Published OnlineFirst November 29, 2017; DOI: 10.1158/2326-6066.CIR-17-0490

Research Article

Cancer Immunology Research

IAP Antagonists Enhance Cytokine Production from Mouse and Human iNKT Cells

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Abstract

Inhibitor of apoptosis protein (IAP) antagonists are in clinical trials for a variety of cancers, and mouse models show synergism between IAP antagonists and anti–PD-1 immunotherapy. Although IAP antagonists affect the intrinsic signaling of tumor cells, their most pronounced effects are on immune cells and the generation of antitumor immunity. Here, we examined the effects of IAP antagonism on T-cell development using mouse fetal thymic organ culture and observed a selective loss of iNKT cells, an effector cell type of potential importance for cancer immunotherapy. Thymic iNKT-cell development probably failed due to increased strength of TCR signal leading to negative selection, given that mature iNKT cells treated with IAP antagonists were not depleted, but had enhanced cytokine production in both mouse and human *ex vivo* cultures. Consistent with this, mature mouse primary iNKT cells and iNKT hybridomas increased production of effector cytokines in the presence of IAP antagonists. *In vivo* administration of IAP antagonists and α-GalCer resulted in increased IFNγ and IL-2 production from iNKT cells and decreased tumor burden in a mouse model of melanoma lung metastasis. Human iNKT cells also proliferated and increased IFNγ production dramatically in the presence of IAP antagonists, demonstrating the utility of these compounds in adoptive therapy of iNKT cells. *Cancer Immunol Res; 6(1); 25–35. ©2017 AACR.*

Introduction

The inhibitor of apoptosis proteins (IAP) are an evolutionarily conserved family of proteins with diverse biological functions. Although initially identified as potent caspase inhibitors, IAP proteins play a critical role in regulation of NF-κB signaling (1–4). The IAP family is defined by the presence of at least one baculovirus inhibitory repeat (BIR) domain, and four IAPs conserved in both mice and humans contain an additional RING finger E3 ubiquitin ligase domain; these include X-linked (X)IAP, cellular (c)-IAP 1 and 2, and melanoma associated (ML)-IAP (1–4).

The IAP E3 ligases are regulated endogenously by the second mitochondrial activator of caspases (SMAC), an inhibitory protein released from the mitochondria that binds to the IAPs through the BIR domain. Several pharmaceutical companies have developed small-molecule SMAC mimetics that act as IAP antagonists and have some activity against tumor cell lines (5). These compounds have proved useful in elucidating many of the endogenous functions of the IAPs, in part through their ability to modulate the function of multiple IAPs simultaneously, circumventing functional redundancy within the family (1–4). Binding of the IAP antagonists to cIAP1/2 induces a conformation change that leads to auto-ubiquitination and degradation, with subsequent modulation of NF-κB1 and NF-κB2 signaling. In addition, cIAP1/2 loss leads to assembly of an RIP1–FADD–caspase 8 complex, sensitizing some tumor cells to TNF superfamily ligand–mediated cell death.

IAP antagonism can modulate the immune system, which raises the possibility of a cancer immunotherapeutic (6). LCL161, an IAP antagonist, was efficacious in multiple myeloma, through the activation of type I interferon signaling by tumor cells, which led to myeloid cell activation and tumor cell phagocytosis (7). IAP antagonism conferred immunological memory and long-term survival in a small fraction of mice; survival could be increased via combination with PD-1 blockade (7). Similarly, another study in glioblastoma showed that LCL-161 treatment caused enhanced production of TNFα from intratumoral T cells, and simultaneously enhanced sensitivity to TNFα induced apoptosis in the malignant cells (8). These studies agree with previous work from our group showing that IAP antagonists have potent costimulatory activity in effector T cells, likely through the inhibition of c-IAP1 and c-IAP2 and downstream activation of NF-κB2 signaling (6).

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Note: Supplementary data for this article are available at Cancer Immunology Research Online (http://cancerimmunolres.aacrjournals.org/).

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Mice deficient in each of the IAPs have been generated experimentally; however, only XIAP deficiency has been reported in humans (9, 10). XIAP-deficient patients develop X-linked lymphoproliferative syndrome (XLP), an immunodeficiency characterized by chronic infection with Epstein–Barr virus, although the specificity of this finding has been questioned (11). A potential cause of the phenotype associated with XLP is a loss of invariant natural killer T (iNKT) cells, a subset of T cells that recognize lipid antigens in the context of the MHC-homologue CD1d. iNKT cells orchestrate the early phases of an immune response through the elaboration of cytokines and have been implicated in antiviral immunity (12). In contrast to human XIAP deficiency, XIAP knockout mice are relatively immunologically intact and have equivalent numbers of iNKT cells compared with wild-type mice (9, 10). Given the structural similarity among the RING domain containing IAPs, failure to observe an iNKT-cell defect in XIAP knockout mice could indicate functional redundancy among the IAPs in mice.

iNKT cells primarily express a semi-invariant T-cell receptor (TCR) comprised of Vα14Jα18 in mice and Vα24Jα18 in humans, as well as a restricted subset of Vβ genes: Vβ8.2, Vβ7, or Vβ2 in mice, and Vβ11 in humans (13). Due to the semi-invariant nature of the TCR, iNKT cells act as a preexpanded T-cell pool capable of rapid and diverse cytokine production early in an immune response, including production of both IFNγ and IL-4 (12). Like peptide-reactive T cells, iNKT cells develop in the thymus, and several distinct signaling pathways have been identified that are specifically required for the generation of iNKT cells (7, 8, 14–20).

iNKT cells may also play a role in antitumor immunity, particularly through their secretion of IFNγ to cross-activate NK cells (21–29). iNKT cells can also orchestrate a tumor growth factor (TGF)-β mediated pathway of immune cell inhibition that is less completely understood, but is of potential importance to antitumor immunity (30). The broad iNKT cell–activating ligand α-galactosylceramide (α-GalCer) was originally identified in a small molecule screen for compounds with antitumor activity (31), and clinical trials using either α-GalCer–pulsed dendritic cells, infusion of iNKT cells expanded *in vitro*, or iNKT cell–based CAR-T therapies are underway in are variety of cancers (26, 32–35).

We show here that IAP antagonists block iNKT-cell development in fetal thymic organ cultures (FTOC), possibly through alterations in TCR signal strength. Conversely, in mature iNKT cells, IAP antagonists act as pharmacological costimulators, enhancing cytokine responses to α-GalCer. IAP antagonism of iNKT cells *in vivo* results in enhanced IFNγ and IL-2 production in response to α-GalCer, and decreased tumor burden in mice inoculated intravenously with B16 melanoma. Human iNKT cells also respond to IAP antagonism; addition of IAP antagonists to α-GalCer–stimulated human peripheral blood mononuclear cells similarly enhances Th1 cytokine production, while also increasing the yield and purity of iNKT cells upon *in vitro* culture, making this approach a viable strategy for augmenting current techniques used in iNKT-cell infusion therapies.

