Inhibitor of Apoptosis protein (IAP) antagonists demonstrate divergent immunomodulatory properties in human immune subsets with implications for combination therapy

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ABSTRACT

Inhibitor of apoptosis proteins (IAPs) are critical in regulating apoptosis resistance in cancer. Antagonists of IAPs, such as LCL161, are in clinical development and show promise as anti-cancer agents for solid and hematological cancers, with preliminary data suggesting they may act as immunomodulators. IAP antagonists hypersensitize tumor cells to TNF-α-mediated apoptosis, an effect which may work in synergy with that of cancer vaccines. This study aimed to further investigate the immunomodulatory properties of LCL161 on human immune subsets. T lymphocytes treated with LCL161 demonstrated significantly enhanced cytokine secretion upon activation, with little effect on CD4 and CD8 T cell survival or proliferation. LCL161 treatment of peripheral blood mononuclear cells significantly enhanced priming of naïve T cells with synthetic peptides *in vitro*. Myeloid dendritic cells underwent phenotypic maturation upon IAP antagonism and demonstrated a reduced capacity to cross-present a tumor antigen-based vaccine. These effects are potentially mediated through an observed activation of the canonical and non-canonical NF-kappa B pathways following IAP antagonism with a resulting upregulation of anti-apoptotic molecules. In conclusion, this study demonstrated the immunomodulatory properties of antagonists at physiologically-relevant concentrations and indicates their combination with immunotherapy requires further investigation.

INTRODUCTION

Following a long period with little clinical success, immunotherapy is finally delivering on decades of promise. Recently, two immune-based therapies have demonstrated significant clinical benefit [1, 2]. However, for future success with immune therapies such as therapeutic cancer vaccines, significant hurdles still need to be overcome. Where successful therapies may emerge is through the combination of targeted small molecule agents and immunotherapy, to transiently reduce tumour growth and survival whilst creating a permissive environment for the enhancement of immunotherapy. However, given that many of the targeted molecules are critically involved in the survival and function of immune cells, a careful evaluation is required to understand potential 'off-target' effects of these agents on immune cells [3, 4].

An attractive class of small molecule agents are antagonists of the Inhibitor of Apoptosis (IAP) proteins. The IAP family of proteins has diverse roles in regulating apoptosis, acting as regulators of caspase activation and through control of NFκB signaling. IAP proteins are regulated by the second mitochondrial activator of caspases (SMAC)/DIABLO [5, 6], and synthetic small molecule mimetics of SMAC (IAP antagonists) demonstrate potent anti-cancer activity [7, 8]. Although originally hypothesized to mediate tumor apoptosis through direct caspase activation, IAP antagonists have been shown to function through the auto-ubiquitination and degradation of cIAPs. This results in activation of the canonical and noncanonical NF-κB signaling pathways, leading to production of TNF-α and, in the absence of cIAPs, apoptosis induction through the consequent autocrine and paracrine TNF receptor signaling [9-11]. In addition, IAP antagonists may sensitize tumor cells to FAS and TRAIL-mediated apoptosis [12-15]. Therefore, the combination of an anti-cancer vaccine with an agent able to potentiate the activity of immune effector cells is a promising strategy. Successful combination of IAP antagonists with immune therapy will depend on potential off-target effects. A number of studies have demonstrated a role for IAPs in leukocyte biology. cIAPs form part of a multi-protein complex regulating CD40 signalling [16, 17] and survival and responsiveness [18] in B lymphocytes. They are involved in toll-like receptor signaling in macrophages [19] and the differentiation of monocytes into macrophages [20], the protection of macrophages from apoptosis following lipopolysaccharide (LPS) stimulation [21], and the protection of CD8+ T cells from apoptosis during Human T cell leukemia virus type 1 (HTLV-1 infection) [22, 23]. A recent report demonstrated that IAP antagonists mediate co-stimulation to promote anti-tumor immunity [24]. In that study, IAP antagonists were shown to augment murine lymphocyte proliferation, functioning through activation of NFκB2, and to potentiate antitumor vaccines *in vivo* using a B16 mouse melanoma model.

In this study, we investigate the immunomodulatory properties of the IAP antagonist LCL161 on human immune subsets and effects that may influence its combination with immune therapies. This orally administered IAP antagonist has been shown to be well tolerated in cancer patients in a phase I study with no associated dose-limiting toxicities [25]. We demonstrate that at the clinically achievable concentrations IAP antagonists have little direct effect on lymphocyte viability or proliferation, but significantly increase the cytokine secretion by effector and helper T cells and enhance the *in vitro* expansion of antigen-specific naïve and memory T cells. Finally, we demonstrate that IAP antagonism results in the phenotypic maturation of human dendritic cells, with an associated activation of the canonical and non-canonical NFκB pathways and a reduction in the capacity to cross-present a cancer vaccine.

METHODS

Reagents

IAP antagonists (IAP-As) LBW242 and LCL161[25-27] were kindly provided by Novartis Pharma Ag and solubilized in 100% DMSO at 10mM . All phenotyping antibodies were from BD Biosciences, unless indicated otherwise. NY-ESO-1 specific CD8+ T cell clones were generated with synthetic peptides as previously described [28]. Complete media contains RPMI 1640 with 20 mM HEPES, 60 mg/L penicillin, 12.5 mg/L streptomycin, 2 mM L-glutamine, 1 % non-essential amino acids and 10 % heat-inactivated foetal calf serum (Gibco). All flow cytometry was performed on a BD FACS Canto II, and data analyses were performed using the FlowJo software (TreeStar Inc).

Lymphocyte functional assays

Viability

T cells were isolated with MACS positive selection (Miltenyi Biotech) (>96% purity), un-stimulated / stimulated with microbeads coated with anti-CD3/CD28 antibodies (Invitrogen) at a 2:1 ratio, with or without the indicated concentration of IAP antagonist for the indicated time in complete media. Viability was assessed using anti-annexin V-FITC and propidium iodide (Sigma). To evaluate viability, both Ann-V+/PI+ or Ann-V+/PI- cells were classified as apoptotic. Cells were analysed by flow cytometry.

Proliferation assay

Purified CD4 and CD8 T cells were labeled with 1µM CFSE (invitrogen) , plated at $5x10^4$/well and stimulated as above. After staining with anti-CD3, anti-CD4 and anti-CD8, cells were analyzed on the BD FACSCanto II, gating for lymphocytes with FSC/SSC parameters. Data analysis was performed using FlowJo and the included Proliferation tool. The 'Proliferation index' was used to define a proliferation value. This value, which represents the average number of cell divisions undergone by the responding cells, more faithfully reflects the biology of the system by considering only the fraction of responding cells (http://www.flowjo.com/v7/html/proliferation.html).

Cytokine production assay

Purified CD4 and CD8 T cells were plated at $1x10^5$ cells/well and stimulated as above. After 20, 40 and 68 hours, GolgiPlug (BD Bioscience) was added for the final 4 hours to halt cytokine secretion. Cells were stained with anti-CD3, -CD4, and -CD8 antibodies, permeabilized (Fix/Perm kit, BD) and stained for IFN-gamma (eBioscience). Cells were analysed by flow cytometry.

Peptide stimulation assay

Healthy donor peripheral blood mononuclear cells (PBMCs) were plated at $1x10^6$ cells/well in complete media with 1µg/ml (final concentration) synthetic peptides. HLA-A2-restricted epitopes Flu-M1,58-66; BMLF1,280-288 and Melan-A,26-35 (all synthesized Mimotopes, US) were added with or without IAP antagonist. After 24 hours, 25IU IL-2 (Proleukin®) was added, and subsequently every 3 days till day 10. Then, PE-conjugated HLA-A2 tetramers containing the relevant peptide (Tetramer Production Facility, LICR, Lausanne, Switzerland) were used to evaluate the percentage of specific CD8+ CD3+ T cells. Cells were analysed by flow cytometry.

Dendritic cell culture

PBMCs from healthy donor buffy coats (Red Cross Blood Bank, Melbourne, Australia) were prepared by Ficoll-Paque density gradient centrifugation (GE Healthcare). Monocytes were isolated with anti-CD14 microbeads to a purity >95% (Miltenyi Biotech). Immature monocyte-derived dendritic cells (ImoDCs) were generated from monocytes with GM-CSF (20 ng/ml) and IL-4 (10 ng/ml) for 4-5 days. Where indicated, imoDC were treated with titrating concentrations of IAP antagonist or 500 ng/ml CD40L-trimer (a kind gift from Amgen, CA, USA) for 24 or 48 hours. Phenotypic analysis was performed using the following antibodies: Anti-Annexin V, anti B7-DC, anti B7-H1, anti B7-1, anti B7-2 anti HLA-DR, and anti CD14 in combination with a violet live/dead fluorescent viability dye (Invitrogen). Cells were analysed by flow cytometry.

Tumor antigen cross-presentation assay

ImoDCs were plated at 1 x 10^5 cells/well in 96 well U-form plate in 200 µl fresh culture media supplemented with GM-CSF. Where indicated, ImoDC were treated with titrating concentrations of IAP antagonist or 500 ng/ml CD40L-trimer for 24 or 48 hours before pulsing with antigen. DCs plated in culture media were then pulsed with recombinant NY-ESO-1/ ISCOMATRIX vaccine formulation (as previously described [29]) for 18 hours with or without the IAP antagonist, as indicated. DCs were then washed and used as targets for NY-ESO-1 specific CD8+ T cell clones and T cell activation measured by intracellular cytokine staining (ICS). T cells were added at > 1:10 effector-to-target ratio

with GolgiPlug for 4 hours, stained for CD3 and CD8, then permeabilized and stained for IFN-gamma. Cells were analysed by flow cytometry. Where indicated, prior to the ICS assessment, DCs were pulsed for 1 hour with 10µg/ml of the NY-ESO-1 157-165 peptide followed by washing.

Endocytosis assay

This assay was a modification of a published protocol [30]. Briefly, 1µM Fluoresbrite® yellow green carboxylated latex microspheres (Polysciences, Inc) were coated overnight with 1 mg/mL mouse IgG (ES121 antibody, LICR Melbourne) or BSA (Sigma). ImoDCs were plated at 1 x 10^5 cells/well in 96 well in fresh culture media supplemented with GM-CSF and where indicated with IAP antagonist at 10µM. After 24 hours, DCs were pulsed with 9.1 × 10^7 coated beads/mL at 37ºC for 45 minutes and washed. Cells were analysed on a BD FACS Canto II and data analyses were performed using FlowJo. As a control, PHA activated, purified CD4 T cells were also pulsed for 45 mins at 37º C to control for non-specific cell adhesion versus endocytosis.

