Stromal fibroblasts support dendritic cells to maintain IL-23/Th17 responses after exposure to ionizing radiation

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Summary sentence

Crosstalk between stromal fibroblasts and dendritic cells negates the immune suppressive effect of ionizing radiation.

Running Title: Dendritic cell–fibroblast crosstalk

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Abbreviations:

APC Antigen presenting cell, **ATM** Ataxia-telangiectasia mutated kinase, **cAMP** Cyclic adenosine 3′,5′-monophosphate, **CCL2** Chemokine (C-C Motif) Ligand 2, **CCL20** Chemokine (C-C Motif) Ligand 20, **COPD** chronic obstructive pulmonary disease, **COX2** cyclooxygenase 2, **CXCL16** Chemokine (C-X-C Motif) Ligand 16, **DC** dendritic cell, **DNA-PK** DNA-dependent protein kinase, **FB** fibroblast**, iDC** immature DC, **IR** ionizing radiation, **LN** lymph node, **LPS** lipopolysaccharide, **mDC** mature DC, **MoDC** monocyte-derived DC, **PGE_2** prostaglandin E2, **PKA** Protein Kinase-A, **PP2A** Protein phosphatase 2A, **rh** recombinant human, **TAF** Tumor associated FB, **TNFα** tumor necrosis factor α, **TLR** Toll-Like Receptor

Abstract

Dendritic cell (DC) function is modulated by stromal cells including fibroblasts (FB). Whilst poorly understood, the signals delivered through this cross-talk substantially alter DC biology. This is well illustrated with release of TNFα/IL-1β from activated DC, promoting PGE_2 secretion from stromal FB. This instructs DC to up-regulate IL-23, a key Th17-polarising cytokine. We previously showed ionizing radiation (IR) inhibited IL-23 production by human DC *in vitro*. In the present study we investigated the hypothesis that DC-FB cross-talk overcomes the suppressive effect of IR to support appropriately polarized Th17 responses.

Radiation (1-6Gy) markedly suppressed IL-23 secretion by activated DC ($p<0.0001$) without adversely impacting their viability, and consequently inhibited the generation of Th17 responses. Cytokine suppression by IR was selective as there was no effect on IL-1β, -6, -10, -27 or TNFα, and only a modest (11%) decrease in IL-12p70 secretion. Co-culture with FB augmented IL-23 secretion by irradiated DC and increased Th17 responses. Importantly, in contrast to DC, irradiated FB maintained their capacity to respond to TNFα/IL-1β and produce PGE_2, thus providing the key intermediary signals for successful DC-FB crosstalk.

In summary, stromal FB support Th17 polarizing cytokine production by DC that would otherwise be suppressed in an irradiated microenvironment. This has potential ramifications for understanding the immune response to local radiotherapy. These findings underscore the need to account for the impact of micro-environmental factors, including stromal cells, in understanding the control of immunity.

Introduction

Cross-talk between DC and stromal FB has a marked impact on DC function with regards to their ability to mature, migrate, and trigger appropriate adaptive responses [1-3]. Whilst the importance of cross-talk between FB and the immune system for the onset and maintenance of cancer and autoimmunity was recently recognized, the exact mechanisms governing these interactions and the extent to which stromal cells affect the outcome of therapeutic interventions like radiotherapy remain poorly understood [4-7].

Fibroblasts play important roles in the pathology of a wide range of diseases. Tumor associated FB (TAF) promote tolerogenic DC in hepatic, breast and ovarian cancers while in pancreatic cancer they skew DC to promote Th2 responses [8-11]. Pulmonary FB are involved in the pathology of COPD (chronic obstructive pulmonary disease) and asthma by secreting CCL2 (Chemokine (C-C Motif) Ligand 2) and CCL20 (Chemokine (C-C Motif) Ligand 20) to attract DC to airways and thus maintain chronic inflammation [12]. Similarly, synovial FB are implicated in the perpetuation of rheumatoid arthritis through recruitment and activation of leucocytes including T cells, macrophages and DC [7, 13]. Recently we established that FB modulate IL-23 secretion by DC to promote Th17 responses and this process was implicated in the maintenance and progression of psoriatic lesions [3]. This model proposes that dermal FB respond to TNFα (tumor necrosis factor α) and IL-1β, secreted from activated DC, by producing PGE_2 (prostaglandin E2). Fibroblast-derived PGE_2 acts in a juxtacrine manner to amplify IL-23 release from DC thus supporting the generation of Th17 responses. The IL-23/Th17 axis is important for the development and maintenance of autoimmune disorders including rheumatoid arthritis, psoriasis and colitis [14-17]. IL-23 promotes tumour growth directly and indirectly through Th17 responses that drive proliferation, invasion, metastasis and angiogenesis [18-23]. Furthermore, it is implicated in the development of idiopathic and radiation-induced fibrosis [24, 25].

Radiotherapy (RT) is a major tool for the treatment and palliation of tumours including squamous cell carcinoma of the skin, breast and primary brain tumours and brain metastases [26-28]. Current protocols employ fractionated RT typically comprising relatively low doses (1-6Gy) of ionising radiation (IR) administered over a period of weeks [26, 29] This allows tumour cells to be targeted with sufficient cumulative dose to deliver therapeutic benefit whilst restricting side effects to a tolerable minimum. However, during radiotherapy, immune and stromal cells residing in and adjacent to the tumour niche are also affected by IR through both direct and bystander effects. Irradiated tumour cells up-regulate MHC I expression and secrete chemokines and cytokines such as CXCL16 (Chemokine (C-X-C Motif) Ligand 16), TNFα, IL-1β and IL-6 which in turn stimulate infiltration and activation of DC [30]. DC are relatively resistant to radiation-induced apoptosis maintaining viability at doses up to 30Gy [31]. Their radio-resistance is a result of constitutively expressed DNA repair systems including ATM (ataxia telangiectasia mutated) kinase and DNA-PK (DNA-dependent protein kinase) [32]. However, despite this, direct exposure of DC to IR suppresses their function. Irradiation of DC down-regulates the production of the T cell polarising cytokine IL-12 without affecting IL-10 thus changing the ratio between pro and anti-inflammatory stimuli and shifting the balance from T cell activation to tolerance [31]. Furthermore, we recently described that IR inhibits secretion of IL-23, another T-cell polarising cytokine, by DC [33]. Taken together this suggests that, in the setting of local radiotherapy, the cytokine “signal 3” from activated DC is substantially modified, thus altering the nature of subsequent T-cell responses.