Materials and Methods

Animals

C57BL/6 mice were purchased from The Jackson Laboratory or bred in house. CD1d-deficient mice were purchased from The Jackson Laboratory. iNKT transnuclear (Vα14, Vβ7A;RAG2$^{-/-}$, Vβ7C;RAG2$^{-/-}$, and Vβ8.2;RAG2$^{-/-}$) mice were generated by somatic cell nuclear transfer and bred in house (36). All animal experimentation was done in accordance with institutional guidelines and the review board of Harvard Medical School, which granted permission for this study, and was approved by the AAALAC-accredited Dana-Farber Cancer Institute IACUC.

Fetal thymic organ culture

Embryonic day 16 fetal thymic lobes were harvested from timed pregnant C57BL/6 mice. Three to six fetal thymic lobes per well were cultured in Transwell plates (Corning). Lobes were cultured for 18 to 20 days in 700 μL DMEM containing 20% fetal bovine serum per well in 12-well tissue culture plates. IAP inhibitors (500 nmol/L) or control compound were added to the media throughout the culture period or only during the final 48 hours. Media were changed every 2 to 3 days. Cells were harvested by mechanical disruption of the thymic lobes and passage through a 70-μm cell strainer.

Antibodies and reagents

Antibodies to mouse CD3 (hamster mAb clone 145-2C11) and mouse CD28 (hamster mAb clone 37.15) for T-cell stimulation were purchased from BD Biosciences. Fluorescent antibodies for flow cytometry, including mouse IL-2 (clone JES6-5H4), mouse IFNγ (clone XMG1.2), mouse CD3 (clone 17A2), mouse CD4 (clone RM4-5), mouse CD8 (clone 53-6.7), human CD3 (clone OKT3), and human Vα24/Jα18 (clone 6B11), were purchased from BioLegend. CD1d-PBS57 (CD1d-αGal) tetramers were obtained from the NIH Tetramer Core Facility, and αGalCer was purchased from Avanti. IAP antagonists were provided by Novartis Pharmaceuticals.

Cell culture

Ld cells (gift from Dr. Michael Brenner) were cultured in DMEM with 10% FBS, 1% PenStrep, and 2 mmol/L L-glutamine. CD1d expression on Ld cells was confirmed by inclusion of a no αGalCer condition in each experiment, but no further authentication was performed. Ld cells were kept in culture for no more than 4 weeks and were tested for *mycoplasma* every 4 months. For cocultures, total FTOC cells were added to Ld cells pulsed with 200 ng/mL α-GalCer (Avanti Lipids), and IL-2 production was measured by ELISA (BD Pharmingen).

For culturing of spleen cells from iNKT transnuclear mice (36), whole spleens from transnuclear mice (Vβ7A;RAG2$^{-/-}$, Vβ7C;RAG2$^{-/-}$, and Vβ8.2;RAG2$^{-/-}$) were inflated with PBS before homogenization and plating at 2×10^5 cells per well in a 96-well plate, with the indicated concentrations of α-GalCer with or without 500 μmol/L LCL-161, as indicated. Supernatants were collected after 24 hours and analyzed by ELISA (BioLegend). In some cases, CD8 and CD4 T cells were isolated from C57BL/6 spleens by magnetic bead separation (Thermo Fisher Dynabeads, Untouched CD8 and Untouched CD4). CD8 and CD4 T cells were plated at 1.5×10^5 cells per well in a 96-well plate with 4×10^5 anti-CD3/anti-CD28 beads/mL (Gibco). Whole spleens from Vα14 transnuclear mice (36) were inflated with PBS, homogenized, and plated at 1.5×10^5 cells per well in a 96-well plate with 100 ng/mL α-GalCer. Production of IL-4, IL-2, GM-CSF, and IFNγ of 24 hour culture supernatants was measured by ELISA as indicated (BioLegend) or by cytokine bead array (Eve Technologies).

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Experimental lung metastasis model

C57BL/6 mice were treated with 75 mg/kg vehicle or LCL-161 by oral gavage at day −2 and day 0 of analysis. On day 0, mice were injected with 3×10^5 B16F10 cells intravenously. On days 0, 4, and 8, mice were treated with 1 μg α-GalCer intraperitoneally. On day 14, mice were sacrificed and downstream analyses performed.

Human iNKT-cell isolation and culturing

Peripheral blood was obtained from healthy human volunteers. Volunteers were screened for the presence of CD1d-tetramer$^+$ cells, and only volunteers with iNKT cells at >0.1% frequency were included. For cytokine analysis, whole peripheral blood mononuclear cells were cultured at 5×10^6 cells per well in a 96-well plate with 200 ng/mL α-GalCer and/or 500 nmol/L LBW-242 as indicated. IFNγ and IL-4 were measured by ELISA of 48-hour culture supernatants. Human iNKT-cell lines were generated as previously described (33).

Statistical analysis

Two sample comparisons used the *t* test with pooled variance if there was no evidence of inhomogeneity of variances between groups. If the variances were unequal, the exact Wilcoxon rank sum test, a nonparametric alternative to the *t* test, was used. Every effort was made to keep testing consistent across related experiments. For comparisons of more than two groups, analysis of variance (ANOVA) was used if there was no evidence of inhomogeneity of variance; the Kruskal–Wallis test was the nonparametric alternative.

Results

IAP antagonists block iNKT-cell development in FTOC

Moderate TCR signals are required for CD4$^+$ and CD8$^+$ T cells to develop in the thymus. Given previous work by us and others showing that IAP antagonists enhance activation of mature T cells via increased NF-kB2 signaling downstream of TNF family receptors (6), we asked whether enhanced costimulatory signals would alter T-cell development. In order to study thymic development *ex vivo*, we used FTOC, in which thymic lobes can be grown in tissue culture to recapitulate T-cell development *ex vivo*. We chose to use this system rather than systemically delivering IAP antagonists to whole mice due to potential confounding effects on thymic development resulting from alterations in peripheral immune cell function (6). Using a panel of three structurally distinct IAP antagonists (control: LCV-843; IAPi: LBW-242, LCL-161, and LCJ-917; Supplementary Fig. S1 and ref. 18), we found that total thymocyte numbers were reduced by about 50% in treated FTOCs, accompanied by a decrease in the percentage of CD4$^+$ T cells (Supplementary Fig. S2A) and a corresponding decrease in the CD4$^+$ to CD8$^+$ T-cell ratio (Supplementary Fig. S2B). The overall percentage of single positive cells (SP) remained constant (Supplementary Fig. S2C).