Immunoblotting

Cells were lysed in RIPA buffer (Sigma-Aldrich) supplemented with protease inhibitor cocktail (Roche) and for anti-phospho antibody analysis with PhosSTOP (Roche). SDS-PAGE analyses were performed using 4-12% NUPAGE gels (invitrogen). Membranes were blocked with 5% BSA/TBS (dilutant for antibodies plus 0.1% Tween 20). All primary antibodies were used at 1:1000 overnight at 4°C. Anti-Bcl-xL (54H6), anti-IκBalpha (44D4), anti-phospho Rel-A (93H1), anti-p52/p100 (18D10), anti-cIAP1 (R.pAb) and cIAP-2 (58C7) all Cell Signaling Technology. Anti β-tubulin / β-actin (Sigma-Aldrich). For anti-IκBalpha stains, a peroxidase-labeled anti-rabbit immunoglobulin (Sigma) secondary antibody was used at 1:20,000 dilution, and bands were visualized using chemiluminescence (ECL Plus) using a STORM imager (GE Healthcare). For all other blots Ir-dye secondary antibodies were used at 1:15,000-1:25,000 dilution, and membranes were imaged on an Odyssey Imager (LI-COR Biosciences). Quantification was performed using the incorporated Odyssey Software.

Quantitative RT-PCR

qRT-PCR was performed with a Stratagene Mx3005 (Stratagene). RNA isolation was undertaken using the RNeasy kit (Qiagen). cDNA was prepared using the ABI High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) with random primers and RNA purified from control or drug-treated imoDC as indicated. qPCR was performed using the indicated primers, designed to span across intron/exon boundaries using the Primer design software from Roche &

verified using BLAST. QuantiFast SybrGreen MasterMix was used according to the manufacturer's protocol. Expression was calculated as transcript numbers of the gene of interest per β-actin transcript using ($2^{(\beta\text{actin CT} - \text{gene of interest CT})}$).

Multiplex cytokine/chemokine analysis

MoDC were plated at 100,000 cells per well in 200ul of media in a 96-well plate and treated with 10μM of drug for 48 hours. Cell free supernatants were collected and frozen at -80 degrees C for further analysis. The samples were diluted, acquired and analysed using a Luminex200 plate reader and Xponent Version 3.1 software, respectively. Standard curves contained 7 points, ranging from 0.64, 3.2, 16, 80, 400, 2000, and 10000 pg/ml for the indicated cytokines/chemokines. Sample concentrations were calculated based on the standard curve with a 4-parameter logistic analysis and Y weighted as 1/Y^2. TNF-alpha ELISA was performed on supernatants using the BD OptEIA human TNF ELISA set (BD Bioscience) according to the manufacturer's protocol.

RESULTS

The IAP antagonist, LCL161, enhances T lymphocyte cytokine secretion but has a negligible effect on proliferation and apoptosis induction

Degradation of IAPs by small molecule inhibitors has been shown to induce apoptosis in solid [9, 10] and hematopoietic tumour cells [27, 31]. To explore if the investigational IAP-antagonist (IAP-A), LCL161, exhibited any effects on human lymphocytes, we assessed apoptosis induction, cell proliferation and cytokine secretion in CD8+ and CD4+ T cells with and without T cell activation. The production of TNF-α and IL-2 was studied in purified CD4+ and CD8+ T cells following non-specific activation with anti-CD3/CD28 beads in the presence of titrating concentrations of LCL161 (0.1, 1.0 and 10 μM). ICS was performed on T cells 24, 48 and 72 hours post treatment/activation. A concentration-dependent increase in cytokine production was observed upon drug treatment and TCR stimulation, with a significant increase in the frequency of cytokine producing cells demonstrated at the 10μM concentration ($P < 0.05$); an example of IL-2 production in CD4+ T cells from 3 donors is shown (Figure 1A) and representative ICS data for IL-2 and TNF-α are shown (Online resource Figure 5). However, due to large inter-donor variation in the percentage of cytokine-producing cells following activation (in the absence of drug), fold-increase in percentage of cytokine-producing cells (with drug) versus the untreated control was determined. Thus normalizing the response for effect of the IAP-A. Treatment with LCL161 results in a significant and concentration-dependent increase in the number of TNF-α and IL-2 producing T cells following activation at all time points (except for IL-2 production by CD8+ T cells at 24 hours) ($P <0.05$) (Figure 1B). The largest effect was an increase in IL-2 producing cells 72 hours following activation. No significant cytokine secretion was detected in T cells following drug treatment in the absence of cellular activation via the T cell receptor (data not shown).

To assess viability and apoptosis induction, purified CD4+ and CD8+ T cells were unstimulated or non-specifically activated in the presence of titrating concentrations of LCL161 (0.1, 1.0 and 10 μM). Apoptosis induction was assessed 24, 48 and 72 hours following treatment. Although overall modest, the largest effect observed was for unstimulated CD8+ T cells at 24 hours with an increase in the mean percentage of apoptotic cells of just 11% at 10μM LCL161. For all conditions excepting activated CD8+ T cells, a small but statistically significant increase ($P <0.05$) in apoptosis induction was observed with 10μM LCL161 at the 24 and 48 hour time points (Online Resource Figure 1A). To investigate the effects of the investigational drug on lymphocyte proliferation, purified CD4+ and CD8+ T cells were

labeled with the membrane-incorporating dye CFSE to track cellular divisions and left unstimulated or activated in the presence of titrating concentrations of LCL161 or an analogue IAP-A compound, LBW242, described to modulate murine lymphocyte proliferation [24]. After 96 hours, proliferation was assessed and a proliferation index for each treatment condition and population was determined (detailed in materials & methods). No proliferation was observed for either population in the absence of activation with or without IAP antagonist treatment (data not shown). Representative proliferation plots for activated CD8+ T cells are shown (Online Resource Figure 1B). No significant effect was observed at any concentration of LCL161 or LBW242, although a trend for a concentration-dependent decrease in proliferation following treatment with inhibitors for both T cell subsets was observed (Online Resource Figure 1C). Taken together, these data demonstrate that treatment of human T lymphocytes with IAP antagonists increases the frequency and duration of cytokine production in CD4 and CD8 T cells, while having negligible effects on lymphocyte proliferation and viability. Without concurrent T cell activation, IAP-As have little effect on T lymphocytes for the parameters studied.

IAP antagonism enhances the stimulation of antigen specific T lymphocytes

To investigate the effect of IAP-As on lymphocyte activation, *in vitro* peptide stimulation experiments were performed using antigens that would stimulate both memory and naïve CD8+ T cells in whole PBMC cultures. Immunodominant epitopes from the influenza matrix antigen and the EBV BMLF1 protein were selected to stimulate memory responses. Melan-A, a melanocyte differentiation antigen, was shown to a high precursor frequency of naïve CD8+ T cells in healthy donors [32]. Healthy donor PBMCs were stimulated *in vitro* with each of these peptides in the presence of titrating concentrations of LCL161 (0, 0.1, 1.0 & 10μM) and the frequency of specific CD8+ T cells was assessed using recombinant MHC tetramers. A concentration-dependent increase in the frequency of antigen-specific T cells was observed for all peptides; tetramer staining is shown for a representative donor for each epitope (Figure 2A). The greatest effect was observed with the Melan-A peptide at 10μm LCL161 (mean 3.8%, n=6) in which the smallest amplification was observed following peptide stimulation without drug (mean 0.26%, n=6); thus representing a 15-fold increase in specific cells with drug addition. A summary of the mean response in multiple donor PBMC for Flu-M1, BMLF1 and Melan-A demonstrates a statistically significant increases in the frequencies of expanding BMLF-1 and Melan-A specific cells at 10μM LCL161 treatment (Figure 2B). No statistically significant increase for the Flu-M1 epitope was observed, to which a large frequency of responding cells was detected in the absence of drug (mean 23.5%, n=5). A trend for increase in response magnitude was observed however, with the greatest effect at the 1μM

concentration (33.2%, n=5). These data are contradictory to the results obtained in the short-term proliferation assays employing a strong, non-specific TCR stimulus, where the IAP-As had minimal or even negative effect on T cell proliferation. The peptide stimulation results may reflect a difference in the response to the IAP-As that is dependent on the strength of the T cell receptor signal received by the T cell or the degree and breadth of co-stimulatory signals received. Therefore, the results may have been influenced by IAP-As acting on other cell subsets within the culture, such as antigen presenting cells.

IAP antagonists promote dendritic cell maturation

IAPs have been described to play a number of roles that influence myeloid cell biology, partially resulting from the ubiquitin ligase activity of the members cIAP1 and cIAP2. Studies have demonstrated a role for cIAP1/2 in toll-like receptor (TLR) signaling by regulating MyD88-dependent activation in murine macrophages [19]. Dupoux et al have demonstrated that cIAP1-dependent degradation of TRAF2 regulates the differentiation of monocytes into macrophages [20]. These studies imply that IAP-As may have significant effects on inflammatory APCs, such as mo-DC, which may represent an important population for vaccine biology [29, 33, 34]. Therefore, maturation of immature moDCs (imoDC) was evaluated as a result of IAP-A treatment. *In vitro* differentiated moDC were treated with titrating concentrations of LCL161 (0, 0.1, 1.0 & 10μM) for 24 and 48 hours. Viability and phenotypic markers of maturation were assessed by flow cytometry. Soluble CD40L was employed as a comparator for induction of maturation. Following 4 days differentiation and a further 24-48 hours culture without treatment, imoDC upregulated annexin V and started to undergo apoptosis. Treatment with sCD40L abrogated surface expression of annexin V ($P < 0.001$), protecting the cells from apoptosis. This effect was paralleled by the action of LCL161, with 10 μM LCL161 at 48 hours significantly decreasing annexin V expression ($P < 0.01$) (Figure 3A). To address the effect of LCL161 on maturation, expression of co-stimulatory molecules of the B7 family (B7-1, B7-2, of B7-H1 [PD-L1] and B7-DC [PD-L2]) on imoDC were measured following treatment. The greatest effect was observed with up-regulation of B7-DC in a time and concentration dependent manner (48 hours, 10μM: $P <0.001$) (Figure 3B). No significant upregulation of B7-H1 was observed with treatment (data not shown). B7-1 was also significantly upregulated as a result of LCL161 treatment ($P <0.05$) (Figure 3C). B7-2 and HLA-DR were expressed on ~100% of imoDC at all drug concentrations, thus changes in molecule density were measured by FACS. A significant increase in both molecules was observed at 48-hours post treatment ($P <0.01$) (Figure 3D, E). Representative flow cytometry stains are shown for Donor 1 at the 48-hour time

point (Online resource Figure 6). Taken together these data indicate that IAP antagonists protect immature DC from apoptosis and promote maturation, similar to the results observed with CD40L stimulation.

