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Antitumor cytotoxicity induced by bone-marrow-derived antigen-presenting cells is facilitated by the tumor suppressor protein p53 via regulation of IL-12

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ABSTRACT
Activated antigen-presenting cells (APC) deliver the three signals cytotoxic T cells require to differentiate into effector cells that destroy the tumor. These comprise antigen, co-stimulatory signals and cytokines. Once these cells have carried out their function, they apoptose. We hypothesized that the tumor suppressor protein, p53, played an important role in generating the antitumor response facilitated by APC. CD11c+ APC derived from p53 wild-type (wt) mouse (wt p53) GM-CSF bone marrow cultures (BMAPC) and activated had reduced survival compared to BMAPC from p53 null consistent with p53-mediated apoptosis following activation. There was a lower percentage of antigenic peptide/MHC I complexes on antigen-pulsed p53 null cells suggesting p53 played a role in antigen processing but there was no difference in antigen-specific T cell proliferative responses to these cells in vivo. In contrast, antigen-specific cytotoxicity in vivo was markedly reduced in response to p53 null BMAPC. When these cells were pulsed with a model tumor antigen and delivered as a prophylactic vaccination, they provided no protection against melanoma cell growth whereas wt BMAPC were very effective. This suggested that p53 might regulate the requisite third signal and, indeed, we found that p53 null BMAPC produced less IL-12 than wt p53 BMAPC and that p53 bound to the promoter region of IL-12. This work suggests that p53 in activated BMAPC is associated with the generation of IL-12 required for the differentiation of cytotoxic immune responses and an effective antitumor response. This is a completely new role for this protein that has implications for BMAPC-mediated immunotherapy.

Keywords: Cytotoxic immune response; dendritic cells; interleukin 12; p53 tumor suppressor protein; tumor vaccination

Introduction
When APC acquire antigen in the context of an exogenous or an endogenous danger signal, co-stimulatory molecules are upregulated and MHC/peptide complexes interact with complementary receptors on T cells leading to the production of a third signal, cytokine secretion. This drives T cell activation and differentiation in the secondary lymphoid tissue. Dendritic cells (DC), the most potent APC, are known to die by apoptosis within days following this, presumably preventing an ongoing, energy-expensive and potentially detrimental immune response (reviewed in1). A range of consequences may arise if this does not occur (reviewed in2) but to our knowledge the role of the tumor suppressor protein p53 in this process has not been investigated. This is an important area because the tumor suppressor p53 is a master regulator of cellular life and death decisions.

The p53 protein is thought to protect the genome in damaged cells by temporally or permanently arresting cell growth, facilitating DNA repair and inducing apoptosis (reviewed in3). Mutant p53 or p53 deficiency results in the abrogation of apoptosis, an important contributing factor to tumorigenesis.5,6 Although p53 mutations are associated with more than 50% of cancers (National Cancer Institute, http://seer.cancer.gov/(2010)), the effect of p53 dysfunction on DC and its potential effects on the initiation of an immune response to a developing tumor are largely unknown. Earlier work by our group and others established that splenic and thymic cells from p53-deficient mice are defective in their capacity for apoptosis following exposure to DNA-damaging gamma irradiation.7,9 Since these tissues are major repositories of APC, this suggests that p53 may be an important determinant in APC survival and, as a consequence, may influence their function in tumor surveillance.

DC generated in vitro and pulsed with tumor antigen have been used in prophylactic and therapeutic vaccinations that have shown some promise in delaying tumor growth, with one already licensed for use in the clinic (reviewed in10). Indeed, there have been various trials in which DC pulsed with p53 peptides have been tested as potential vaccines against tumors.11,12 The survival of the DC comprising these vaccines may play a role in their ability to protect...
against tumors, so we have used this model to investigate the consequences of p53 deficiency.