We therefore investigated the hypothesis that local FB support IL-23 release by irradiated DC and thereby maintain their ability to generate Th17 responses. Doses of radiation consistent with those employed for fractionated RT, selectively inhibited IL-23 and to a lesser extent IL-12 by DC, without affecting IL-10, IL-6, IL-27, TNFα or IL-1β secretion. Interestingly, IR did not affect the capacity of FB to amplify IL-23 secretion by DC. The co-

culture of irradiated FB with irradiated DC up-regulated IL-23 secretion and increased Th17 responses. We examined the factors by which FB support the function of DC despite the presence of ionizing radiation by employing a DC-FB co-culture system in the presence of IR. The enhancing effect of FB was mimicked by addition of PGE_2 or forskolin to irradiated DC and was abrogated by treating FB with the COX2 (cyclooxygenase 2) inhibitor indomethacin. This effect occurred despite activation of the ATM pathway in irradiated DC which we previously established was involved in IL-23 down-regulation [33]. These findings indicate that even after exposure to ionizing radiation, activated FB act through secretion of PGE_2 to activate the cAMP (Cyclic adenosine 3′,5′-monophosphate) pathway in irradiated DC, leading to increased IL-23 secretion independent of ATM kinase. Our findings not only establish the significance of FB in regulating DC responses but also highlight the complex interplay between DC and their microenvironment, and illustrate the importance of developing more comprehensive cell biology models for understanding immunity.

Materials and methods

Reagents

All reagents were endotoxin-free. Recombinant human GM-CSF and TNFα were from PeproTech, Rocky Hill, NJ; IL-4 and IFNγ were from R&D Systems Europe, Oxford, U.K.; Ultrapure TLR4-agonist (*Salmonella Minnesota LPS)* was from InvivoGen (San Diego, CA); Recombinant human IL-1β and IL-6 were from Immunotools, Friesoythe, Germany; IL-23 was from eBioscience (San Diego, CA); PGE_2, Indomethacin and forskolin were from Sigma-Aldrich (Dorset, U.K.). Mouse anti-human CD4-PE was from BD Biosciences (Oxford, U.K.); mouse anti-human CD4-PECy7, mouse anti-human CD45RA-FITC, mouse anti-human CD14-Pe-Cy5.5 and matching isotype controls were from eBioscience (San Diego, CA). For CD4 activation, mouse anti-human CD28 was obtained from BD Biosciences, IL-2 (R&D systems), and CD3 (OKT3) was produced in-house. AnnexinV/PI staining kit was obtained from BD Bioscience (Oxford, UK).

Generation of monocyte-derived DC

Monocyte-derived DC were generated as previously described [34]. Briefly, fresh blood samples were obtained from healthy volunteers, and buffy coats were obtained from the National Blood Transfusion Service in accordance with the approval of the relevant ethical review boards. PBMC were isolated using endotoxin-free Histopaque 1.077(Sigma Aldrich, Dorset, U.K.) gradient centrifugation. $CD14^+$ monocytes were purified using anti-CD14 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). DC were generated by culture in DC medium (RPMI 1640, 10% fetal calf serum, 1% sodium pyruvate –all from Sigma Aldrich Dorset, U.K- containing recombinant human (rh) GM-CSF (1000 U/mL) and rhIL-4 (1000 U/mL) for 5 days. Additional complete medium was added on day 3. The purity and quality of DC were determined by flow-cytometry and morphologic analysis.

DC-FB co-culture

The human dermal FB cell line BJ6 was obtained from Dr. Lloyd Hamilton (University of Nottingham) whilst primary dermal FB were obtained from Dr. Anja Saalbach (University of Leipzig). All cells were tested free of mycoplasma infection prior to use. For co-culture, FB were seeded in flat-bottomed 96 well plates and rested overnight. DC were added to FB at a 4:1 ratio. Co-cultures were incubated for 24h in a humidified atmosphere of 5% CO_2 in air at 37^{o}C. Supernatants were collected and stored at -20^{o}C. All experimental conditions were performed in biological triplicates and on multiple donors. In some experiments the COX-2 inhibitor indomethacin (2μM) was added to FB prior to co-culture to determine the contribution of PGE_2 synthesis in the induction of IL-23. For assessment of cell-cell interaction Costar Transwell permeable support system was used with FB in the lower and DC in the upper chamber separated by 3μm pores. Primary FB were used up to and including the 4th passage after which they were discarded.

Irradiation of cells

Cells were irradiated in tissue culture plates immediately prior to DC activation with LPS (lipopolysaccharide) (500ng/mL) and IFNγ (1000U/mL). For experiments with indomethacin, DC were activated 3h before irradiation and adding to FB to minimize the effect of COX2 inhibitor on DC maturation. Cells were irradiated (0-6Gy of 195kVp X-rays, 0.87Gy/min, 0.5mm Cu filter, 48.4cm FSD) using a Gulmay Xstrahl cabinet irradiation system. Cell morphology was monitored by phase-contrast microscopy (x40) following IR and again after a further 24h of culture. Cell viability, apoptosis and necrosis were determined 24h after radiation by trypan blue exclusion and AnnexinV/PI FACS using

DMSO as a positive control (not shown). Flow cytometry was performed using Beckmann Coulter FC500 flow cytometer and analyzed with FlowJo software.

Measurement of secreted cytokine

The secretion of IL-23p40/p19 or IL-12p70 was determined by commercial human IL-23 Ready-set-go ELISA (eBioscience, San Diego, CA) and human IL-12p70 ELISA kits (BD Biosciences, Oxford, U.K.). Assays did not significantly react with other proteins, and the sensitivities were 15 and 7.8pg/mL respectively. IL-6 was measured by ELISA (ImmunoTools-Friesoythe, Germany) with a sensitivity of 9pg/mL. IL-1β, TNFα, IL-17, IL-27 and PGE_2 were measured with DuoSet assays (R&D Systems Europe, Oxford, U.K), and assay sensitivity was 3.9, 15.6, 7.8, 156 and 30.9pg/mL respectively. Absorbance was measured at 450nM using a spectrophotometer.

Generation of Th17 responses

DC-FB co-cultures were treated as previously described for 12h then washed with fresh medium to limit the impact of LPS/IFNγ on T-cells. We previously established that IL-23 is not produced by DC until >12h after TLR (Toll-Like Receptor) stimulation [35]. Human naïve $CD4^+$ T cells were obtained from fractionated fresh whole blood and naïve $CD4^+$ cells were obtained via a two-step isolation procedure using the Naïve $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Isolated T cells ($CD4^+CD45RA^+$, purity> 95%) were cultured in 48 well plates at $2x10^5$ cells per well. Allogeneic T cells were cultured in a 1:1 ratio of fresh medium and DC: FB co-culture supernatant for 5 days in the presence of anti-CD3 (OKT3; 1μg/ml) and anti-CD28 (5μg/ml) antibodies with IL-2 (50IU/ml). As controls for Th17 polarization and the

importance of IL-23, T cells were treated with recombinant IL-1β, IL-6 and IL-23. T cells were rested for 2 days and re-stimulated with anti-CD3/anti-CD28. For flow-cytometric assessment of Th17, cells were treated with Brefeldin A (BD Bioscience, Oxford, U.K.) for 20h, fixed with 0.5% formaldehyde, permeabilized (Perm Buffer, Biolegend, San Diego, CA) and incubated with mouse anti-human IL-17 FITC (eBioscience San Diego, CA). Flow cytometry was performed using Beckman Coulter FC500 flow cytometer and analyzed with FlowJo software. Supernatants from parallel cultures without Brefeldin A were harvested after 48h for ELISA.