Induction of NF-κB signaling and expression of anti-apoptotic molecules mediated by IAP-A treatment of dendritic cells

IAP-As have been shown to result in the auto-ubiquitination and degradation of cIAPs in tumour cells, where loss of cIAPs results in activation of both the canonical and noncanonical NF-κB signaling pathways leading to production of TNF-α [11]. Given the importance of NF-κB signaling in DC biology for the expression of proinflammatory cytokines [35] and that CD40 signals through both the canonical and non-canonical NF-κB pathways [36], we investigated if either pathway is modulated in moDC as a result of IAP-A exposure. In the canonical pathway, stimulation leads to activation of the IκB kinase (IKK) complex and degradation of IκB proteins. Expression of IκBα in imoDCs was monitored by western blot analysis at four time points following treatment with 10μM LCL161 (Figure 4A/B). A significant loss of IκBα occurred at the 6 and 24-hour time points following treatment ($P < 0.001$; Figure 4A/B). Degradation of IκB proteins was followed by p65 (RelA) phosphorylation which was assessed after treatment with 10μM LCL161. Phosphorylation was detectable above the untreated control level at 2, 6 and 24-hours following treatment. CD40L served as a positive control with phospho-p65 detectable at 30-minutes (not shown) and 24 hours after stimulation ($P<0.001$) (Figure 4C/D). Although not reaching statistical significance, a mean 2.5 fold increase in phospho-p65 was detected 24 hours following drug treatment. However, LCL161-induced activation was lower in magnitude that that observed with CD40 ligation (Figure 4C/D). To investigate activation of the non-canonical pathway, we assessed degradation of p100 to p52; an event mediated by NIK and the IKK complex. Drug-induced degradation of p100 and a reciprocal p52 expression were observed 24 hours post LCL161 treatment, with CD40L inducing the greatest p52 levels ($P<0.05$) (Figure 4E/F). A ratio of p52 to p100 levels shows a significant increase at 24 hours following LCL161 ($P<0.01$) and with CD40L ($P<0.05$) (Figure 4E/F). Taken together, these data support our hypothesis that IAP antagonism leads to the activation of the canonical and non-canonical NFκB pathways in human APC.

NFκB signaling controls the expression of anti-apoptotic molecules, including members of the Bcl-2 family [37] and has also been described to regulate expression of IAP molecules [21]. Therefore, we assessed the expression of cIAP1/2, Bcl-2 and Bcl-xL in LCL161 treated imoDC by qPCR and western blot analysis. qRT-PCR analysis showed

no significant change in cIAP1 expression with either LCL161 or CD40L. Whereas, CD40L ($P<0.001$) and LCL161 ($P<0.05$) significantly increased the expression of cIAP2. A time-dependant increase in Bcl-2 expression was observed, although only reaching statistical significance with CD40L ($P<0.001$). Bcl-xL expression was significantly increased ($P<0.05$ and $P<0.001$) following LCL161 and CD40L treatment, respectively (Figure 5A). Western blot analysis of cIAP2 confirmed LCL161-mediated degradation up to 2 hours following treatment with a recovery of expression at 6 hours and an increase in protein levels at 24 hours (Figure 5B). Bcl-xL protein levels showed a modest increase at 24 hours (Figure 5C). CD40L induced a significant increase in expression of both proteins at 24 hours ($P<0.001$). The induced expression of anti-apoptotic factors is consistent with the observed survival and loss of annexin-V expression levels and activation of NF-κB following treatment with LCL161.

IAP antagonists influence DC function resulting in reduced cross presentation of a tumour antigen/adjuvant complex

To assess the functional effects of IAP antagonists on DC biology, cytokine secretion and antigen cross presentation were evaluated *in vitro*. TNF-α ELISAs were performed on the culture media of imoDC treated for 24, 48 and 72 hours with titrating concentrations of LCL161. Soluble CD40L stimulation served as a positive control. Although significant quantities of TNF-α were detectable following CD40L stimulation no TNF-α was found following treatment with LCL161 at any concentration suggesting that, IAP-A treatment of immature DC does not activate TNF-α secretion (data not shown). Furthermore, supernatants from 48 hour cultures were assessed for 8 cytokines/chemokines using multiplex technology following LCL161 treatment alone or in combination with CD40L. Again, no induction of any factor (IL-1β, -6, -8, 10, 12p70, TNF-α, MIP-1α, MIP-1β) was observed with treatment, although MIP-1β levels decreased relative to the untreated cells. Interestingly, LCL161 treatment of CD40L activated moDC also showed a trend for a slight reduction in levels for three cytokine/chemokines at 48 hours, although significance was not achieved and donor variability was significant (Online Resource Figure 2). These data demonstrate that the NF-κB signaling observed in LCL161 treated imoDC does not result in the induction of pro-inflammatory cytokines even though phenotypical maturation is observed.

If IAP antagonists are to be employed in combination with therapeutic cancer vaccination, it is critical to understand their potential effect on a DC's capacity to process and present antigen. Therefore, we studied the effect of IAP-A treatment on cross presentation of the previously reported cancer vaccine, NY-ESO-1/ISCOMATRIX (ESO/IMX) [29,

38, 39]. ImoDC were untreated or treated with the IAP antagonist for 24 or 48 hours and pulsed with the ESO/IMX vaccine for a further 18 hours to allow uptake, antigen processing and presentation. Cross presentation of the HLA-A2-restricted epitope 157-165 by the antigen-pulsed DC was assessed using an epitope-specific CD8+ T cell clone. Recognition assays measured specific IFN-γ production in a short-term co-culture experiment. No drug was present in this assay to influence the T cell function. In a representative example, LCL161 treatment prior to ESO/IMX pulsing of DC resulted in a concentration-dependent reduction in the recognition of DC by the specific T cell clone, corresponding to an ~50% reduction in responding cells at 10μM LCL161 (Figure 6A). Due to a large inter-donor variation in percentage response in the untreated control, for each donor the treatment values were normalized against the untreated control value and thus response is expressed as an activation index. This enabled the assessment of the drug effect on recognition of the epitope. Treatment with CD40L prior to antigen loading resulted in a significant mean reduction in recognition by the specific clone of ~40% at the 24 and 48 hour time points ($P < 0.001$) (Figure 6B). LCL161 treatment prior to antigen exposure also resulted in a time and concentration-dependent reduction in recognition by the epitope-specific T cell clone. A significant reduction in the mean value was observed at 24 and 48 hours ($P < 0.05$ and $P < 0.001$, respectively). At 48 hours after 10μM LCL161 treatment, a reduction in magnitude equivalent to that of CD40L treatment was observed. LBW242, the less active analogue compound, resulted in smaller effect than observed with LCL161, with significance only observed 48 hours following treatment ($P < 0.05$) (Figure 6B).

A hallmark of DC maturation is the reduced capacity for antigen endocytosis when compared to immature DC, associated with an increased capacity for antigen processing and presentation [40, 41]. Given our data, which demonstrated that IAP antagonists induce a phenotypic maturation of imoDC, the observed reduction in recognition by the epitope-specific T cell clone may be due to the reduced capacity for treated DCs to uptake the ESO/IMX complex. Endocytosis assays [30] were performed on IAP-A treated imoDC, using 1μm fluorescent latex particles coated in BSA or immunoglobulin. Endocytosis was measured by flow cytometry to quantitate bead-containing cells. No drug-dependent reduction in the endocytosis capacity of treated imoDC was observed in repeated assays (Online Resource Figure 3).

Interestingly, concurrent treatment of imoDC with the ESO/IMX complex and LCL161 for just 18 hours also resulted in a significant decrease in recognition of DC by the NY-ESO-1 specific T cells ($P<0.001$) (Online Resource Figure 4). The ESO/IMX vaccine has been demonstrated to be rapidly endocytosed, processed and resulted in the presentation of NY-ESO-1 epitopes within fewer than 6 hours *in vitro*[42]. Our data indicated that full phenotypic maturation of DCs

induced by LCL161 required 24-48 hours. Together, these pieces of evidence suggest that it is unlikely that the reduced recognition in the presence of LCL161 was a result of reduced antigen uptake through maturation, given the kinetics of the experiment. Importantly, IAP-A treated DC loaded exogenously with synthetic peptide show no reduction in their capacity to stimulate specific T cells, suggesting that the IAP-A effects are also associated with antigen processing and presentation (Online Resource Figure 4A).

Discussion

In this study we sought to address the potential of IAP antagonists to modulate human immune cells with a view to their combination with immunotherapy for cancer. Our data indicate that an IAP-A in clinical development, LCL161, positively impacted on T lymphocyte function with regard to enhanced cytokine production; specifically TNF-alpha, a know potentiator of tumour cell apoptosis in the presence of IAP-As. The compound demonstrated little effect on human lymphocyte viability. IAP antagonist treatment of moDC resulted in protection from apoptosis, similar to that observed with CD40 ligation. NFκB signaling is shown to result in the upregulation of anti-apoptotic factors [37] and canonical NFκB signaling has been reported to be required for protection from apoptosis and to ensure cell survival in developing and differentiated mo-DC [43]. Loss of cIAPs has been reported to result in the accumulation of NIK and activation of the alternative NFκB pathways in other cell types [9, 10], and indeed we observed concurrent activation of NFκB signaling in moDC following treatment with LCL161, a transient loss and then recovery of cIAP2 and further upregulation of anti-apoptotic molecules. Regulation of cIAP via NFκB signaling has been previously described in myeloid cells, where by LPS signaling upregulates cIAP2 in macrophages [21], and thus is a possible explanation for our observed results. However, the LCL161-induced loss of cIAP2 and its subsequent recovery (as seen on the mRNA and protein level) may have been the result of a feedback loop following the transient drop in protein levels and thus independent of the NFκB signaling concurrently induced by the IAP antagonists. Further studies are required to elucidate this direct connection as observed in mDC.