In all IAP antagonist-treated cultures, we consistently observed a 95% to 100% loss of iNKT cells, which can be identified as CD3$^+$ T cells that bind CD1d tetramers loaded with α-GalCer (refs. 37–39; Fig. 1A and B). Across several experiments, iNKT cells reliably developed in untreated FTOCs and FTOCs treated with an inactive compound; in contrast, treatment with

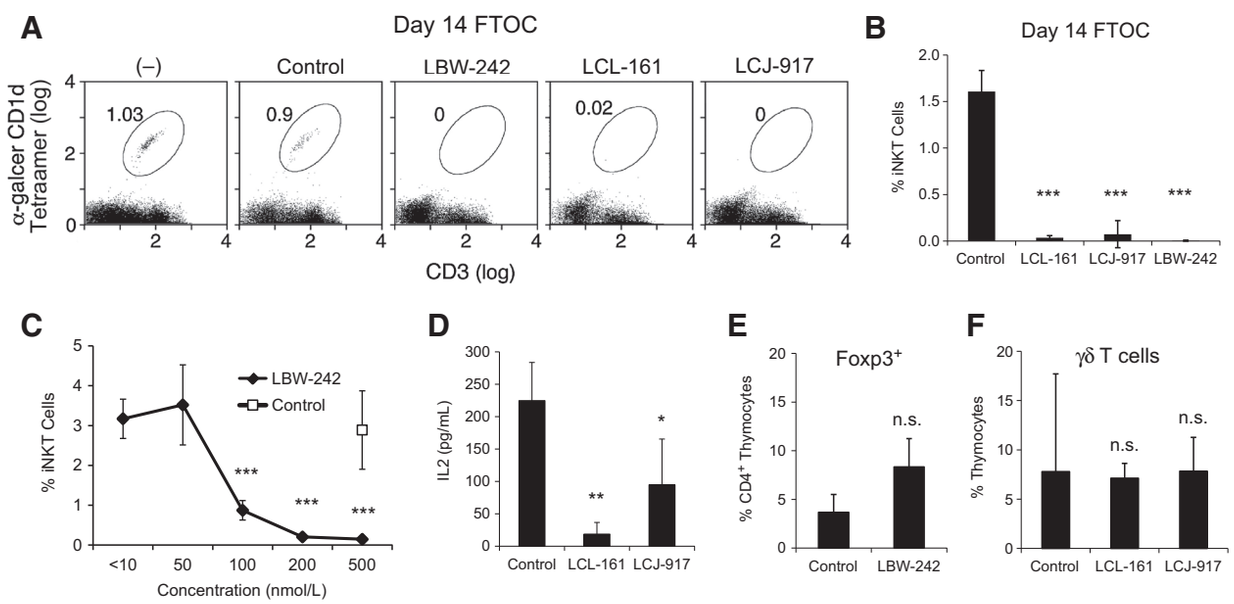

Figure 1.

iNKT-cell development in FTOC is blocked by IAP antagonists. **A–D,** Thymii from E16.5 embryos were cultured in the presence of IAP antagonists (LBW-242, LCL-161, LCJ-917), vehicle (–), or control compound (LCV-843) for 14 days. Control and LBW-242 were used at 500 nmol/L or as indicated; LCL-161 and LCJ-617 were used at 200 nmol/L. **A,** α-GalCer-loaded CD1d tetramer$^+$ iNKT cells were identified in FTOC using flow cytometry. **B,** Quantification of results from three independent experiments performed and analyzed as in **A**. **C,** Dose–response curve for LBW-242 in FTOC. **D,** Cells recovered from FTOC were stimulated with anti-CD3 without further exposure to IAP antagonists; labels indicate the compounds added during thymic culture. IL2 was measured in the culture supernatant by ELISA. **E,** Foxp3-positive thymocytes were identified in LBW-242–treated cultures using thymi harvested from Foxp3-GFP knock-in embryos. **F,** γδ T cells were identified in LCL-161– and LCJ-917–treated cultures by δ chain expression. **D–F,** Error bars represent SEM. Results are representative of at least two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant versus control.

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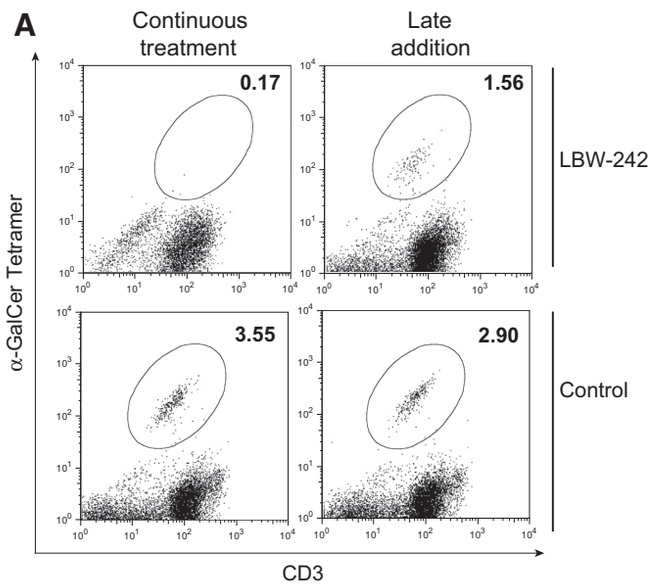

Figure 2.
Mature iNKT cells persist in IAP antagonist–treated cultures. **A,** FTOC were performed as in Fig. 1 in the presence of LBW-242 or control compound (continuous treatment) or were cultured in the absence of compound until day 14 when LBW-242 or control compound were added (late addition). All cultures were harvested at day 16 and analyzed by flow cytometry. **B,** Spleen cells (5×10^5) were cultured in the presence of LBW-242 or control compound; after 24 hours, total viable cells were quantified by Trypan blue exclusion, and T cells and iNKT cells were identified by flow cytometry. Error bars present SEM. **A** and **B,** Results are representative of two independent experiments.

pharmacologically relevant doses of multiple distinct IAP antagonists (LBW-242, LCL-161, and LCJ-917) prevented iNKT-cell development in a dose-dependent fashion (Fig. 1B and C). The absence of iNKT cells was not the direct result of reduced $CD4^+$ T-cell numbers, as iNKT cells were still absent in IAP antagonist–treated cultures that had relatively preserved $CD4^+$ T cell numbers. Given that iNKT cells can escape detection by T-cell receptor downregulation, we also confirmed the absence of iNKT cells by cytokine production in response to α-GalCer. Consistent with a substantial decrease in iNKT-cell numbers, cells from FTOCs treated with IAP antagonists were impaired in their ability to produce IL-2 when cocultured with CD1d transduced fibroblasts (Ld cells) pulsed with α-GalCer (Fig. 1D). Loss of iNKT cells was not associated with a general inability to detect rare thymic populations in FTOC, as both $Foxp3^+$ T cells and γδ T cells persisted at normal frequencies in IAP antagonist–treated cultures (Fig. 1E and F). These findings indicate that murine iNKT cells have a particular sensitivity to IAP antagonism, consistent with involvement of the IAP proteins in normal iNKT-cell development (9).