Measurement of intracellular phospho-ATM

For measurement of intra-cellular phospho-ATM (pATM) co-cultures were separated using 0.3μm membrane Transwell plates (Corning, NY, USA). FB were seeded in the lower chamber and allowed to rest overnight before DC were added to the upper chambers. Cultures were irradiated and immediately activated with LPS/IFNγ. After 12h, DC were fixed in cold 2% formaldehyde/PBS and permeabilized with cold methanol. Binding of primary pATM (Ser1981) (eBioscience San Diego, CA) antibody was detected with a FITC-conjugated secondary Ab (Dako, Glostrup, Denmark). Cells were acquired using a MACSQuant cytometer and analyzed using FlowJo.

Quantitation of mRNA level for IL23A, IL12B with real-time RT-PCR

Real-time PCR was performed as previously described [33, 34]. In short cells were plated in DC medium, rested for 1h, irradiated and immediately stimulated with LPS/IFNγ. RNA was isolated (Nucleospin RNAII kit, Macherey-Nagel Düren, Germany) and cDNA prepared using GoScript Reverse Transcription system, Promega Madison, WI. Taqman

quantitative PCR was carried out for IL12B and IL23A with TOP1 as housekeeping gene (Applied Biosystems Paisley, UK, TOP1 Hs00243257_m1, Hs00168405_m1, *IL-12B/IL-12p40* Hs00233688_m1, *IL-23A/IL-23p19* Hs00372324_m1), with Mastermix (Primer Design Southampton, UK) on a Stratagene MX3005P and analyzed with Stratagene software. Quantification was done by δδCT method where δCT = (gene of interest CT) − (TOP1 CT), δδCT calculated with mDC at 0Gy as reference condition.

Statistical analysis

Results were statistically analyzed using student's t-test or 2-way ANOVA in GraphPad® Prism software and figures annotated as follows: *:0.05>p≥0.01, **:0.01p≥0.001, ***:0.001>p≥0.0001, ****:p<0.0001. Results are presented as mean ± standard deviation (SD).

Results

Irradiated FB recover IL-23 secretion from irradiated DC

The impact of stromal cells on the response of DC to ionizing radiation has not previously been studied. Therefore, we tested the hypothesis that DC-FB cross-talk overcomes the immune-inhibitory effect of IR. We recently established that exposure of DC to IR inhibits TLR4-dependent IL-23 secretion through the activation of ATM kinase [33]. In agreement with this, irradiation of DC significantly suppressed TLR-dependent IL-23 secretion. Radiation induced suppression of IL-23 was dose-dependent and maximal at 6Gy ($p<0.001$ Figure 1A) and occurred in all donors tested ($p<0.05$) (Figure 1B & C, Table 1).

Co-culture of FB with TLR4-activated DC (mDC) markedly increased the secretion of IL-23 ($p<0.05$, Figure 1D). Furthermore, we determined the ability of irradiated FB to sustain IL-23 responses from irradiated DC. Importantly, irradiated FB maintained their ability to support increased IL-23 secretion from irradiated DC ($p<0.01$) in all donors (Figure 1D). The levels of IL-23 secreted by irradiated mDC co-cultured with irradiated FB were greater than or equal to those obtained from non-irradiated mDC controls. It should be noted that whilst IL-23 secretion was increased in the presence of FB, the levels were nevertheless lower than those obtained from non-irradiated co-cultures. However, the fold increase of IL-23 secretion by co-cultures was similar regardless of irradiation (Figure 1E). The ability of FB to increase IL-23 secretion by irradiated DC was maintained regardless of whether the BJ6 cell line or primary dermal FB were used (Figure 1F). As expected, neither immature DC (iDC) nor FB secreted IL-23 irrespective of irradiation status or addition of FB (data not shown).

In the presence of IL-6 and IL-1β, IL-23 serves a key role in the polarization of human naïve $CD4^+$ T cells towards a Th17 phenotype [33, 36]. Therefore, we determined whether the protective effect conferred by FB on IL-23 production resulted in the induction of Th17

responses. Stimulation of naïve $CD4^+$ T cells through CD3/CD28 in the presence of conditioned supernatants from mDC cultures elicited Th17 responses as shown by secretion of IL-17A (Figure 1G). Furthermore, the addition of FB to DC enhanced IL-17 secretion by T cells in all donors tested irrespective of exposure to IR.

Irradiated DC maintain their ability to stimulate fibroblasts

To determine the mechanism by which FB increase IL-23 secretion in irradiated DC we tested each step in the DC-FB co-culture. Initially we determined if the secretion of TNFα and IL-1β were reduced by IR [2]. In contrast to IL-23, DC maintained their secretion of TNFα and IL-1β following exposure to IR (Table 1).

In view of the differential effect of IR on cytokine expression we assessed the secretion of other cytokines including IL-12, IL-27, IL-6 and IL-10 (Table 1). The inhibitory effect of IR was restricted to IL-12 and IL-23 which share common p40 subunit. IR strongly down-regulated IL-23 and only suppressed IL-12 to a modest extent. Interestingly another member of IL-12 family, IL-27, was unaffected. Production of the pro-inflammatory cytokine IL-6, as well as anti-inflammatory IL-10 were unaffected by IR. Next we determined whether IR affected transcription of the IL-23-specific *IL-23A* gene or the common *IL-12B* gene that encodes the p40 chain. Irradiation of DC (6Gy) significantly suppressed transcription of *IL-23A* when compared with un-irradiated DC ($p<0.0001$). Although the effect of IR on *IL-12B* was also inhibitory it was nevertheless less marked than on *IL-23A* (Figure 2A, B).

In contrast to many other cell types (e.g. monocytes), DC remain viable when irradiated [37]. This is because DC (but not classical monocytes) have constitutively active DNA-repair systems that repair double-strand DNA-breaks [38]. We therefore confirmed that

the suppression of IL-23 we observed in irradiated DC was not due to apoptosis or necrosis. Ionizing radiation did not impact on the viability of DC. Staining with AnnexinV/PI showed the viability of DC to be to be unaffected by IR (Figure 2C).

FB are functionally unimpaired by IR

Since irradiated DC retained their ability to secrete TNFα/IL1β we next investigated the effect of IR on the response of FB to these cytokines. Co-culture of irradiated FB with non-irradiated DC did not impair their capacity to promote IL-23 release from mDC (Figure 3A, B). Furthermore, irradiated FB retained their ability to respond to signals received from DC and secrete PGE_2 since stimulation of irradiated BJ6 and primary FB with exogenous TNFα/IL-1β elicited PGE_2 secretion which was unaffected by radiation (Figure 3C, D). As expected, exposure of resting FB to radiation did not elicit cytokine release. In the absence of DC, FB did not secrete IL-6, IL-12, IL-10, IL-17, IL-23 or IL-27 in response to activation with TNFα, IL-1β or LPS/IFNγ irrespective of exposure to IR (data not shown). Irradiation had no impact on FB viability as assessed by dye-exclusion (data not shown) and AnnexinV/PI staining (Figure 3E). It is important to stress that we examined the effects of IR on FB within the first 24h of exposure after which time activated DC would be expected to migrate to regional LN [39].