NF-κB signaling is critical in dendritic cell biology [35, 36], where the capacity of CD40 ligation to regulate myeloid DC function is driven by the non-canonical NF-κB pathway [44], and blockade of NFκB can result in the induction of tolerogenic DCs [45, 46]. However, contradictory data exist for the role of NF-κB signaling in the regulation of co-stimulatory molecules by myeloid DC [43, 47, 48]. We obtained clear evidence of upregulation of co-stimulatory molecules of the B7 family as well as HLA-DR following IAP antagonism in the absence of additional maturation factors and concurrent activation of NFκB signaling. Although direct mechanistic evidence is not provided in this study, given the described activation of the signaling pathway by IAP degradation and the importance of the pathway in DC maturation, our data support a role for NF-κB signaling in phenotypic maturation in this setting. An increase in co-stimulatory molecule expression on DC by IAP antagonism would be complementary to the importance of IAPs in regulating signaling down-stream from TNF-family co-stimulatory molecules on T lymphocytes [24], giving IAP antagonists the capacity to greatly enhance priming of naïve T cells reliant on co-stimulatory signals. This would offer

an explanation to support the observed enhancement of *in vitro* priming of naïve T cells in our study. Interestingly we did not observe the induction of pro-inflammatory cytokines in DC classically associated with NF-κB activation. Of note, a study investigating the role of cIAP1/2 in TLR signaling demonstrated that treatment of murine bone marrow-derived macrophages with an IAP antagonist targeting these molecules resulted in the inhibition of pro-inflammatory cytokines production following TLR4 signaling without preventing NFκB activation or the IFN response [19].

A recent report describes the combination of an IAP-A with cancer vaccines in a murine model of melanoma, resulting in enhanced vaccine efficacy *in vivo* [24]. We investigated the effect of IAP-As on a cancer vaccine comprising recombinant NY-ESO-1 protein and the adjuvant ISOMATRIX [29, 34, 38, 42], in an *in vitro* human system. We demonstrate that IAP antagonist treatment of human myeloid dendritic cells, prior to antigen exposure or concurrently, results in a reduced capacity to activate antigen-specific T cells when cross presenting the ESO/IMX vaccine. Dendritic cell maturation has been associated with a decrease in the capacity to uptake and process antigen [41], although potentially not for all forms of antigen [30]. Our results from endocytosis experiments did not indicate that prior IAP antagonist treatment of immature moDC had an effect on particle uptake. However, the kinetics of maturation parallel the level and kinetics of reduced cross presentation and therefore it seems likely the lower activation of T cells is a result of reduced antigen uptake through maturation. Differences in particle sizes may account for the experimental discrepancy. Of note is the reduced cross presentation observed with concurrent IAP-A treatment and antigen exposure for a reduced time period. The previously described rapid kinetics of ISCOMATRIX-mediated antigen uptake, proteasome-independent processing and presentation of NY-ESO-1 epitopes [29, 49] would suggest this process happens before the maturation effects of the IAP-A would take place; also before the observed NFκB signaling would have occurred. The precise mechanism warrants further investigation.

In conclusion, although the data from this study support the combination of IAP antagonists with a number of immunotherapeutic approaches – based on the enhancement of lymphocyte function, maturation of moDC and enhancement in the activation and expansion of antigen-specific T cells – for combination with protein-based cancer vaccines the treatment schedule for vaccination and LCL161 administration may have to be carefully considered. Together with the capacity to enhance T cell mediated secretion of TNF-α, highly beneficial considering IAP antagonists hypersensitize tumour cells to TNF-mediated apoptosis, IAP antagonists should be investigated further as a novel class of immunomodulators for combination with tumour immunotherapy.

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Authorship Contributions

A.K designed, performed and analyzed the experiments and wrote the manuscript. J.F., A.P. and S.K. performed experiments and analyzed data. J.C. contributed to experimental design and manuscript preparation.

Conflict of interest statement

The authors declare that they have no conflict of interest.

References

1. Hodi FS, O'Day SJ, McDermott DF et al. (2010) Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med. 363: 711-23. doi: NEJMoa1003466 [pii]
10.1056/NEJMoa1003466
2. Kantoff PW, Higano CS, Shore ND et al. (2010) Sipuleucel-T immunotherapy for castration-resistant prostate cancer. N Engl J Med. 363: 411-22. doi: 10.1056/NEJMoa1001294
3. Heine A, Held SA, Bringmann A, Holderried TA, Brossart P (2011) Immunomodulatory effects of anti-angiogenic drugs. Leukemia. 25: 899-905. doi: leu201124 [pii]
10.1038/leu.2011.24
4. Ott PA, Adams S (2011) Small-molecule protein kinase inhibitors and their effects on the immune system: implications for cancer treatment. Immunotherapy. 3: 213-27. doi: 10.2217/imt.10.99
5. Du C, Fang M, Li Y, Li L, Wang X (2000) Smac, a mitochondrial protein that promotes cytochrome c-dependent caspase activation by eliminating IAP inhibition. Cell. 102: 33-42. doi: S0092-8674(00)00008-8 [pii]
6. Yang QH, Du C (2004) Smac/DIABLO selectively reduces the levels of c-IAP1 and c-IAP2 but not that of XIAP and livin in HeLa cells. J Biol Chem. 279: 16963-70. doi: 10.1074/jbc.M401253200
M401253200 [pii]
7. Varfolomeev E, Vucic D (2011) Inhibitor of apoptosis proteins: fascinating biology leads to attractive tumor therapeutic targets. Future Oncol. 7: 633-48. doi: 10.2217/fon.11.40
8. Vucic D, Fairbrother WJ (2007) The inhibitor of apoptosis proteins as therapeutic targets in cancer. Clin Cancer Res. 13: 5995-6000. doi: 13/20/5995 [pii]
10.1158/1078-0432.CCR-07-0729
9. Varfolomeev E, Blankenship JW, Wayson SM et al. (2007) IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. Cell. 131: 669-81. doi: S0092-8674(07)01348-7 [pii]
10.1016/j.cell.2007.10.030
10. Vince JE, Wong WW, Khan N et al. (2007) IAP antagonists target cIAP1 to induce TNFalpha-dependent apoptosis. Cell. 131: 682-93. doi: S0092-8674(07)01357-8 [pii]
10.1016/j.cell.2007.10.037
11. Wu H, Tschopp J, Lin SC (2007) Smac mimetics and TNFalpha: a dangerous liaison? Cell. 131: 655-8. doi: S0092-8674(07)01396-7 [pii]
10.1016/j.cell.2007.10.042
12. Dai Y, Liu M, Tang W, Li Y, Lian J, Lawrence TS, Xu L (2009) A Smac-mimetic sensitizes prostate cancer cells to TRAIL-induced apoptosis via modulating both IAPs and NF-kappaB. BMC Cancer. 9: 392. doi: 1471-2407-9-392 [pii]
10.1186/1471-2407-9-392
13. Varfolomeev E, Alicke B, Elliott JM et al. (2009) X chromosome-linked inhibitor of apoptosis regulates cell death induction by proapoptotic receptor agonists. J Biol Chem. 284: 34553-60. doi: M109.040139 [pii]
10.1074/jbc.M109.040139
14. Fakler M, Loeder S, Vogler M, Schneider K, Jeremias I, Debatin KM, Fulda S (2009) Small molecule XIAP inhibitors cooperate with TRAIL to induce apoptosis in childhood acute leukemia cells and overcome Bcl-2-mediated resistance. Blood. 113: 1710-22. doi: blood-2007-09-114314 [pii]
10.1182/blood-2007-09-114314
15. Loeder S, Zenz T, Schnaiter A, Mertens D, Winkler D, Dohner H, Debatin KM, Stilgenbauer S, Fulda S (2009) A novel paradigm to trigger apoptosis in chronic lymphocytic leukemia. Cancer Res. 69: 8977-86. doi: 0008-5472.CAN-09-2604 [pii]
10.1158/0008-5472.CAN-09-2604
16. Matsuzawa A, Tseng PH, Vallabhapurapu S, Luo JL, Zhang W, Wang H, Vignali DA, Gallagher E, Karin M (2008) Essential cytoplasmic translocation of a cytokine receptor-assembled signaling complex. Science. 321: 663-8. doi: 1157340 [pii]
10.1126/science.1157340
17. Vallabhapurapu S, Matsuzawa A, Zhang W, Tseng PH, Keats JJ, Wang H, Vignali DA, Bergsagel PL, Karin M (2008) Nonredundant and complementary functions of TRAF2 and TRAF3 in a ubiquitination cascade that activates NIK-dependent alternative NF-kappaB signaling. Nat Immunol. 9: 1364-70. doi: ni.1678 [pii]
10.1038/ni.1678
18. Gardam S, Turner VM, Anderton H, Limaye S, Basten A, Koentgen F, Vaux DL, Silke J, Brink R (2011) Deletion of cIAP1 and cIAP2 in murine B lymphocytes constitutively activates cell survival pathways and inactivates the germinal center response. Blood. 117: 4041-51. doi: blood-2010-10-312793 [pii]
10.1182/blood-2010-10-312793