IAP antagonists do not induce apoptosis of mature iNKT cells

Although we hypothesize that the effect of IAP antagonism on iNKT-cell development in FTOC occurred through signaling modulation, in principle, the direct induction of apoptosis in iNKT cells by IAP antagonists could account for our results. To validate our hypothesis, we examined the effect of IAP antagonism late during development of the FTOC. The majority of positive selection for iNKT cells in FTOC occurs prior to day 14 of culture; therefore, addition of IAP antagonists on days 14 through 16 would be expected to have minimal effects on iNKT-cell populations, if the principal mechanism of action were through modulation of developmental signaling (37). In contrast, if the IAP antagonists act through the induction of apoptosis, late addition of IAP antagonists should lead to loss of iNKT cells (37). Consistent with a role during developmental signaling at the time of selection, cultures with delayed IAP antagonism on day 14 contained similar iNKT-cell populations as cultures exposed to control compound (Fig. 2A). Similarly, splenic T cells and mature, peripheral iNKT cells show no difference in iNKT-cell numbers after treatment with IAP antagonists, confirming that IAPs do not play a critical role in the survival of mature iNKT cells (Fig. 2B). Further support for a nonapoptotic role was that mice treated with oral LCL-161 showed no effect on peripheral iNKT, CD4, or CD8 T-cell populations (Supplementary Fig. S4). Taken together, these results suggest that IAP antagonists either activate apoptosis in an iNKT-cell precursor, or modulate intrinsic or extrinsic signals that are required for iNKT-positive selection (40). In addition, our results are consistent with the report of iNKT-cell deficiency in humans lacking XIAP, although this finding has not been substantiated by other groups (9, 11).

IAP antagonists enhance cytokine production from mature iNKT cells

iNKT cells are early orchestrators of cytokine responses in mice. iNKT-cell proliferation or cytokine production has been linked to several TNF family receptors whose signaling pathways involve cIAP1/2, including CD40, OX-40, 4-1BB, and GITR (41–44). We previously reported that unfractionated mouse spleen cells stimulated with α-GalCer, which spontaneously loads onto CD1d and acts as a broad iNKT-cell agonist, causes a rapid production of IFNγ that can be augmented by LBW-242 (6). Here, we show that addition of the IAP antagonist LBW-242 to α-GalCer–treated whole splenocyte cultures increased not only IFNγ but also IL-2 production by 3- to 6-fold above cultures treated with a control compound (LCV-843; Fig. 3A; ref. 6); a similar LBW-242-dependent enhancement of IL-2 production was observed from three different iNKT hybridoma cell lines stimulated with α-GalCer (Fig. 3B). Consistent with previous reports, the effect on cytokine secretion resembled true costimulation rather than direct IAP antagonist-mediated cytokine induction, as cytokines were not produced by cultures treated with IAP antagonists in the absence of antigenic stimulation (control; Fig. 3A; ref. 6).

Conventional CD4 T cells show a dramatic enhancement of cytokine production when stimulated in the presence of IAP antagonists (6). To determine how much of the IAP antagonist

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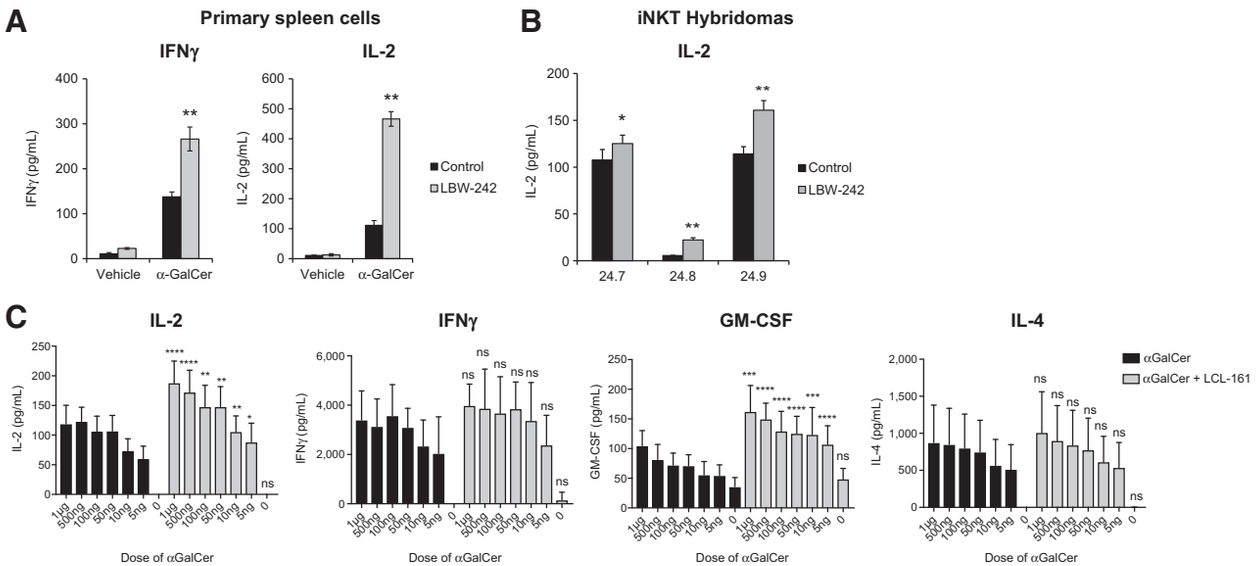

Figure 3.

IAP antagonists enhance cytokine production from mature iNKT cells. **A,** Spleen cells (5×10^5) were treated with (α-GalCer) or vehicle in the presence of LBW-242 or control compound (LCV-843) for 24 hours. **B,** The iNKT-cell hybridomas 24.7, 24.8, and 24.9 were stimulated with anti-CD3 (10 μg/mL) for 24 hours in the presence of LBW-242 or control compound. **C,** Whole spleens from transnuclear mice (Vβ7A, Vβ7C, and Vβ8.2 on a RAG2$^{-/-}$ background) were inflated with PBS before homogenization and plating at 2×10^5 cells per well in a 96-well plate, with 1 μg/mL, 500 ng/mL, 100 ng/mL, 50 ng/mL, 10 ng/mL, 5 ng/mL, or 0 ng/mL α-GalCer with or without 500 nmol/L LCL-161, as indicated. Supernatants were collected after 24 hours. **A–C,** Cytokines were measured by ELISA. Error bars represent SEM. Results represent two independent experiments with six replicas per experiment. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ versus control.

enhancement of the CD4 T-cell response that we previously reported was due to CD4$^+$ iNKT cells, we treated stimulated CD4 T cells from iNKT cell–deficient CD1d-knockout mice with IAP antagonists (Supplementary Fig. S4). Although overall IL4 production was reduced in CD1d-knockout cultures, as has been previously reported (45), the effect of the IAP antagonists was unchanged, with treatment leading to an approximate 2-fold increase in IL4 secretion in both strains (Supplementary Fig. S4). In addition, IAP antagonist treatment enhanced IL-2 secretion to a similar extent in wild-type and iNKT cell–deficient mice (Supplementary Fig. S4). These results show that in spleen cultures stimulated with anti-CD3/CD28, most of the effect of IAP antagonisms is on the peptide-reactive CD4 T cells, which are at much higher abundance than iNKT cells in these cultures.