FB support function of irradiated DC through COX2-dependent PGE_2 release

Previously we established the importance of FB for IL-23 secretion by DC and demonstrated that this was mediated through COX2-dependent PGE_2 [3]. To assess whether this mechanism was involved in irradiated cells we first demonstrated the involvement of FB-derived soluble factors. FB were separated from DC by a 0.3μm

porous membranes in Transwell plates. Despite the lack of cell-cell contact, FB retained their capacity to support IL-23 secretion from irradiated DC (Figure 4A). Therefore, we evaluated the involvement of PGE_2 in up-regulation of IL-23 secretion by irradiated DC. The addition of PGE_2 to DC immediately after irradiation and activation resulted in a significant increase of IL-23 secretion in all donors tested (Figure 4B). Because PGE_2 secretion by FB is usually COX2 dependent [2, 37] we determined the importance of COX2 activation for FB-dependent recovery of IL-23 secretion using the COX2 inhibitor, indomethacin. IL-23 production from irradiated co-cultures was significantly reduced by indomethacin (Figure 4C). In the presence of indomethacin, IL-23 secretion from DC-FB co-cultures was reduced to levels similar to those achieved in the absence of FB.

FB promote irradiated DC IL-23 responses through the cAMP pathway

Irradiation of human DC mono-cultures selectively inhibits IL-23 by phosphorylation of the ATM kinase [33]. However PGE_2 also up-regulates IL-23 through activation of the cAMP-PKA (Protein Kinase-A) signaling pathway [40, 41]. Interestingly, in lung cancer cAMP signaling inhibits IR-induced phosphorylation of ATM [42]. Therefore, we sought to dissect the molecular mechanism responsible for IL-23 secretion in irradiated DC-FB co-cultures.

We initially assessed ATM activation [3] in irradiated DC mono-cultures or DC-FB co-cultures. DC-FB co-cultures were separated by 0.3μm porous membrane to ensure that phospho-ATM (pATM) levels were measured only in DC. As expected, irradiation of mDC resulted in ATM phosphorylation after 2h and this persisted for at least 12h as demonstrated by intracellular staining with flow cytometry (Figure 4D). However co-culture with FB did not suppress the levels of ATM expressed by irradiated DC suggesting that the support of DC function by FB occurred independently of ATM kinase. Lastly, we examined the involvement of the cAMP pathway in regulation of IL-23 from irradiated DC.

The addition of the cAMP active analogue Forskolin to irradiated mono-cultures of mDC resulted in a dose-dependent increase in IL-23 ($p<0.01$) (Figure 4E), implicating a role for the cAMP signal transduction pathway.

Discussion

Studies on the impact of the stromal microenvironment on immunity are important as immune cells are in constant cross-talk with their stroma during each and every stage of the immune response. Stromal cells have the ability to affect a wide range of immune functions including DC maturation, their migration to lymph nodes, and subsequent polarization of T cell responses [1, 2, 12, 43]. The present study investigated the ability of stroma to modulate the outcome of therapeutic interventions directed towards immune system. Our previous work established that IR inhibits the cytokine response of DC and in particular IL-23 [33]. On the other hand, stromal FB support IL-23 production by activated DC [2]. Therefore, we addressed the hypothesis that FB, cells which are known to be relatively radio-resistant [44], continue to support the function of DC in the presence of IR.

The doses of IR used in the current study are similar to those employed during routine radiotherapy of common malignancies [26, 29]. At these doses IR damages transformed cells and initiates their demise by generating free radicals which induce stress responses and consequently cell death if the damage is not repaired [29, 38, 45, 46]. Due to their high proliferative rates and impaired DNA repair mechanisms many tumor types are selectively sensitive to radiation-induced DNA damage [30, 47]. However, whilst tumor cells are the main target for radiotherapy, immune and stromal cells residing in the tumor microenvironment are also exposed to and affected by IR [48]. In this regard, previous work using mono-cultures has shown that IR suppresses IL-12 secretion by moDC between 2 and 20Gy [31] while another report showed increased IL-12 secretion by murine DC at 0.05Gy and this effect was reversed to the level of non-irradiated DC at 1Gy [49]. The disparity between these findings may be caused by differences in the behavior of human and murine DC however they may also reflect different mechanisms of action for

IR at extremely low doses (0.05-1Gy) comparing to higher doses used for radiotherapy (2-6Gy)[26, 50].

We found that the inhibitory effect of IR was restricted to IL-12 family members sharing the common p40 subunit. However, the effect was considerably more pronounced for IL-23 than IL-12p70. Interestingly the maximal effect of IR was exerted at 6Gy with a plateau of effect at higher doses. This may be due to ATM reaching its maximum activation state at this dose. On the other hand, the effect of IR did not extend to the other IL-12 family member, IL-27. IL-27 comprises the IL-27p28 subunit and Epstein–Barr virus-induced gene 3 (EBI3) which are related to p35 and p40 respectively [51]. Those findings demonstrate the highly selective effect of IR on DC functions with prevalence to inhibit Th17 responses. The lack of effect of IR on TNFα and IL-1β had important consequences for our multicellular model as it allowed activated DC to sustain their interactions with FB and generate the PGE_2 feedback loop [3]. Irradiated FB also continued to support the production of IL-23 by DC irrespective of the dose of IR to which FB were exposed. This was observed not only with a FB cell line, but also with primary dermal FB thus supporting the potential physiological importance of this observation.

Fibroblasts (resting, TLR-activated, or irradiated) were not responsible for the secretion of any of the cytokines affected by IR. Previous reports describe FB expression of the IL23p19 subunit upon stimulation with IL-1β [52] but to the best of our knowledge there are no reports of IL-23 heterodimer secretion [53]. It is important to recognize the difference between biologically active IL-23 heterodimer secreted by APC, and IL-23p19 monomer and in the current study we measured heterodimeric IL-23 secretion [53].

It is known that FB can enter a senescent phase when exposed to IR yet remain viable [18, 44, 54]. This represents one of the important mechanisms of tumor development as in this state senescent FB alter their phenotype and promote tumor growth, invasion and

render adjacent tumor cells increasingly radio-resistant [44]. Therefore, we assessed the effect of IR on FB in functional assays. PGE_2 stimulates IL-23 secretion in human and murine DC [2, 41, 55, 56]. Previous reports have published conflicting data in regard to regulation of PGE_2 secretion by gingival FB irradiated with low-level diode laser [57, 58]. In our model, irradiation of dermal FB did not alter PGE_2 secretion in response to exogenous TNFα and IL-1β, and this likely accounts for their continued capacity to augment IL-23 release by non-irradiated DC. Therefore, irradiated FB were unimpaired in their ability to secrete soluble immune modulators and maintained their capacity to respond to environmental stimuli.

According to recent reports PGE_2 stimulates IL-23 secretion from DC through activation of the cAMP/PKA pathway [40, 41] and we previously described that IR down-regulates IL-23 through phosphorylation of ATM kinase [33]. Interestingly, cAMP signaling inhibits radiation induced phosphorylation of ATM in lung cancer [42]. Addition of forskolin increased IL-23 secretion by irradiated DC to levels commensurate with those from irradiated DC-FB co-cultures, suggesting a role for cAMP. However, in contrast to Cho and colleagues, co-culture of irradiated DC with FB did not affect pATM levels despite up-regulated IL-23. This disparity may be due to differences in timing of cAMP and ATM activation. Cho and co-workers activated cAMP before irradiation of cells thus preventing phosphorylation of ATM by activated PP2A (Protein phosphatase 2A). In our study DC were irradiated before activation and subsequent stimulation from FB. Since ATM is phosphorylated in DC within 15min of IR [33], signals from FB are received by DC too late to inhibit ATM activation. It is therefore even more notable that up-regulation of IL-23 secretion by FB occurred despite ATM activation.