19. Tseng PH, Matsuzawa A, Zhang W, Mino T, Vignali DA, Karin M (2010) Different modes of ubiquitination of the adaptor TRAF3 selectively activate the expression of type I interferons and proinflammatory cytokines. Nat Immunol. 11: 70-5. doi: ni.1819 [pii]
10.1038/ni.1819
20. Dupoux A, Cartier J, Cathelin S, Filomenko R, Solary E, Dubrez-Daloz L (2009) cIAP1-dependent TRAF2 degradation regulates the differentiation of monocytes into macrophages and their response to CD40 ligand. Blood. 113: 175-85. doi: blood-2008-02-137919 [pii]
10.1182/blood-2008-02-137919
21. Conte D, Holcik M, Lefebvre CA, Lacasse E, Picketts DJ, Wright KE, Korneluk RG (2006) Inhibitor of apoptosis protein cIAP2 is essential for lipopolysaccharide-induced macrophage survival. Mol Cell Biol. 26: 699-708. doi: 26/2/699 [pii]
10.1128/MCB.26.2.699-708.2006
22. Waldele K, Silbermann K, Schneider G, Ruckes T, Cullen BR, Grassmann R (2006) Requirement of the human T-cell leukemia virus (HTLV-1) tax-stimulated HIAP-1 gene for the survival of transformed lymphocytes. Blood. 107: 4491-9. doi: 2005-08-3138 [pii]
10.1182/blood-2005-08-3138
23. Zane L, Sibon D, Legras C et al. (2010) Clonal expansion of HTLV-1 positive CD8+ cells relies on cIAP-2 but not on c-FLIP expression. Virology. 407: 341-51. doi: S0042-6822(10)00476-9 [pii]
10.1016/j.virol.2010.07.023
24. Dougan M, Dougan S, Slisz J et al. (2010) IAP inhibitors enhance co-stimulation to promote tumor immunity. J Exp Med. 207: 2195-206. doi: jem.20101123 [pii]
10.1084/jem.20101123
25. Infante JR, Dees EC, Burris HAI et al. (2010) A phase I study of LCL161, an oral IAP inhibitor, in patients with advanced cancer. The 101st Annual Meeting of the American Association for Cancer Research., Washington, DC, USA
26. Houghton PJ, Kang MH, Reynolds CP et al. (2011) Initial testing (Stage 1) of LCL161, a SMAC mimetic, by the pediatric preclinical testing program. Pediatr Blood Cancer. doi: 10.1002/pbc.23167
27. Weisberg E, Ray A, Barrett R et al. (2010) Smac mimetics: implications for enhancement of targeted therapies in leukemia. Leukemia. 24: 2100-9. doi: leu2010212 [pii]
10.1038/leu.2010.212
28. Knights AJ, Nuber N, Thomson CW et al. (2009) Modified tumour antigen-encoding mRNA facilitates the analysis of naturally occurring and vaccine-induced CD4 and CD8 T cells in cancer patients. Cancer Immunol Immunother. 58: 325-38. doi: 10.1007/s00262-008-0556-8
29. Robson NC, McAlpine T, Knights AJ, Schnurr M, Shin A, Chen W, Maraskovsky E, Cebon J (2010) Processing and cross-presentation of individual HLA-A, -B, or -C epitopes from NY-ESO-1 or an HLA-A epitope for Melan-A differ according to the mode of antigen delivery. Blood. 116: 218-25. doi: blood-2009-10-249458 [pii]
10.1182/blood-2009-10-249458
30. Platt CD, Ma JK, Chalouni C, Ebersold M, Bou-Reslan H, Carano RA, Mellman I, Delamarre L (2010) Mature dendritic cells use endocytic receptors to capture and present antigens. Proc Natl Acad Sci U S A. 107: 4287-92. doi: 0910609107 [pii]
10.1073/pnas.0910609107
31. Chauhan D, Neri P, Velankar M et al. (2007) Targeting mitochondrial factor Smac/DIABLO as therapy for multiple myeloma (MM). Blood. 109: 1220-7. doi: blood-2006-04-015149 [pii]
10.1182/blood-2006-04-015149
32. Zippelius A, Pittet MJ, Batard P et al. (2002) Thymic selection generates a large T cell pool recognizing a self-peptide in humans. J Exp Med. 195: 485-94.
33. Duewell P, Kisser U, Heckelsmiller K et al. (2011) ISCOMATRIX Adjuvant Combines Immune Activation with Antigen Delivery to Dendritic Cells In Vivo Leading to Effective Cross-Priming of CD8+ T Cells. J Immunol. 187: 55-63. doi: jimmunol.1004114 [pii]
10.4049/jimmunol.1004114
34. Klein O, Schmidt C, Knights A, Davis ID, Chen W, Cebon J (2011) Melanoma vaccines: developments over the past 10 years. Expert Rev Vaccines. 10: 853-73. doi: 10.1586/erv.11.74
35. Ouaaz F, Arron J, Zheng Y, Choi Y, Beg AA (2002) Dendritic cell development and survival require distinct NF-kappaB subunits. Immunity. 16: 257-70. doi: S1074761302002728 [pii]
36. Hayden MS, Ghosh S (2004) Signaling to NF-kappaB. Genes Dev. 18: 2195-224. doi: 10.1101/gad.1228704
18/18/2195 [pii]
37. Bonizzi G, Karin M (2004) The two NF-kappaB activation pathways and their role in innate and adaptive immunity. Trends Immunol. 25: 280-8. doi: 10.1016/j.it.2004.03.008
S1471490604001000 [pii]

38. Davis ID, Chen W, Jackson H et al. (2004) Recombinant NY-ESO-1 protein with ISCOMATRIX adjuvant induces broad integrated antibody and CD4(+) and CD8(+) T cell responses in humans. Proc Natl Acad Sci U S A. 101: 10697-702. doi: 10.1073/pnas.0403572101
0403572101 [pii]
39. Nicholaou T, Ebert L, Davis ID, Robson N, Klein O, Maraskovsky E, Chen W, Cebon J (2006) Directions in the immune targeting of cancer: lessons learned from the cancer-testis Ag NY-ESO-1. Immunol Cell Biol. 84: 303-17. doi: ICB [pii]
10.1111/j.1440-1711.2006.01446.x
40. Garrett WS, Chen LM, Kroschewski R, Ebersold M, Turley S, Trombetta S, Galan JE, Mellman I (2000) Developmental control of endocytosis in dendritic cells by Cdc42. Cell. 102: 325-34. doi: S0092-8674(00)00038-6 [pii]
41. Hickman-Miller HD, Yewdell JW (2006) Youth has its privileges: maturation inhibits DC cross-priming. Nat Immunol. 7: 125-6. doi: ni0206-125 [pii]
10.1038/ni0206-125
42. Schnurr M, Orban M, Robson NC, Shin A, Braley H, Airey D, Cebon J, Maraskovsky E, Endres S (2009) ISCOMATRIX adjuvant induces efficient cross-presentation of tumor antigen by dendritic cells via rapid cytosolic antigen delivery and processing via tripeptidyl peptidase II. J Immunol. 182: 1253-9. doi: 182/3/1253 [pii]
43. van de Laar L, van den Bosch A, van der Kooij SW, Janssen HL, Coffer PJ, van Kooten C, Woltman AM (2010) A nonredundant role for canonical NF-kappaB in human myeloid dendritic cell development and function. J Immunol. 185: 7252-61. doi: jimmunol.1000672 [pii]
10.4049/jimmunol.1000672
44. O'Sullivan BJ, Thomas R (2002) CD40 ligation conditions dendritic cell antigen-presenting function through sustained activation of NF-kappaB. J Immunol. 168: 5491-8.
45. Li M, Zhang X, Zheng X et al. (2007) Immune modulation and tolerance induction by RelB-silenced dendritic cells through RNA interference. J Immunol. 178: 5480-7. doi: 178/9/5480 [pii]
46. Carreno LJ, Riedel CA, Kalergis AM (2011) Induction of tolerogenic dendritic cells by NF-kappaB blockade and Fcgamma receptor modulation. Methods Mol Biol. 677: 339-53. doi: 10.1007/978-1-60761-869-0_22
47. Hernandez A, Burger M, Blomberg BB et al. (2007) Inhibition of NF-kappa B during human dendritic cell differentiation generates anergy and regulatory T-cell activity for one but not two human leukocyte antigen DR mismatches. Hum Immunol. 68: 715-29. doi: S0198-8859(07)00132-2 [pii]
10.1016/j.humimm.2007.05.010
48. Gasparini C, Foxwell BM, Feldmann M (2009) RelB/p50 regulates CCL19 production, but fails to promote human DC maturation. Eur J Immunol. 39: 2215-23. doi: 10.1002/eji.200939209
49. Schnurr M, Chen Q, Shin A et al. (2005) Tumor antigen processing and presentation depend critically on dendritic cell type and the mode of antigen delivery. Blood. 105: 2465-72. doi: 2004-08-3105 [pii]
10.1182/blood-2004-08-3105

Figure legends

Figure 1

Significant increase the frequency of cytokine-secreting T cells and the duration of secretion following treatment with the IAP antagonist LCL161

Production of the cytokines IL-2 and TNF-α by purified CD4+ and CD8+ T cells was assessed by ICS flow cytometry at 24, 48 and 72 hours following activation with anti-CD3/CD28 micro particles and titrating concentrations of LCL161. (A) A representative example for IL-2 production by CD4+ T cells from 3 donors is shown. Percentage of cytokine producing cells (y-axis) is shown as a fraction of total CD3+CD4+ T cells in the FACS lymphocyte gate. Each point represents the mean from triplicate wells for each donor, with the grand mean and SEM shown at each concentration of LCL161 (x-axis). (B) To normalise for inter-donor variation, the fold-increase in percentage of cytokine-producing cells was calculated for each concentration in relation to the untreated control and plotted for each time point. Mean values with SEM from the results obtained with 6 donors are shown for IL-2 (upper histograms) and TNF-α (lower histograms) production in CD4+ (left histograms) and CD8+ (right histograms) T cells. One-way ANOVA with a Dunnetts post-test against the untreated control was performed, * = P <0.05, ** = P <0.01, *** = P <0.001.

Figure 2

A significant increase in the frequency of antigen-specific T cells stimulated in *in vitro* peptide sensitisation assays is mediated by IAP antagonist treatment

The effects of LCL161 treatment on *in vitro* stimulation of peptide-specific CD8+ T cells was assessed 10-days after stimulation of $1x10^6$ PBMC with an HLA-A2-restricted synthetic peptide for either Flu-M1 (58-66), EBV BMLF1 (280-288) or melan-A (26-35). Specific cells were detected using fluorescently-labelled recombinant HLA-A2 MHC-peptide multimers containing the relevant peptide. (A) Representative contour plots are shown for each peptide with titrating concentrations of LCL161. The percentage of MHC/peptide multimer-positive (y-axis) CD8+ (x-axis) CD3+ T cells in the FACS lymphocyte gate is shown. (B) Percentage of tetramer + CD8+ cells (y-axis) are shown for each

peptide and LCL161 concentration (x-axis). Each point represents a mean value from multiple replicate stimulations performed with 5 donors (Flu, EBV BMLF1) or 6 donors (melan-A) and the grand-mean (horizontal bar) with SEM is indicated. One-way ANOVA with a Dunnetts post-test against the untreated control was performed, * = P <0.05, *** = P <0.001.

Figure 3

IAP antagonism induces phenotypic maturation of monocyte-derived dendritic cells

The effects of LCL161 treatment on maturation of immature monocyte-derived dendritic cells was assessed *in vitro* 24-hours (left scatter plots) and 48-hours (right scatter plots) following treatment with LCL161. Mo-DC were treated on day 4 following differentiation from monocytes, seeded in triplicate wells per concentration and treated with LCL161 at the concentrations indicated (x-axis). Soluble CD40L was used as a maximal control for induction of maturation. Each point represents the mean value obtained from triplicate wells per donor. Horizontal bars indicate the grand-mean derived from the results obtained with 4 donors and error bars show the SEM. (A) For Annexin-V, staining was performed in combination with a viability dye. Ann-V positive, viability dye negative (viable cells) were counted as apoptotic cells (y-axis). (B/C) For B7-DC and B7-1 the percentage of viable (Live/Dead fluorescent dye negative) positive cells (y-axis) within the monocyte gate of the FACS plot is indicated. (D/E) For B7-2 and HLA-DR the mean fluorescence intensity (MFI) of the positive cells (>95%) is indicated (y-axis) for the viable cells within the monocyte gate of the FACS plot. One-way ANOVA with a Dunnetts post-test against the untreated control was performed, * = P <0.05, ** = P <0.01, *** = P <0.001.