Recent work suggests that a major conventional method for generating DC (defined principally by expression of the integrin CD11c as well as MHC and co-stimulatory molecules) using mouse bone marrow cultured with GM-CSF actually results in a heterogeneous mixture mainly comprising DC and macrophages. Since we used this in vitro methodology in our work, we shall now term these cells simply bone-marrow-derived antigen-presenting cells (BMAPC). Here, we show for the first time that CD11c+ BMAPC express p53 when they are activated by microbial danger signals that ligate toll-like receptors (TLR). Their subsequent apoptosis is p53 dependent since, in the absence of p53, they survive considerably longer than BMAPC expressing p53. This deficiency has no distinguishable effect on their phenotype and although antigenic peptide loading onto MHC by p53 null BMAPC is reduced, antigen-specific T cell proliferation is not affected. However, we show that BMAPC require p53 to generate tumor-specific cytotoxic responses necessary to provide effective prophyllactic immunization against a tumor. Our findings suggest that this is because activated wt p53 BMAPC normally produce IL-12 whereas the production of this cytokine by p53 null BMAPC is much lower. Delayed transcription of IL-12 in p53 null BMAPC and a chromatin immunoprecipitation assay (ChIP) showing that p53 binds to the IL-12 promoter implicate p53 in the regulation of IL-12 production, the necessary third signal.

Results

**Stressed and activated BMAPC express p53 leading to apoptotic death**

Immature BMAPC, identified on the basis of CD11c and low expression of the co-stimulatory markers CD40 and CD86, were used to investigate the effect of p53 on BMAPC following an activation signal known to upregulate CD40 and CD86 required for the delivery of the second signal for T cell activation. Immature BMAPC exposed to lipopolysaccharide (LPS), a ligand for the pathogen recognition receptor toll-like receptor 4 (TLR4), became activated (Fig. S1, Fig. 1A), and upregulated CD40 and CD86. Using intra-nuclear staining of BMAPC, we were able to visualize a corresponding increase in intra-nuclear p53 expression in CD11c+ LPS-treated cells compared with untreated CD11c+ cells (Fig. 1B) both in terms of the percentage of cells positive for p53 and the degree of expression (LPS MFI 2589 ± SEM 22.85, n = 6 vs. untreated MFI 2059 ± SEM 27.55, n = 6). Replicate Western blots also demonstrated the presence of small amounts p53 in activated BMAPC. These data suggested that p53 might be implicated in apoptotic death known to follow BMAPC activation (Fig. 1C).

**p53 influences BMAPC survival in vitro and antigen processing but does not affect post-activation phenotype**

We found that the yield of bone marrow cells from p53 null mice was higher than that from wt p53 mice and a higher percentage differentiated into BMAPC after culture in GM-CSF (Fig. 2A). A median of 68% of bone marrow cells seeded from p53 null donors generated BMAPC compared with 54% of those from wt p53 donors (Fig. 2A). This suggested that less apoptosis was occurring in the bone marrow and that increased numbers of BMAPC precursor cells were surviving in culture. To investigate p53-mediated apoptosis further, BMAPC from p53 null and wt p53 donors were activated with LPS or CpG, a TLR9 ligand, and apoptotic death was assayed by caspase activation 4 d later. A marked trend toward lower apoptosis and increased survival was observed in p53 null BMAPC: only 12.2% of CpG-activated BMAPC from these donors had succumbed to apoptosis compared with 68.8% from wt p53 mice (exemplified in Fig. 2B flow cytometric histogram) at 96 h post stimulation. A similar result was recorded using LPS-activated BMAPC (8.24% ± SD 4.5, n = 3 vs. 36.0% ± SD 8.0, n = 3) (Fig. 2B bar graph). It seemed possible that p53 deficiency may affect the capacity of these BMAPC to respond to activation signals by upregulating cell surface co-stimulatory molecules. To test this, p53 null and wt p53 BMAPC were activated with LPS or CpG and phenotyped for MHCII, CD80, CD86 and CD40. There were no distinguishable differences in expression of these, either in the percentage of cells expressing the marker or in the degree of expression (Fig. 2C). This does not necessarily mean that antigen will be presented with similar efficacy because the capacity to process antigen for loading onto MHC molecules influences the magnitude of T cell response. Therefore, BMAPC were pulsed with ovalbumin protein, conjugated to virus like particles (VLP/ova), that are known to be processed by BMAPC, to cross present the MHCII peptide SIINFEKL on CD80. Subsequent staining with antibody to detect MHCII/SIINFEKL peptide complexes showed that there were fewer detectable MHCII/peptide complexes on p53 null than on wt p53 BMAPC (p53 null 4.26% ± SEM 0.35 n = 3; wt 7.51% ± SEM 0.058 n = 3) (Fig. 2D; gating procedure Fig. S2). Thus, these in vitro studies suggested that although p53 expressed in activated BMAPC did not affect the cell phenotype, it did appear to influence the processing of antigen and was an important factor in cell survival.