The impact of FB on the ability of irradiated DC to promote Th17 responses was examined. Previously we and others established the role of IL-23 in the generation of

Th17 [2, 33, 56]. In agreement with this, IL-17 secretion from T-cells conditioned with supernatants from DC-FB co-cultures was enhanced as compared to supernatants from DC mono-culture. PGE_2 was shown to promote Th17 directly and in conjunction with IL-23 [59, 60]. In our model the concentration of PGE_2 secreted by FB was unaffected by IR. On the other hand, changes in IL-17 reflected differences in IL-23 levels, demonstrating the biological importance of FB-dependent IL-23 secretion in regulation of adaptive immunity.

In summary we show that although IR inhibits IL-23 secretion from DC mono-cultures, the inclusion of FB provides a positive feedback loop that serves to maintain IL-23 secretion by DC. We propose that this translates to enhanced Th17 responses in a post-RT environment (Fig 5) and studies to investigate this are underway. Since IL-17 is a critical factor driving post-radiation fibrosis [24] this work identifies a potential mechanism for the pathological consequences of radiation and chemotherapy [25, 61]. Importantly it highlights the need for multicellular models of the immune microenvironment. Insight into the complex interactions between the immune system and stroma is necessary to our understanding of pathology and for development of novel therapeutic interventions.

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Authorship

AM undertook or supervised all experimental work, interpreted the data and wrote the manuscript.

QW, SS, RVS and HAF undertook specific components of experimental work.

AS generated and characterized primary dermal fibroblasts and wrote the manuscript.

IS, JMR, JG and MJG undertook statistical analysis and wrote the manuscript.

MJG provided advice on radiation responses.

PMP conceived the original project idea and supervised the research.

AMJ conceived the original project idea, supervised the research and wrote the manuscript.

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Conflict of interests disclosure

The authors declare no conflict of interest.

Figure legends

Figure 1. FB promote IL23-Th17 axis during irradiation. IR suppresses IL-23**.** DC were irradiated prior to addition of LPS/IFNγ and IL-23 release measured by ELISA; **A** – IL-23 suppression was dependent on the dose of IR (a representative donor from 3). **B**- shows a representative experiment at 6Gy and **C** - shows a summary of 18 donors. **D -** FB rescue IL-23 secretion by irradiated DC. Co-culture of irradiated FB with irradiated DC up-regulated IL-23 secretion (summary of 8 donors). **E** - shows fold change of IL-23 secretion by irradiated DC/FB co-cultures at 0 and 6Gy. **F** - Primary dermal FB demonstrate similar IL-23-enhancing activity to the BJ6 cell line (data from a representative donor or 5). **G** - FB permit irradiated DC to promote Th17 responses. IL-17A secretion by naïve $CD4^+$ T cells activated with anti-CD3/anti-CD28 in the presence of supernatants of the indicated DC/FB cultures. T-cells were stimulated for 5 days and IL-17A secretion determined after re-stimulation (collective data from 3 donors). Error bars indicate standard deviation (SD) of triplicate experiments.

Table 1. Effect of IR on cytokine secretion by mDC. DC were irradiated (6Gy) or non-irradiated before activation with LPS/IFNγ. Supernatants were collected following 24h incubation and secreted IL-23, IL-12, IL-27, IL-1β, TNFα, IL-6 and IL-10 were measured by ELISA. All experiments were repeated at least 4 times in triplicate and significance was assessed with 2-way ANOVA.

Figure 2. Effect of IR on DC. Changes in IL-23 secretion were associated with decreased transcription of the; **A** - *IL-23A* gene and **B** - *IL-12B* gene as shown using Q-RT-PCR in 3 donors. Statistical significance was measured by the student *t*-test. **C** – Despite exposure

to up to 6Gy IR, there was no decrease in viability of DC as assayed by AnnexinV /PI staining with flow-cytometry up to 72h after irradiation. Representative result for 1 of 3 donors tested.

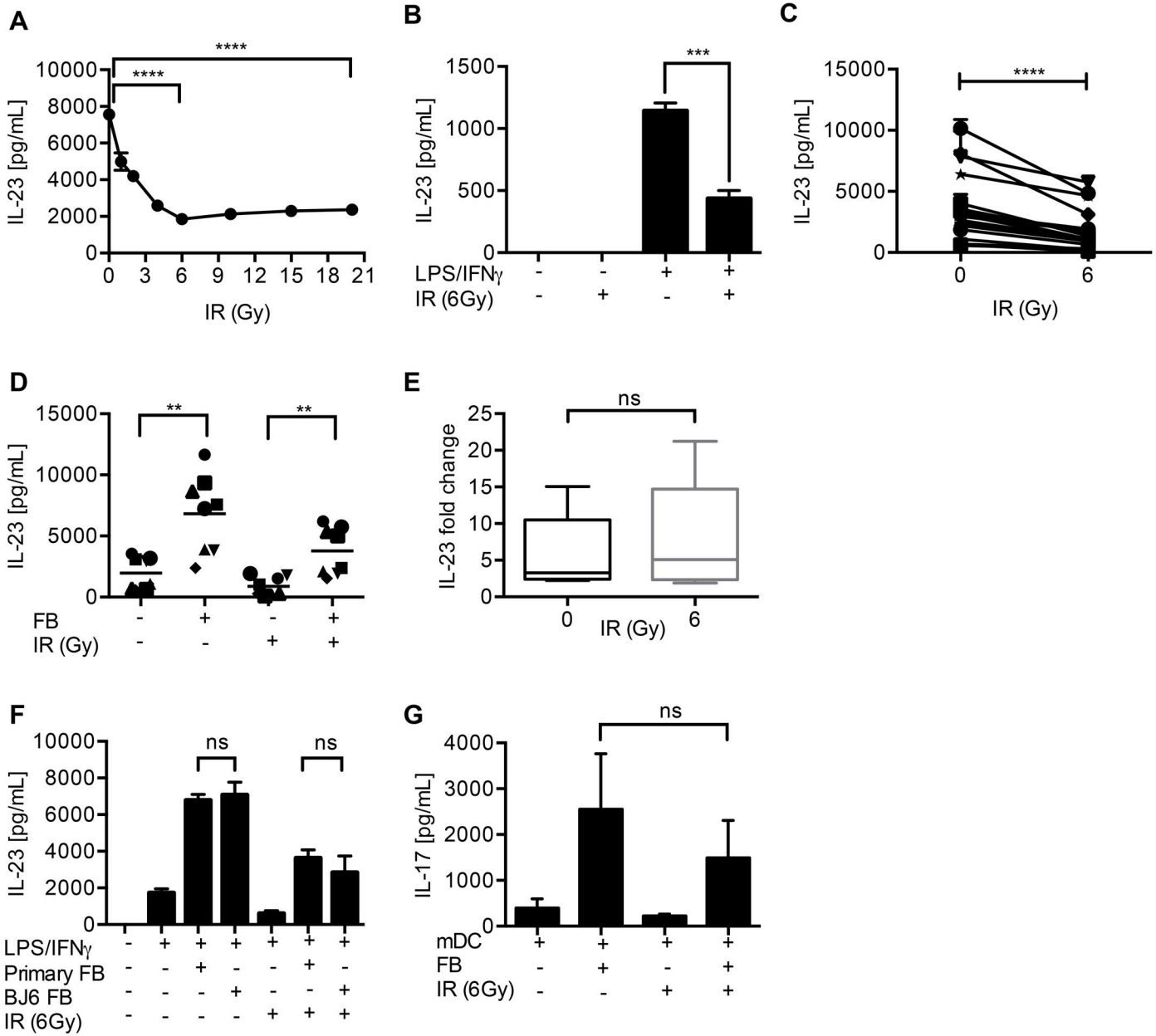
Figure 3. FB are functionally resistant to IR. Irradiation of FB did not impair their ability to up-regulate IL-23 secretion from non-irradiated DC; **A** - shows a representative donor, **B** - summary of 3 donors. **C** - PGE_2 secretion (determined by ELISA) by FB stimulated with recombinant TNFα and IL1β (1ng/mL) is unaffected by IR; C - BJ6 cell line and **D**-representative results for 1 of 2 primary FB donors. **E** - IR (6Gy) does not affect FB viability up to 24h after irradiation as assessed by AnnexinV/PI staining; representative data from 3 experiments.