Figure 4

Exposure of moDC to the IAP antagonist LCL161 activates the canonical and non-canonical NFκB pathways

MoDC were left untreated (UT) or treated with 10μM LCL161 and assessed for activation of the NFκB pathway by Western blot analysis. Whole cell lysates were prepared 30 minutes and 2, 4, 6 and 24 hours following drug treatment. CD40L treatment for 24 hours represents the positive control. Equal quantities of protein were evaluated on a 4-12% SDS-PAGE gel, transferred to membrane and probed with antibodies to IκBα (A,B) phospho-p65/Rel-A (C,D) and

p52/p100 (E,F); antibodies to β-tublin (A,B) and β-actin (C-F) were used for reference. Chemiluminescent imaging was used for (B) and near-infrared fluorescence detection was used for (D) and (F). (A) and (C) show quantified percentage expression levels as a percentage of the respective untreated control, whereas (E) shows a ratio for expression levels of p52 to p100 at each time point; all values were first normalised to the respective house keeping protein (β-tublin and β-actin). (A, C, E) Each point represents the result per donor with the mean (horizontal bar) and SEM shown from 4 donors. One-way ANOVA with a Dunnetts post-test against the untreated control was performed, * = P <0.05, ** = P <0.01, *** = P <0.001.

Figure 5

Treatment of moDC with the IAP antagonist LCL161 induces expression of anti-apoptotic factors

MoDC were left untreated (UT) or treated with 10μM LCL161 and assessed for expression of anti-apoptotic molecules by qPRC and Western blot analysis. Whole cell lysates and mRNA were prepared from cells treated for 2, 4, 6 and 24 hours. CD40L treatment for 24 hours represents the positive control. Untreated cells were cultured in parallel for 24 hours. (A) Semi-quantitative RT-PCR was performed on cDNA from the treated imoDC with specific primer pairs for cIAP1, cIAP2, Bcl-2 and Bcl-xL using SYBRgreen chemistry. Relative transcript copy numbers are expressed as copy number of NY-ESO-1 transcripts per β-actin transcript using $2^{(\beta\text{-actin CT} - \text{gene of interest CT})}$. For Western blot analysis, equal quantities of protein were loaded and ran on 4-12% SDS-PAGE gels, transferred to membrane and probed for the indicated proteins. Antibodies to cIAP2 (B) and Bcl-xL (C) were used at a 1:500 dilution. Near-infrared fluorescence detection was used with the appropriate secondary antibodies, and membranes were imaged accordingly. For each condition values were normalised to the expression of β-actin and quantified percentage expression levels are shown as a percentage of the respective untreated control. Each point represents the result per donor with the mean (horizontal bar) and SEM shown from 4 donors. One-way ANOVA with a Dunnetts post-test against the untreated control was performed, * = P <0.05, ** = P <0.01, *** = P <0.001.

Figure 6

IAP antagonists significantly decrease cross-presentation of tumour antigen by monocyte-derived dendritic cells

MoDC plated in triplicate were treated for 24 or 48 hours with titrating concentrations of LCL161 or LBW242 and rNY-ESO-1/ISCOMATRIX was added for an additional 18 hours following treatment. An antigen specific CD8+ T cell clone was used to assess cross-presentation of MHC-class I associated epitopes in a standard recognition assay measuring specific cytokine secretion by the T cells upon activation. (A) An NY-ESO-1-specific (157-165/HLA-A2) CD8+ T cell clone was incubated for 4 hours with the previously treated and antigen-pulsed DC and the percentage of activated, IFN-γ positive CD8+ CD3+ cells (y-axis) within the lymphocyte FACS gate (x-axis) was assessed at each drug concentration used to treat the moDC (indicated above contour plots). (B) All values were normalised against the untreated control to compensate for inter-donor variation in the % IFN-γ+ cells responding within the untreated group, and therefore allowing assessment of drug effect. This value is referred to as an 'activation index' (y-axis) and indicates relative activation (IFN-γ positivity) of the specific T cell clone following a 4 hour incubation with the 24 or 48 hour drug-treated and antigen-pulsed DC at the concentration indicated (x-axis) for LCL161 (Upper scatter plots) and LBW242 (lower scatter plots). Each point represents the mean from triplicate results per donor, per concentration with the grand-mean (horizontal bar) and SEM shown from 4 donors. One-way ANOVA with a Dunnetts post-test against the untreated control was performed, * = P <0.05, *** = P <0.001.

Figure 1

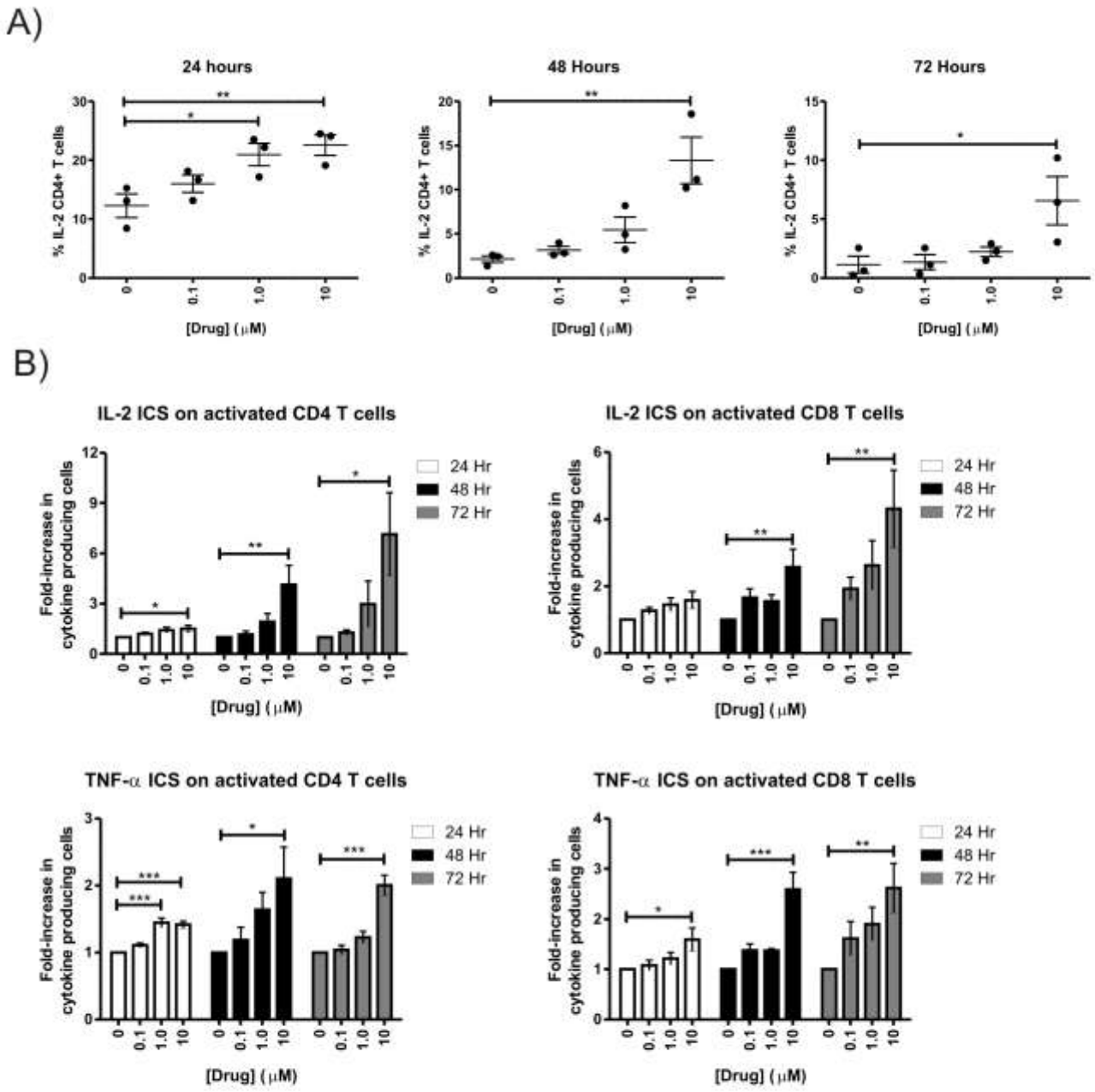

A)
24 hours
48 Hours
72 Hours
% IL-2 CD4+ T cells
[Drug] (μM)
B)
IL-2 ICS on activated CD4 T cells
IL-2 ICS on activated CD8 T cells
TNF-α ICS on activated CD4 T cells
TNF-α ICS on activated CD8 T cells
Fold-increase in cytokine producing cells
24 Hr
48 Hr
72 Hr
[Drug] (μM)

