Beyond establishing targeting of myeloid subsets with different nanoformulations,¹¹⁴ a major effort will be to incrementally improve drug loading with subset targeting. Overall, we expect nanoformulations to play an important role in enhancing the

efficacies of small molecule immunostimulatory drug candidates by directly targeting them to tumor-associated myeloid cells.

Concluding remarks

It is increasingly clear that local tumoral production of IL-12 is an attractive option for cancer immunotherapy, and has particular promise in synergizing with checkpoint blockade. In this review, we have highlighted the non-canonical NFκB pathway as one source of targets that could be pharmacologically modulated for IL-12 production. At the time of writing, therapeutics targeting this pathway include agonists of TNFSFRs as well as inhibitors of cIAP. Clinical trials are ongoing to evaluate these therapeutics but more research is needed to clarify how these drugs work in man. In parallel, it is important to further study non-canonical NFκB signaling to identify new promising approaches, as well as analyze whether any existing drugs have effects on this pathway. Finally, advances in localized delivery of drugs to the tumor microenvironment will be pertinent for IL-12 therapeutics, as it can aid efforts in mitigating immunotoxicity.

Conflicts of interest

RW is a consultant for Tarveda Pharmaceuticals, Moderna, Alivio Therapeutics, Lumicell, Accure Health, and Aikili Biosystems. These commercial relationships are unrelated to the current study. MJP is a consultant for AstraZeneca, Elstar Therapeutics, KSQ Therapeutics, Merck, and Siamab Therapeutics. These commercial relationships are unrelated to the current study. PDK: no relevant disclosures.

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