In whole spleen cultures, cytokine production could be coming from multiple cellular sources. To obtain pure populations of naïve iNKT cells, we pooled spleens from 3 different monoclonal lines of transnuclear (TN) iNKT-cell mice crossed onto a RAG2$^{-/-}$ background to eliminate any other T-cell specificities (36). TN iNKT cells showed a dose-dependent release of cytokine in response to α-GalCer (Fig. 3C). iNKT-cell production of IL-2 and GM-CSF was significantly enhanced by the addition of LCL-161, whereas production of IFNγ and IL-4 was not (Fig. 3C).

To compare the effects of IAP antagonism on iNKT cells versus conventional CD4$^+$ and CD8$^+$ T cells, we isolated CD8$^+$ and CD4$^+$ T cells from C57BL/6 mice, as well as homogenized whole splenocyte cultures from transnuclear iNKT-cell mice on a RAG-sufficient background (Vα14). This latter culture allowed us to determine the role of iNKT-cell enhancement of cytokine production in a physiologically relevant coculture of mixed splenocytes. IL-2 and GM-CSF production was significantly costimulated by the addition of LCL-161 to CD4$^+$ T cells and Vα14 cultures, but not from CD8$^+$ T cells (Fig. 4). IFNγ production was significantly costimulated by the addition of LCL-161 to CD8$^+$ T cells and Vα14 cultures, but not from CD4$^+$ T cells (Fig. 4). IL-4 production was only significantly costimulated by the addition of LCL-161 to CD4$^+$ T cells alone (Fig. 4). In addition, IAP antagonism altered the production of chemokines; LCL-161 costimulated the production of CXCL-10, while decreasing concentrations of CXCL9, CCL5, and MIP-1β from Vα14 cultures (Fig. 4). IAP antagonism also costimulated the production of multiple other cytokines from Vα14 cultures, including IL-13, TNF-α, VEGF, LIF, IL-1β, and IL-3, while decreasing IL-10, and having no effect on IL-17 (Fig. 4). Therefore, IAP antagonism has costimulatory effects on cytokine production by multiple T-cell subsets, including CD8$^+$ T cells, CD4$^+$ T cells, and iNKT cells (Figs. 3 and 4).

IAP antagonism plus iNKT-cell activation enhances immunity to B16F10 lung metastases

To determine the effects of IAP antagonists on cytokine production from mature iNKT cells *in vivo*, we treated C57BL/6 mice with vehicle or LCL-161 by oral gavage, with or without the addition of α-GalCer to stimulate iNKT cells (Fig. 5). We assessed cytokine production in the serum 2 hours after stimulation (Fig. 5A), and then used intracellular cytokine staining 4 hours after stimulation to determine changes in cytokine production on a per cell basis (Fig. 5B). In both instances, LCL-161 costimulated the production of IL-2 and IFNγ *in vivo*, with increased percentages of iNKT cells secreting each of the cytokines (Fig. 5A and B).

Due to the increased Th1 cytokine production driven by IAP antagonism, we hypothesized that treatment with LCL-161 and

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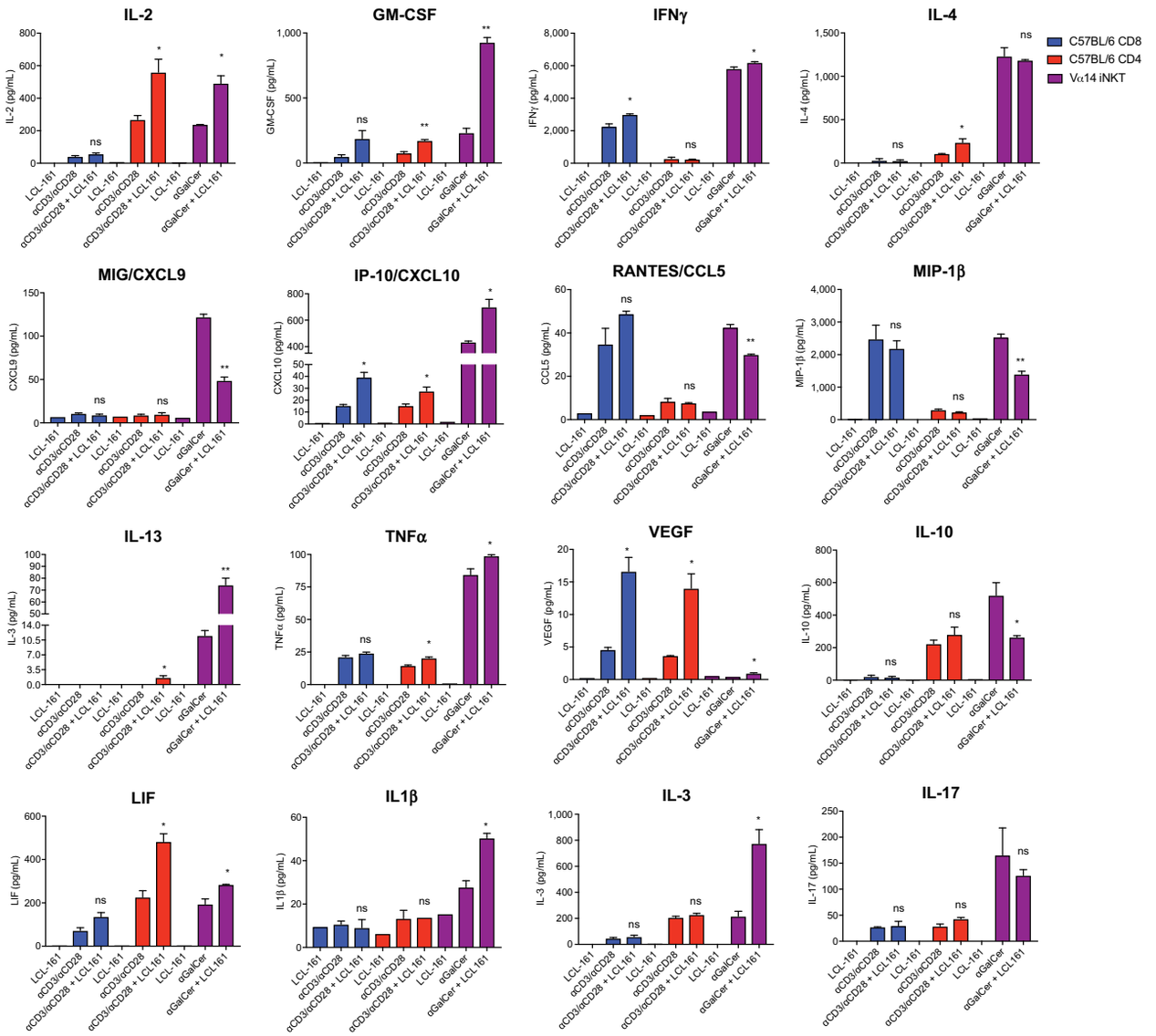

Figure 4.