**Survival of BMAPC in vivo is p53 dependent**

The relatively short survival of activated DC in vivo is important for limiting the duration of the immune response. To confirm that the p53-dependent survival we observed in vitro was reflected in vivo, activated BMAPC from both donor strains were injected intra-dermally into p53 wt C57BL/6 mice. Skin harvested from this site and stained for apoptotic donor BMAPC, showed that lower numbers of caspase 3 stained p53 null BMAPC cells were observed compared with wt BMAPC (Fig. 3A). Donor BMAPC were easily distinguishable from recipient cells in the surrounding tissue on the basis of their large nuclei (assessed by an anatomical pathologist). This suggested that larger numbers of p53 null BMAPC might be available to migrate to the draining lymph node. Migration requires the upregulation of the chemokine receptor CCR7 on mature DC to ligate CCL19 and CCL21 to form the chemokine gradient guiding mature DC. Analysis of the expression of this marker showed that BMAPC from wt donors...
discernibly upregulated CCR7 following activation with CpG (Fig. 3B) but this did not occur in p53 null BMAPC that appeared to have upregulated this marker without activation. Nonetheless, these data do show that BMAPC from both donors expressed CCR7 suggesting that both were capable of trafficking to the draining nodes (Fig. 3B). To compare migration in vivo, both sources of BMAPC, stained with different concentrations of CFSE, were co-administered intra-dermally and the draining nodes were subsequently harvested at various intervals. Individual draining lymph nodes were analyzed for the presence of BMAPC from both donors (a representative FACS profile of a single node is shown in Fig. S3). At 8 h post administration, substantially larger numbers of p53 null cells were detected (75%± SD 14.6, n = 3) compared with co-administered wt p53 BMAPC (30% ± SD 14.5%, n = 3) (Fig. 3C). This is probably attributable to the greater number of viable BMAPC at the injection site available to traffic to the draining lymph nodes rather than faster trafficking. This differential was maintained for 72 h (Fig. 3C). This prolonged survival of BMAPC in the absence of p53 seemed likely to influence the initiation of the immune response in vivo.

**Antigen-specific CD8⁺ T cell proliferation in vivo in response to activated, antigen-pulsed BMAPC is p53 independent but induction of cytotoxic effector cells is strongly influenced by p53 expression**

DC pulsed with model tumor antigen and used as experimental vaccines generate potent cell-mediated responses capable of
Figure 2 For figure legend, see page 5.
preventing the growth of subsequently implanted tumors. An initial step in this process is the induction of T cell proliferation.

To investigate the potential differential capacity of p53 null vs. wt BMAPC to activate T cell proliferation, CFSE-labeled OTI CD8+ T cells were adoptively transferred into wt recipients. A day later, p53 null or wt p53 BMAPC, pulsed with SIINFEKL peptide or left untreated, and were administered intra-derrmally into the right and left flanks, respectively. CFSE labeled cells were retrieved from the draining nodes 72 h later. In this assay, flow cytometric analysis is used to detect progressive dilution of the dye with each round of proliferation, indicated by serial peaks. When the harvested cells were analyzed, no difference in the percentage of proliferating cells in the respective nodes could be distinguished (Fig. 4A) although there were variations in the degree of proliferation occurring in individual mice. This occurred irrespective of the antigen used to pulse the BMAPC i.e. ovalbumin protein or derivative MHCI SIINFEKL peptide. Thus, a p53 null, antigen-pulsed BMDC ‘vaccine’ was as proficient as the wt counterpart in the generation of antigen-specific T cell proliferation.

Because the induction of a proliferative response does not necessarily lead to the generation of cytotoxic effector cells, the induction of cytotoxicity by antigen-pulsed p53 null vs. wt BMAPC was compared using an in vivo assay. Mice were vaccinated with either p53 deficient or wt BMAPC pulsed with either SIINFEKL (BMAPC/SIINFEKL) or OVA (BMAPC/OVA) and a week later injected with a 1:1 ratio mixture of SIINFEKL-pulsed and unpulsed splenocytes as target cells. When recipient blood and spleen were harvested from the vaccinated mice 48 h later, the percentage of specific lysis of SIINFEKL-pulsed target cells generated by immunization with antigen-pulsed p53 null BMAPC (assessed by flow cytometry with populations gated as shown in Fig. S4) was found to be dramatically lower than with wt BMAPC (Fig. 4B). Mice immunized with p53 null BMAPC/SIINFEKL vaccine induced 34% cytotoxicity in the spleen and 14% in the blood compared with wt p53 BMAPC/SIINFEKL where 84% cytotoxicity was induced in the spleen and 93% in the blood. BMAPC/OVA from p53 deficient mice induced 3% cytotoxicity in the spleen but no detectable killing was detected in the blood. By comparison, wt BMAPC/OVA induced 44% cytotoxicity in the spleen and 51% in blood. This strongly suggested that p53 expression by BMAPC influenced signals important in the generation of the cytotoxic cell differentiation pathway.