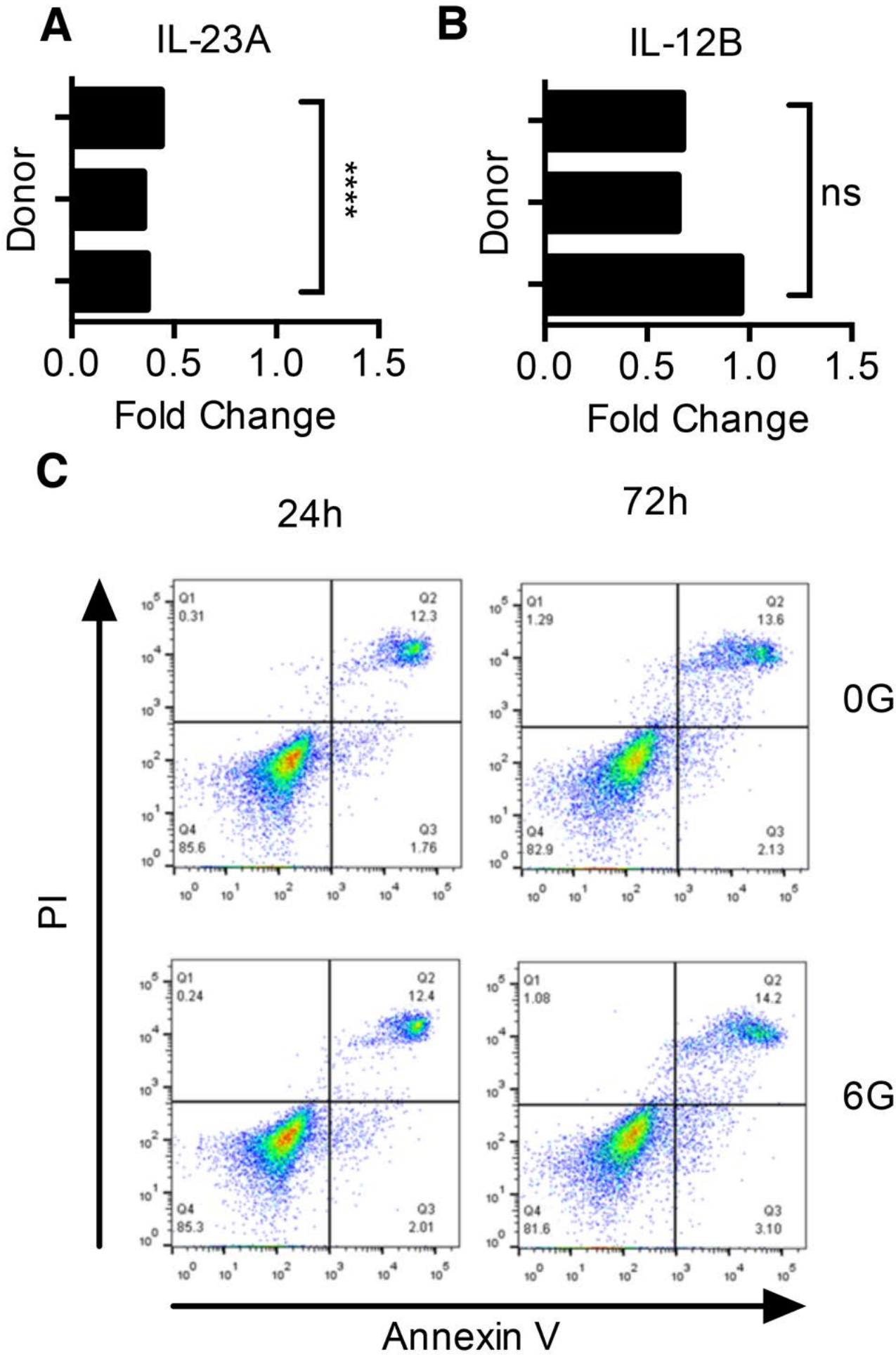
Figure 4. Mechanisms of IL-23 recovery from irradiated DC by FB. FB-dependent IL-23 secretion from irradiated DC is mediated by soluble factors and was independent of cell-cell contact. **A** - Shows fold increase in IL-23 secretion by DC separated from FB with Transwell (0.3μm) treated with IR (summary of 3 donors). **B** - Addition of PGE_2 to irradiated DC up-regulates IL-23 secretion **(**summary of 5 donors). **C** - Up-regulation of IL-23 secretion from irradiated DC by FB is COX2 dependent. FB were treated with Indomethacin for 24h prior to (and throughout) co-culture with irradiated DC **(**data from 3 experiments). **D** - FB did not affect ATM phosphorylation in irradiated DC. Irradiated mDC cultured with FB (blue line) or without FB (orange line) show similar levels of ATM phosphorylation. In both these settings, IR increased ATM activation when compared with non-irradiated mDC (red line) as demonstrated by intracellular staining for flow-cytometry at 2 and 12 h after IR (6Gy, representative donor of 3). **E** - Stimulation of TLR4-activated

DC with the c-AMP agonist forskolin immediately following exposure to IR increased IL-23 secretion (data from 3 representative donors.)

Figure 5. Model of DC-FB crosstalk governing IL-23 dependent Th17 responses after irradiation.

A: TLR-activation of DC in mono-culture elicits secretion of IL-1β, -6 and -23 which prime Th17 responses. **B**: The addition of FB to DC provides an important feedback loop that serves to enhance IL-23 secretion and thus augments Th17 responses. **C**: Irradiation of DC mono-cultures selectively inhibits IL-23 secretion. **D**: However, the presence of FB ensures that irradiated DC continue to secrete sufficient IL-23 to generate Th17 responses. Importantly, the irradiation of FB does not hinder their reinforcement of IL-23 responses.