IAP antagonism costimulates cytokine production from conventional $CD8^+$ and $CD4^+$ T cells, as well as invariant NKT cells. $CD8^+$ and $CD4^+$ T cells were isolated from C57BL/6 mice by magnetic bead separation and plated at 1.5×10^5 cells per well in a 96-well plate. Whole spleens from Vα14 transnuclear mice were inflated with PBS, homogenized, and plated at 1.5×10^5 cells per well. LCL-161 depicts cells treated with 500 nmol/L LCL-161 without stimulation. C57BL6 CD4 and CD8 T cells were stimulated with anti-CD3/anti-CD28 beads, whereas Vα14 spleen cells were stimulated with 100 ng/mL α-GalCer. Supernatants were collected after 48 hours of culture and analyzed by cytokine bead array. Results are representative of two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

α-GalCer would have efficacy against murine tumors. We inoculated C57BL/6 mice intravenously with B16F10 cells. Mice were treated with vehicle or LCL-161 by oral gavage days −2 and 0. Mice also received vehicle or α-GalCer intraperitoneally to stimulate iNKT cells on days 0, 4, and 8 after tumor inoculation, and tumors were harvested on day 14 (Fig. 5C). Combination of α-GalCer with LCL-161 enhanced antitumor responses to B16 lung metastases compared with vehicle treatment or α-GalCer alone (Fig. 5C). Analysis of the splenic T-cell compartments of these mice shows that LCL-161 treatment alone does not affect the population size of iNKT cells, $CD4^+$ T cells, or $CD8^+$ T cells (Supplementary Fig. S4). Therefore, IAP antagonism does not directly affect apoptosis of iNKT cells and conventional T cells *in vivo*, and, importantly, can be used to enhance the efficacy of iNKT cell–based immunotherapy.

Effects of IAP antagonists on human iNKT cells in culture

Collectively, our results indicate a specific sensitivity of developing murine iNKT cells to IAP antagonism, while confirming that treatment of mature iNKT cells augments antigen-mediated cytokine production in a manner analogous to peptide-responsive cells. However, human iNKT cells may respond differently to

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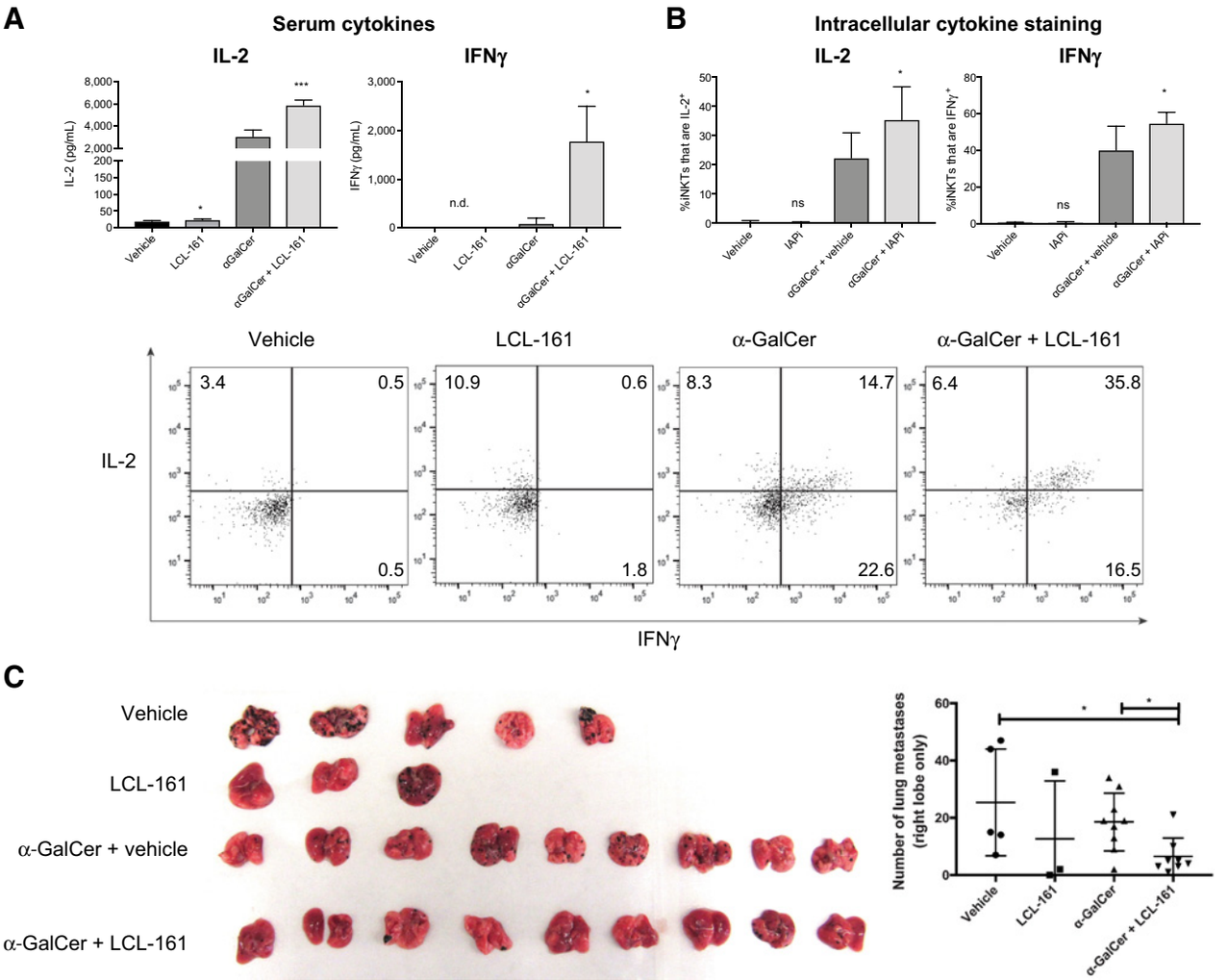

Figure 5.