Figure 2

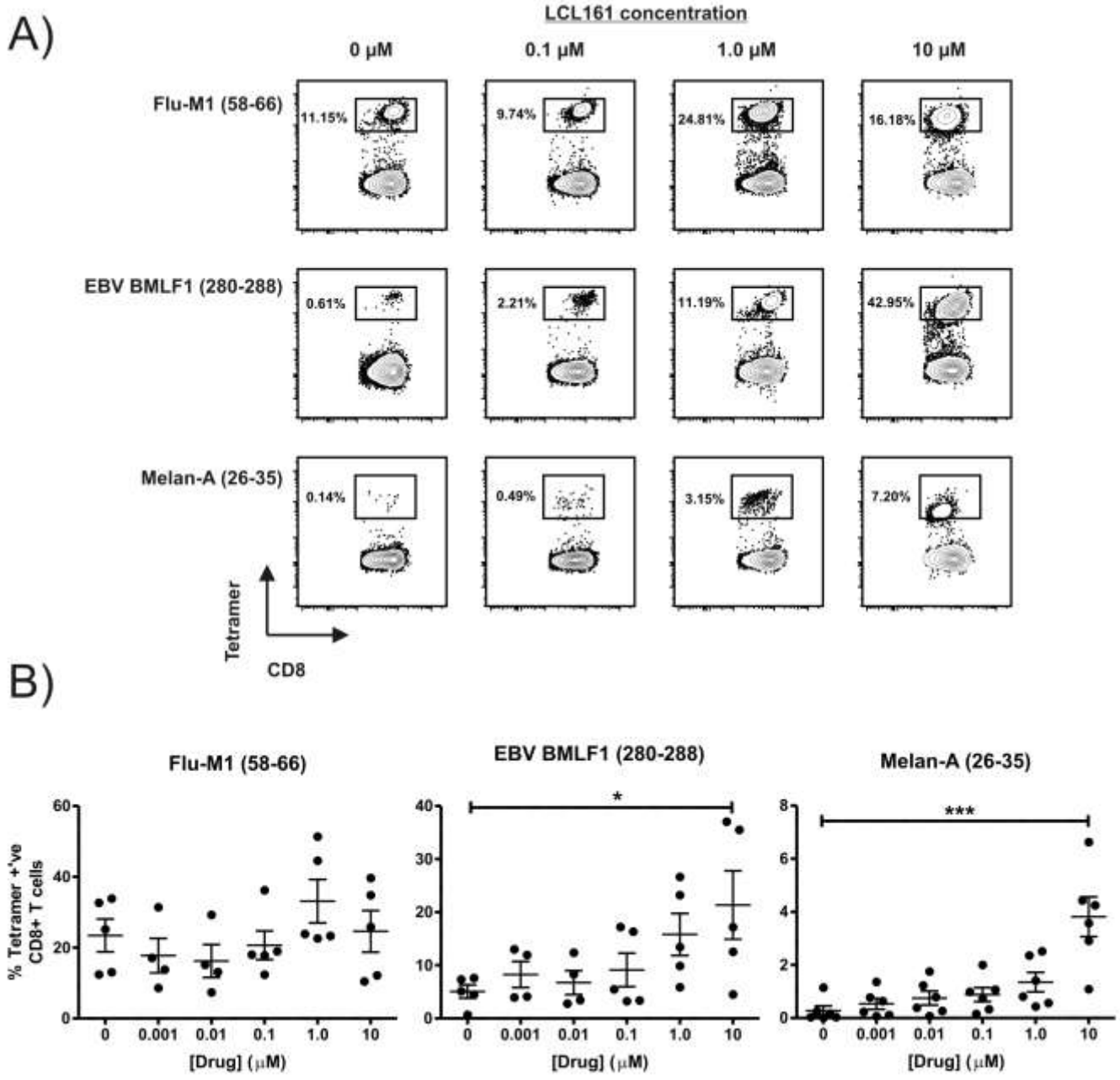

A)
LCL161 concentration
0 µM
0.1 µM
1.0 µM
10 µM
Flu-M1 (58-66)
11.15%
9.74%
24.81%
16.18%
EBV BMLF1 (280-288)
0.61%
2.21%
11.19%
42.95%
Melan-A (26-35)
0.14%
0.49%
3.15%
7.20%
Tetramer
CD8
B)
Flu-M1 (58-66)
EBV BMLF1 (280-288)
Melan-A (26-35)
% Tetramer +'ve CD8+ T cells
[Drug] (µM)
0
0.001
0.01
0.1
1.0
10
*
***

Figure 3

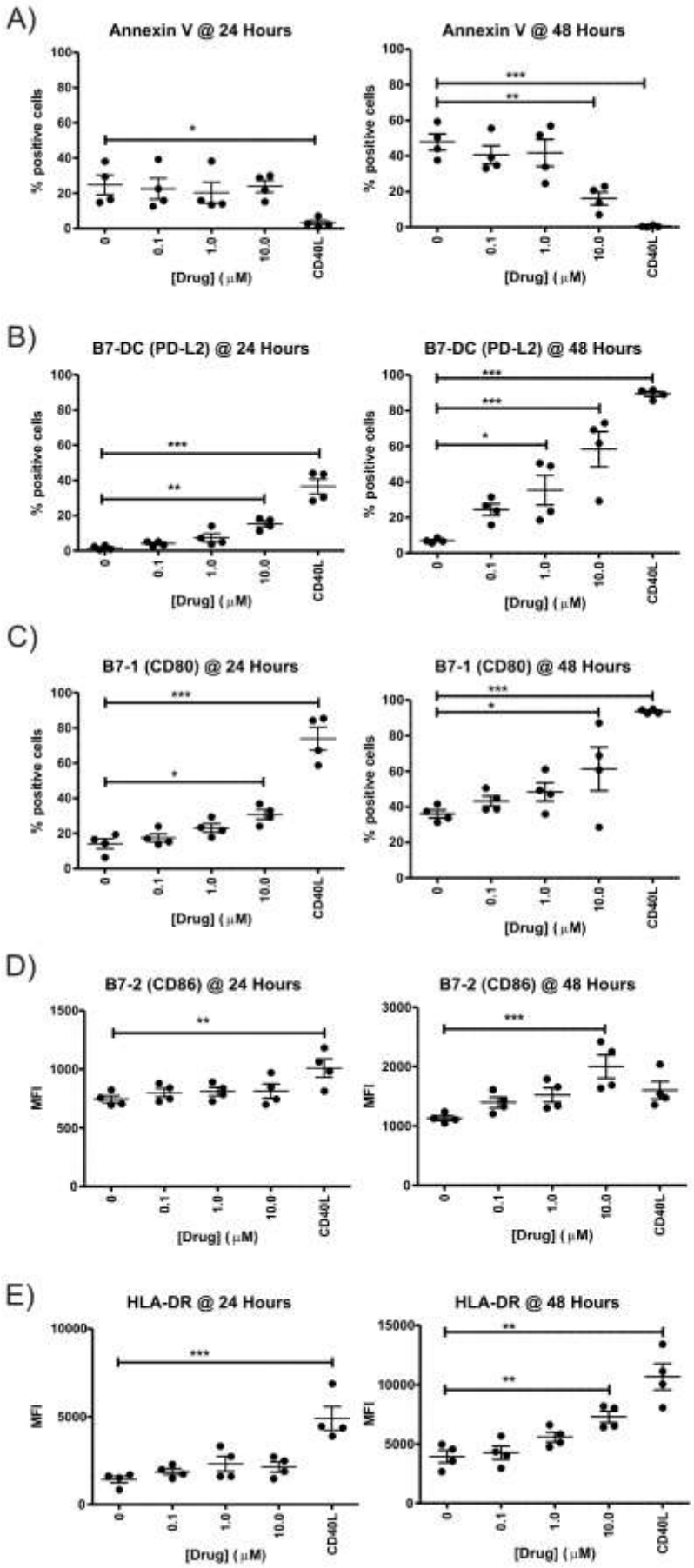

A)
Annexin V @ 24 Hours
Annexin V @ 48 Hours
% positive cells
[Drug] (μM)
B)
B7-DC (PD-L2) @ 24 Hours
B7-DC (PD-L2) @ 48 Hours
C)
B7-1 (CD80) @ 24 Hours
B7-1 (CD80) @ 48 Hours
D)
B7-2 (CD86) @ 24 Hours
B7-2 (CD86) @ 48 Hours
MFI
E)
HLA-DR @ 24 Hours
HLA-DR @ 48 Hours
0
0.1
1.0
10.0
CD40L

Figure 4

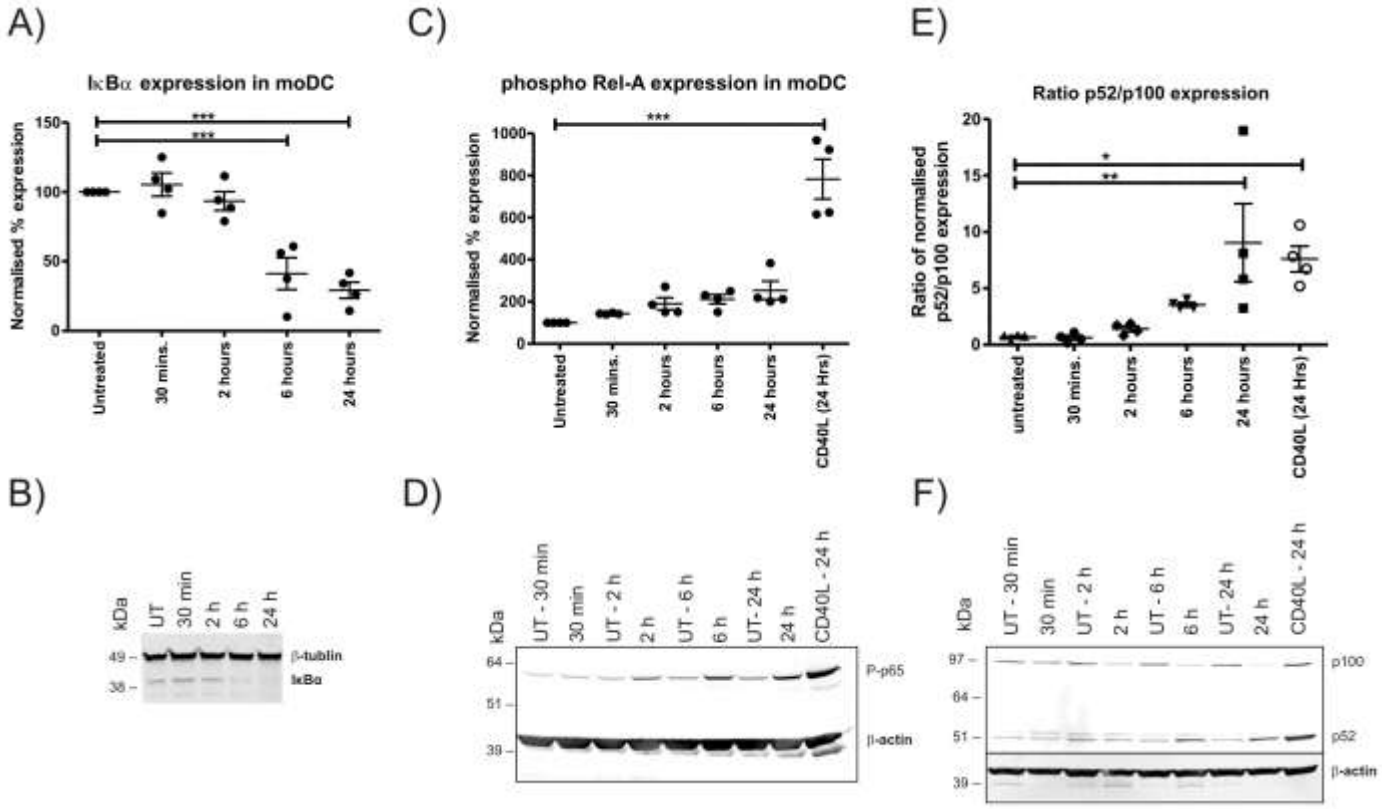

A)
IκBα expression in moDC
Normalised % expression
Untreated
30 mins.
2 hours
6 hours
24 hours
C)
phospho Rel-A expression in moDC
Normalised % expression
Untreated
30 mins.
2 hours
6 hours
24 hours
CD40L (24 Hrs)
E)
Ratio p52/p100 expression
Ratio of normalised p52/p100 expression
untreated
30 mins.
2 hours
6 hours
24 hours
CD40L (24 Hrs)
B)
kDa
UT
30 min
2 h
6 h
24 h
β-tublin
IκBα
D)
kDa
UT - 30 min
30 min
UT - 2 h
2 h
UT - 6 h
6 h
UT- 24 h
24 h
CD40L - 24 h
P-p65
β-actin
F)
kDa
UT - 30 min
30 min
UT - 2 h
2 h
UT - 6 h
6 h
UT- 24 h
24 h
CD40L - 24 h
p100
p52
β-actin