A
B
C
D
E
F
G
IL-23 [pg/mL]
IR (Gy)
LPS/IFNγ
IR (6Gy)
FB
IL-23 fold change
IL-17 [pg/mL]
Primary FB
BJ6 FB
mDC
ns


A
IL-23A
Donor
****
0.0
0.5
1.0
1.5
Fold Change
B
IL-12B
Donor
ns
0.0
0.5
1.0
1.5
Fold Change
C
24h
72h
0Gy
6Gy
PI
Annexin V
Q1
0.31
Q2
12.3
Q4
85.6
Q3
1.76
Q1
1.29
Q2
13.6
Q4
82.9
Q3
2.13
Q1
0.24
Q2
12.4
Q4
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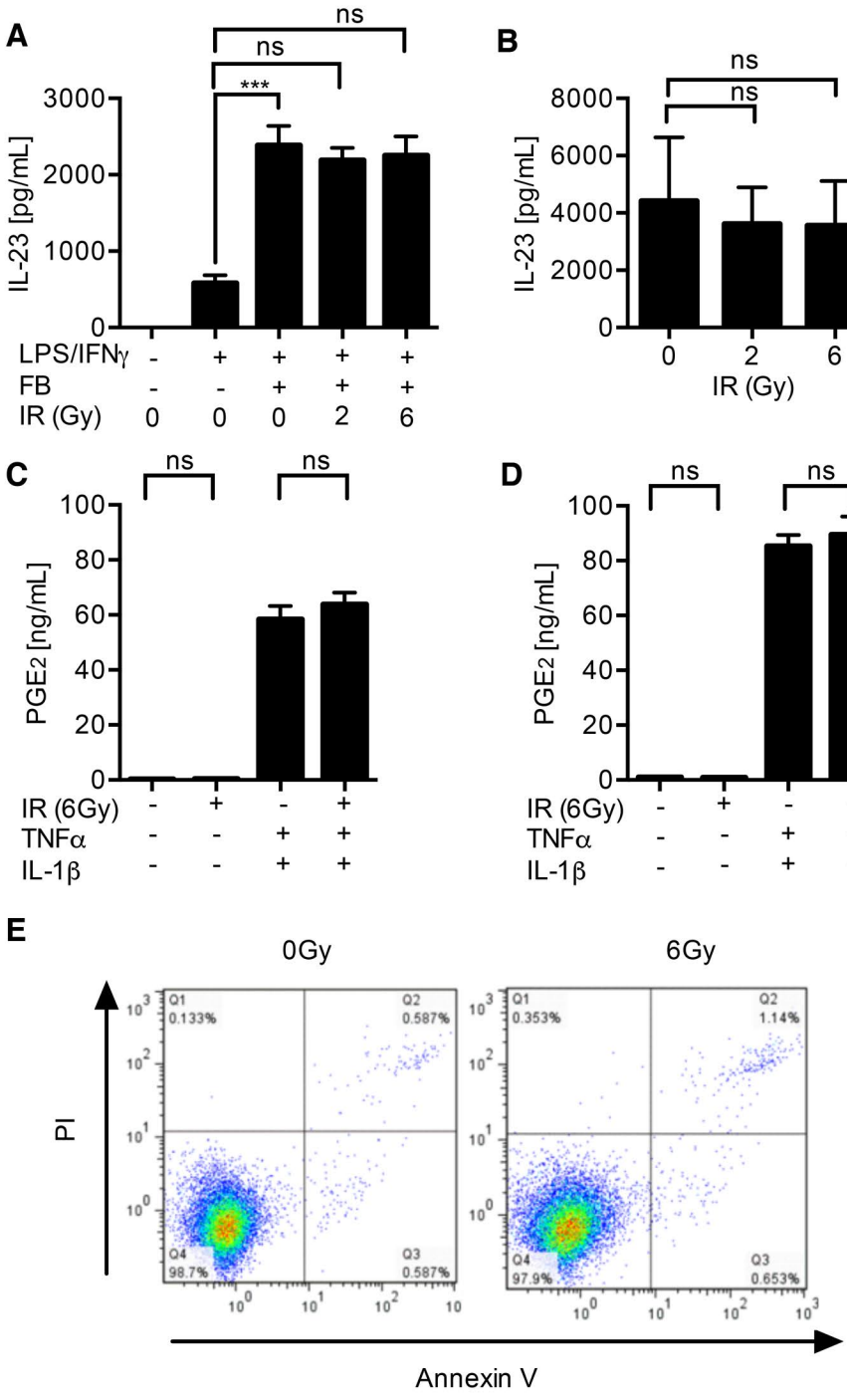
Q3
2.01
Q1
1.08
Q2
14.2
Q4
81.6
Q3
3.10


A
ns
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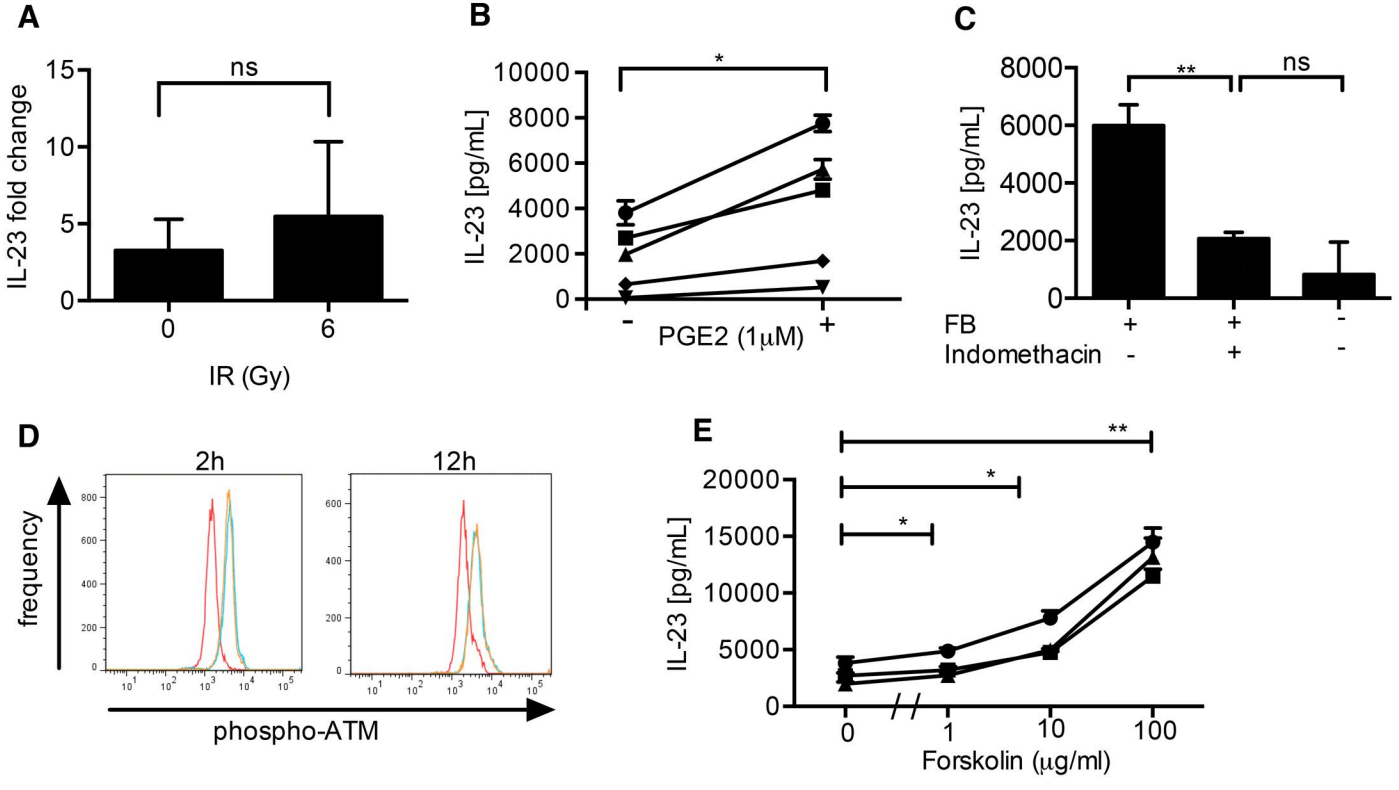
3000
2000
1000
0
IL-23 [pg/mL]
LPS/IFNγ - + + + +
FB - - + + +
IR (Gy) 0 0 0 2 6
B
ns
ns
8000
6000
4000
2000
0
IL-23 [pg/mL]
0 2 6
IR (Gy)
C
ns
ns
100
80
60
40
20
0
PGE2 [ng/mL]
IR (6Gy) - + - +
TNFα - - + +
IL-1β - - + +
D
ns
ns
100
80
60
40
20
0
PGE2 [ng/mL]
IR (6Gy) - + - +
TNFα - - + +
IL-1β - - + +
E
0Gy
6Gy
Q1 0.133%
Q2 0.587%
Q4 98.7%
Q3 0.587%
Q1 0.353%
Q2 1.14%
Q4 97.9%
Q3 0.653%
PI
Annexin V