In vivo effects of IAP antagonist plus α-GalCer on cytokine production and on B16F10 lung metastases. **A–C,** All C57BL/6 mice were treated with vehicle or LCL-161 (75 mg/kg) by oral gavage at days −2 and day 0 of analysis. **A,** On day 0, LCL-161 or vehicle-treated mice were injected intraperitoneally with 1 μg α-GalCer and bled 2 hours after injection. Serum was collected and analyzed by ELISA. **B,** On day 0, LCL-161 or vehicle-treated mice were treated with 1 μg α-GalCer intraperitoneally; 4 hours after stimulation, mice were sacrificed and their spleens were homogenized and stained with anti-CD3 and PBS57-CD1d tetramer before being fixed, permeabilized, and stained intracellularly with anti-IL2 and anti-IFNγ. Representative flow plots are gated on $CD3^+$Tetramer$^+$ iNKT cells. **C,** Mice were treated with vehicle or LCL-161 (75 mg/kg) by oral gavage 2 days prior to, and on the day of tumor inoculation. Mice were injected with B16F10 cells (3×10^5) intravenously. Four hours after inoculation, and again on days 4 and 8, mice were treated with vehicle or 1 μg α-GalCer intraperitoneally. On day 14, mice were sacrificed, and lung nodules in the right lobe were enumerated and quantified. **A–C,** Results are representative of three independent experiments. *, $P < 0.05$; ***, $P < 0.001$.

IAP antagonism, and establishing a role for IAP antagonists in modulating human iNKT cells activation could have implications for IAP antagonist-based therapies, as well as the use of IAP antagonists in the *ex vivo* culture and expansion of iNKT cells. Several T-cell infusion therapies, including iNKT-cell therapies, are under investigation as potential treatments for cancer (25, 26, 29). These therapies seek to augment nascent antitumor responses to tumor antigens or to harness the intrinsic antitumor effects of iNKT cells through the *ex vivo* culture and expansion of patient T cells, which can then be reinfused with the goal of boosting antitumor responses (25).

Human iNKT cells can be expanded in culture and are identified by the presence of the invariant T-cell receptor comprised of Vα24 and Vβ11 (33). One of the limitations of iNKT-cell culture, however, is the overall low purity of the resultant iNKT-cell product (33). This is, in part, due to the difficulty of specifically expanding these rare cells from the larger pool of patient T cells (33). Because IAP antagonists act through costimulation, we hypothesized that adding IAP antagonists to human PBMC undergoing stimulation with α-GalCer would produce specific effects only in α-GalCer reactive iNKT cells, namely, cytokine production, as well as expansion and maintenance in culture. To test this hypothesis, human peripheral blood T-cell cultures were enriched for T cells by depleting $CD14^+$ cells, and cultured with or without the IAP antagonist LBW-242, in the presence or absence of α-GalCer. After 48 hours,

Published OnlineFirst November 29, 2017; DOI: 10.1158/2326-6066.CIR-17-0490

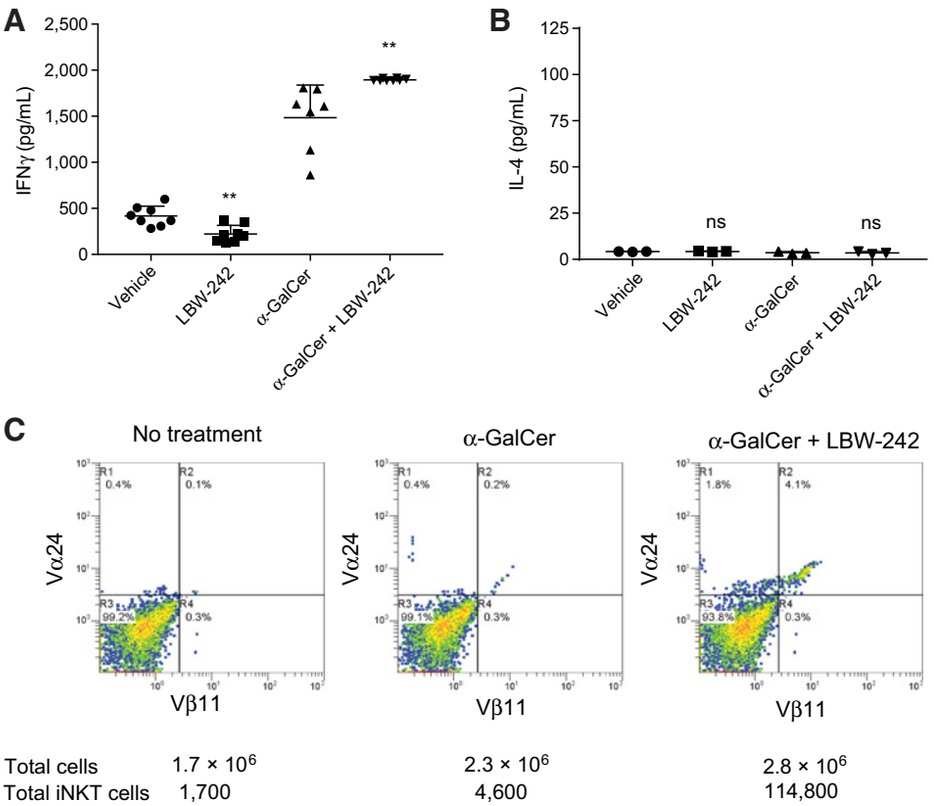

Figure 6.

Effect of IAP antagonist treatment on cultured human invariant NKT-cell IFNγ production and overall yield. **A, B,** Human peripheral blood mononuclear cells were depleted of $CD14^+$ cells and cultured with α-GalCer (1 μg/mL) and LBW-242 (500 nmol/L) as indicated. IFNγ (**A**) and IL-4 (**B**) production were determined by ELISA of 48-hour culture supernatants. **C,** Human peripheral blood mononuclear cells were cultured with α-GalCer with or without LBW-242 or were left untreated. After 2 weeks, invariant NKT cells were identified by flow cytometry using coexpression of Vα24 and Vβ11, and total cultured cells were quantified by Trypan blue exclusion. Results are representative of three independent experiments. **, $P < 0.01$; ****, $P < 0.0001$.

human iNKT cells produced significantly greater amounts of IFNγ as shown by ELISA of culture supernatants using peripheral blood from healthy donors (Fig. 6A). Human iNKT cells did not produce IL-4 upon α-GalCer stimulation (Fig. 6B).

In addition, human iNKT cells could be expanded in culture using IAP antagonism. After 2 weeks, α-GalCer and LBW-242 cotreatment led to increased purity of iNKT-cell cultures, with 4.1% of the cells representing iNKT cells ($Vα24^+Vβ11^+$ T cells) compared with 0.2% of α-GalCer single-treatment controls (Fig. 6C). This increased purity corresponded to an increase in iNKT-cell numbers with an overall 20-fold expansion of iNKT cells in α-GalCer and LBW-242–cotreated cultures compared with α-GalCer–treated controls (Fig. 6C). These findings indicate that IAP antagonists can be used to facilitate *ex vivo* culture of iNKT cells and may be an efficient method for improving the purity and quantity of iNKT cells retrieved for use as immunotherapy.

Discussion

Small-molecule IAP antagonists have been reported in several recent studies to modulate immune cell function, and these compounds are in early clinical trials for the treatment of cancer (6–8). Although the IAPs have been implicated in the regulation of apoptosis through caspase inhibition, most recent evidence suggests that, physiologically, these proteins predominantly function as regulators of cell signaling, largely through their ability to modulate both NF-κB1 and NF-κB2 pathways. Indeed, the mechanism of cell death in tumor cells exposed to IAP antagonists is through NF-κB modulation (46–48), sensitization to killing by TNFα, and/or enhanced uptake by phagocytic cells (7).