Figure 5

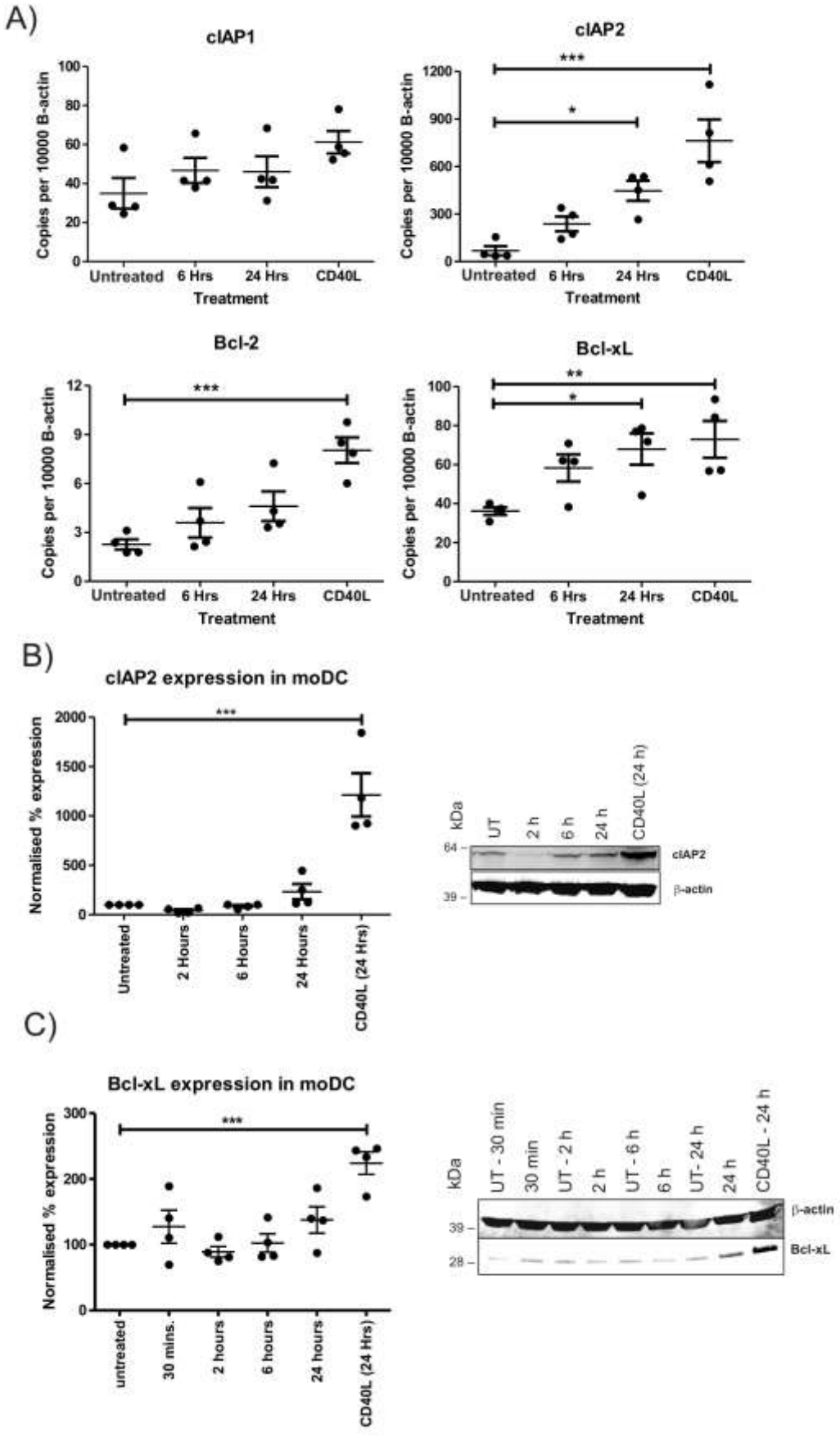
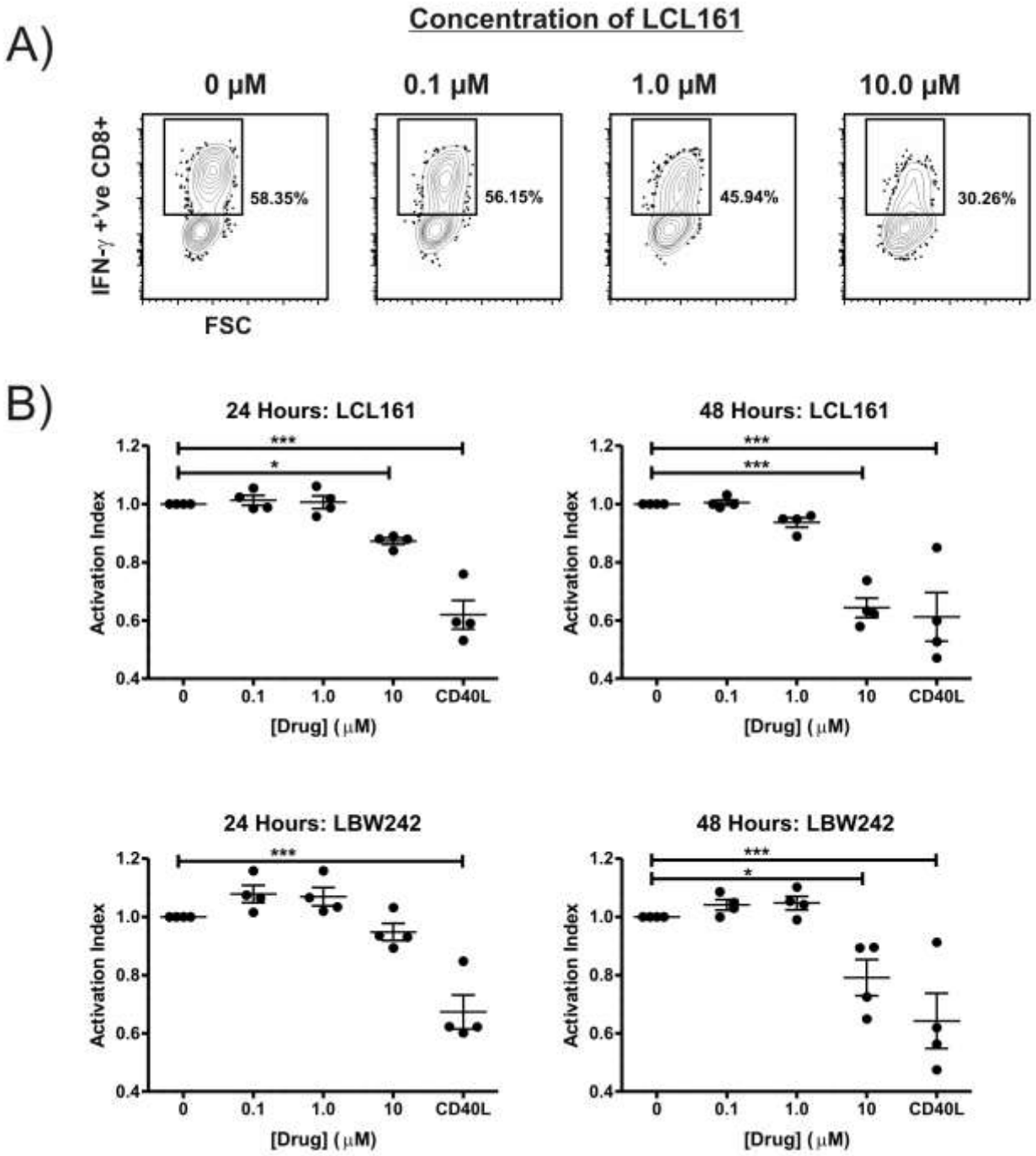

A)
cIAP1
Copies per 10000 B-actin
Untreated
6 Hrs
24 Hrs
CD40L
Treatment
cIAP2
***
*
Bcl-2
Bcl-xL
**
B)
cIAP2 expression in moDC
Normalised % expression
2 Hours
6 Hours
24 Hours
CD40L (24 Hrs)
kDa
UT
2 h
6 h
24 h
64
39
β-actin
C)
Bcl-xL expression in moDC
untreated
30 mins.
2 hours
6 hours
UT - 30 min
30 min
UT - 2 h
UT - 6 h
UT- 24 h
28

Figure 6


A)
Concentration of LCL161
0 µM
0.1 µM
1.0 µM
10.0 µM
IFN-γ +'ve CD8+
58.35%
56.15%
45.94%
30.26%
FSC
B)
24 Hours: LCL161
48 Hours: LCL161
24 Hours: LBW242
48 Hours: LBW242
Activation Index
[Drug] (µM)
0
0.1
1.0
10
CD40L
***
*

Author/s:
Knights, AJ; Fucikova, J; Pasam, A; Koernig, S; Cebon, J

Title:
Inhibitor of apoptosis protein (IAP) antagonists demonstrate divergent immunomodulatory properties in human immune subsets with implications for combination therapy

Date:
2013-02-01

Citation:
Knights, A. J., Fucikova, J., Pasam, A., Koernig, S. & Cebon, J. (2013). Inhibitor of apoptosis protein (IAP) antagonists demonstrate divergent immunomodulatory properties in human immune subsets with implications for combination therapy. CANCER IMMUNOLOGY IMMUNOTHERAPY, 62 (2), pp.321-335. https://doi.org/10.1007/s00262-012-1342-1.