A
IL-23 fold change
15
10
5
0
ns
0
6
IR (Gy)
B
IL-23 [pg/mL]
10000
8000
6000
4000
2000
0
*
-
PGE2 (1μM)
+
C
IL-23 [pg/mL]
8000
6000
4000
2000
0
**
ns
FB
Indomethacin
D
2h
12h
frequency
phospho-ATM
E
IL-23 [pg/mL]
20000
15000
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Forskolin (μg/ml)

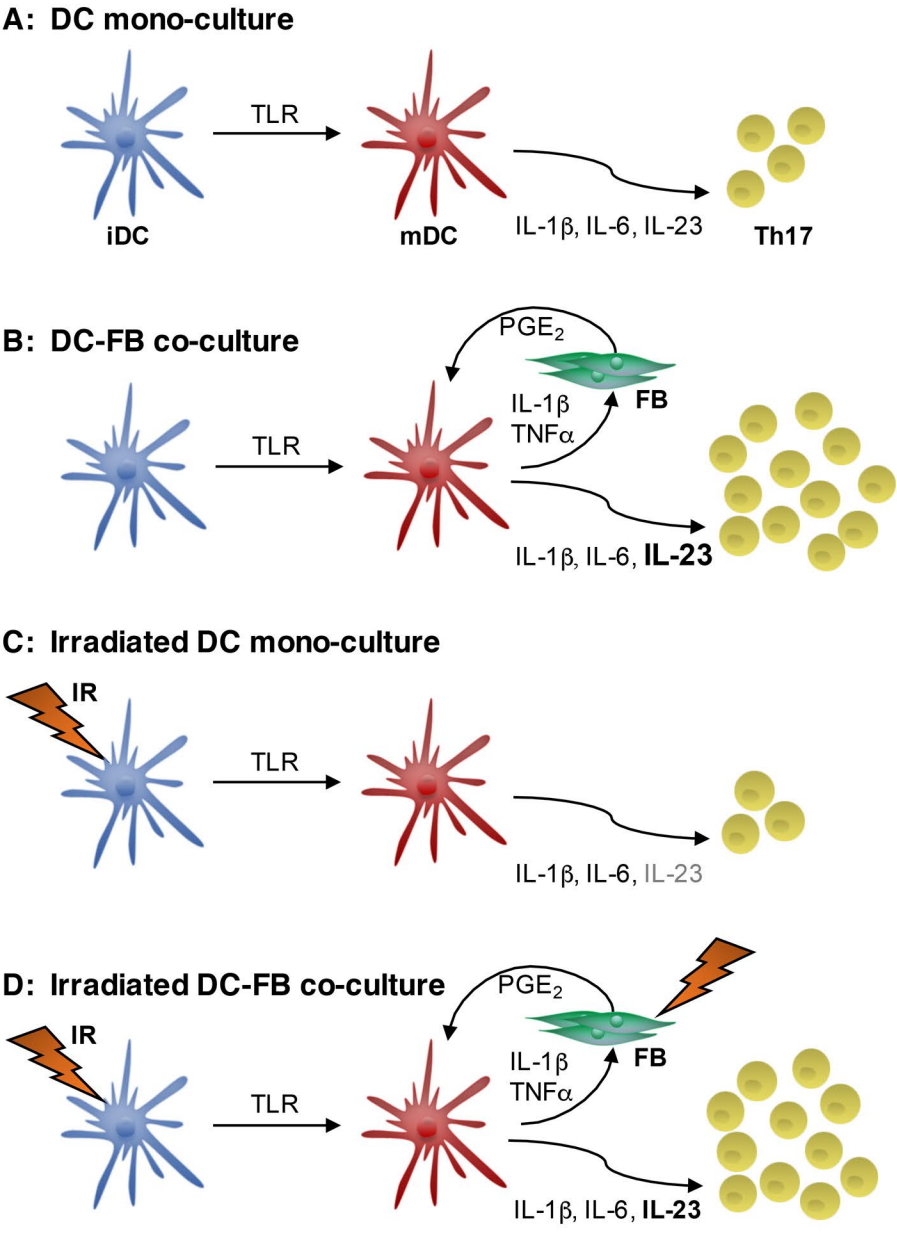
A: DC mono-culture
TLR
iDC
mDC
IL-1β, IL-6, IL-23
Th17
B: DC-FB co-culture
PGE2
FB
IL-1β
TNFα
TLR
IL-1β, IL-6, IL-23
C: Irradiated DC mono-culture
IR
TLR
IL-1β, IL-6, IL-23
D: Irradiated DC-FB co-culture
PGE2
IR
FB
IL-1β
TNFα
TLR
IL-1β, IL-6, IL-23

Table 1.

Cytokine	% remaining secretion	SD	P value
IL-23	43	11	<0.0001
IL-12	89	16.3	0.0005
IL-27	100.9	36.6	ns
IL-1β	93.5	34.4	ns
TNFα	111.8	29.6	ns
IL-6	174.1	114.4	ns
IL-10	95.9	6.2	ns