Data from both mouse and human studies suggest that the immune-modulating effects of IAP antagonism are critical for their antitumor efficacy (6–8). We show here that IAP antagonism enhanced cytokine production from both mouse and human iNKT T cells, and that selective activation of human iNKT cells in the presence of IAP antagonists favored the production of IFNγ. These findings suggest nonuniform responses to IAP antagonism for distinct T-cell subsets and may have implications for modulating immune responses using IAP antagonists.

In thymic cultures, exposure to IAP antagonists led to an overall reduction in developing thymocytes and appeared to select against the production of CD4 T cells. IAP antagonist treatment had a substantial effect on the development of iNKT cells, fully abrogating their development in FTOC. We found that iNKT cells were dramatically more sensitive than other T-cell subsets to IAP antagonism during thymic development, and whether this effect was through changes in NF-κB signaling remains to be determined. Several signaling pathways are known to play an important and selective role in iNKT-cell development. Unlike conventional CD4 T cells, iNKT cells require surface interactions between SLAM family members to activate the intracellular SAP/Fyn signaling pathway (14–16). iNKT-cell development is also more sensitive to alterations in thymic signaling than are conventional T cells; mice deficient in the transcription factors T-bet, PLZF, or c-Myc, for example, have a selective loss of iNKT cells (7, 8, 17, 18). iNKT-cell development has also been linked to the ubiquitin-editing enzyme A20

(49), which directly binds to cIAP1 to prevent interaction with TRAF2/TRAF3 and abrogates NF-κB2 signaling (50).

Collectively, our findings suggest that the RING-containing IAPs have an important role in iNKT-cell biology, with divergent effects on mature and developing cells. Although we did not directly examine signaling here, the two principal observations made through these studies could be explained by a role for the IAPs in modulating the strength of signals received by iNKT cells through their T-cell receptors (TCR). Normally, iNKT cells develop in the thymus from $CD4^+CD8^+$ DP precursors and are positively selected by CD1d expressed on other DP thymocytes (29, 41, 43). Unlike conventional T cells, which undergo negative selection through encounters with peptide–MHC on thymic epithelial cells, negative selection of iNKT cells is mediated primarily by CD1d on dendritic cells (42, 44, 49, 50). Both positive and negative selection of iNKT cells have been convincingly shown in FTOC using titrated addition of α-GalCer (37, 44, 49) and via analysis of retrogenic mice with iNKT TCRs of different affinity for CD1d–lipid complexes (51). In developing iNKT cells, an enhanced TCR signal could lead to negative selection, as is observed when thymocytes are exposed to high concentrations of α-GalCer (38). In mature cells, strong signaling through the TCR leads to activation and cytokine production, rather than cell death. Alternatively, the effect observed in FTOC may be mediated by another thymic cell type. Although thymic stromal cells do not directly present ligands for iNKT-cell selection, these cells do play critical accessory roles in the development of iNKT cells. In particular, NF-κB2 signaling in thymic stromal cells is required for iNKT-cell development, and the IAP antagonists can modulate this pathway, suggesting that the IAP antagonists may act on these cells (40, 52).

IAP antagonism offers an attractive target as part of combination antitumor therapies with the potential to act synergistically with chemotherapy, death receptor agonists, radiation, and kinase inhibitors (53). Combination of LCL-161 with the immunotherapeutic agent anti–PD-1 extends survival in mouse models of multiple myeloma and glioblastoma and is more effective than combination of LCL-161 with chemotherapy (7, 8). Here, we show that combination of LCL-161 with the iNKT-cell agonist lipid α-GalCer reduces growth of melanoma lung metastases compared with either treatment alone.

Regardless of the specific mechanism, our findings have therapeutic implications for iNKT cell–based therapies for cancer. iNKT cells develop early in life (54), making potential effects of IAP antagonism on thymic development an irrelevant concern for adult humans. IAP antagonists enhance cytokine production from mouse and human α-GalCer-stimulated iNKT cells, and facilitate the difficult process of *ex vivo* culturing human iNKT cells. Together, these effects may increase the yield of *ex vivo*-expanded iNKT cells and enhance the potency of Th1-type iNKT-cell activation following α-GalCer transfusion, both of which are important aspects of current approaches for iNKT-cell therapy (26, 34, 55). The absence of appreciable cytokine production from cultures lacking α-GalCer suggests that IAP antagonists could augment therapies using activated iNKT cells without producing additional side effects from nonspecific alterations in immune cell function. These results, therefore, provide further evidence that IAP antagonism represents a new class of immunotherapy with broad therapeutic potential.

Disclosure of Potential Conflicts of Interest

M.A. Exley is currently VP Cellular Immunology at Agenus Inc. G. Dranoff is currently an employee of Novartis, Inc. and has ownership interest (including patents) in the same. M. Dougan reports receiving a commercial research grant from Novartis and has ownership interest (including patents) in the same. S.K. Dougan reports receiving a commercial research grant from Novartis and is a consultant/advisory board member for the same. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: M.A. Exley, M. Dougan, S.K. Dougan
Development of methodology: M.A. Exley, M. Dougan
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Ali, M.A. Exley, M. Dougan
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): E. Clancy-Thompson, L. Ali, M. Dougan, S.K. Dougan
Writing, review, and/or revision of the manuscript: E. Clancy-Thompson, M.A. Exley, G. Dranoff, M. Dougan, S.K. Dougan
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P.T. Bruck
Study supervision: G. Dranoff, M. Dougan
Other (provided laboratory facilities for study conduction and supervised S.K. Dougan): R.S. Blumberg

Acknowledgments

S.K. Dougan and G. Dranoff were funded by the Dana-Farber Cancer Institute/Novartis Program in Drug Discovery. S.K. Dougan was funded by the Claudia Adams Barr Foundation and the Melanoma Research Alliance. M. Dougan was funded by an NIH Ruth L. Kirschstein National Research Service Award (1F32CA210568-01) and the AGA Research Scholars Award. E. Clancy-Thompson was funded by NIH T32CA207021. R.S. Blumberg was funded by NIH DK44319. M. Exley was funded by NIH CA143748 and CA170194.

The authors thank Simon C. Yue for culturing human iNKT-cell lines.

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Received September 4, 2017; revised November 18, 2017; accepted November 22, 2017; published OnlineFirst November 29, 2017.

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Published OnlineFirst November 29, 2017; DOI: 10.1158/2326-6066.CIR-17-0490

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Published OnlineFirst November 29, 2017; DOI: 10.1158/2326-6066.CIR-17-0490

Cancer Immunology Research

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Cancer Immunol Res 2018;6:25-35. Published OnlineFirst November 29, 2017.