Protection against tumor growth by activated, antigen-pulsed BMAPC vaccination is p53 dependent

Since the presence of p53 influenced the ability of BMAPC to induce in vivo cytotoxicity, the next step was to investigate the efficacy of prophylactic antitumor vaccination with BMAPC in the presence and absence of p53 (Fig. 5). As expected, vaccination with CpG activated wt p53 BMAPC/OVA or wt p53 BMAPC/SIINFEKL prior to the administration of B16/OVA melanoma cells resulted in greatly enhanced survival of the recipients compared with the unvaccinated control animals 60 d post tumor cell administration (80% and 70% survival respectively compared with the wt control group where none survived beyond 40 d). However, when mice were vaccinated with CpG-activated p53 null BMAPC/SIINFEKL and implanted with B16. OVA melanoma cells, only 40% survived at 60 d post tumor implantation (median survival 25 d, $p = 0.045$ in comparison with CpG activated wt p53 BMAPC/SIINFEKL; Fig. 5). Even more markedly, and possibly attributable to a defect in the processing of OVA protein observed in earlier in vitro experiments (Fig. 1e), vaccination with p53 null BMAPC/OVA DC was completely unable to protect against tumor growth; the survival of these animals was no different from untreated controls (mean survival 22 d, $p = 0.0012$, in comparison with CpG activated wt p53 BMAPC/OVA and Fig. 5). The apparent necessity for p53 expression in BMAPC vaccines suggested that this might play a role in the control of another signal produced by BMAPC.

IL-12p70 production is higher and IL-12p40 transcription occurs earlier in activated p53 wild type compared with p53 deficient BMAPC

Fully activated DC produce IL-12 regarded as a third signal necessary for cytotoxic T cell activation. Recent work has shown that the cis-imidazoline compound nutlin that blocks MDM2-mediated inhibition of p53, results in an increase in IL-12p40 in macrophages suggesting that IL-12p40 production is regulated by p53. It seemed possible that IL-12 production by BMAPC after activation, necessary to drive the differentiation of cytotoxic effector cells, was p53 dependent. Analysis of CD11c+ BMAPC from p53 null and wt p53 donors for intracellular IL-12p40/70 using flow cytometry showed that while a similar percentage of un-activated cells expressed IL-12 (Fig. 6 A and B), when these cells were exposed to CpG a higher percentage of wt BMAPC produced IL-12 than p53 null cells and the mean fluorescence intensity (MFI) was considerably higher in the presence and absence of p53.
higher in CpG-activated wt p53 BMAPC than in p53 null cells (e.g. MFI 2735 vs. 838; Fig. 6A). When the kinetics of IL-12 production was investigated in supernatants from wtp53 and p53 null BMAPC cultures, considerably more IL-12p40/70 was detected in the supernatant from activated wt p53 BMAPC at all time points (Fig. 6C). These data suggested that p53 might facilitate early transcription of IL-12 following BMAPC activation. Indeed, IL-12 message was 3-fold higher in p53 wt BMAPC 1 h after activation than in untreated controls whereas there was very little difference in p53 null BMAPC with or without activation (Fig. 6C). Finally, a ChIP assay was carried out using p53 null and wt MEFs, which clearly showed that p53 bound to the IL-12 p40 promoter (Fig. 6D). These findings show that when BMAPC are activated via TLR signaling, p53

**Figure 3.** Survival of BMAPC in vivo is p53 dependent. (A) wt p53 adoptively transferred BMAPC have a greater percentage of apoptotic cells compared with p53 null BMAPC. BMAPC from wt p53 and p53 null mice were injected intra-dermally into wt p53 hosts. Skin was harvested 4 h post injection in close proximity to the injection site (within 0.2 mm) and stained with an antibody to detect active caspase 3 to identify apoptotic cells. Positive cells were identified using DAB and light microscopy (top panel) and the percentage of positive cells per 200 total cells was calculated and compared between genotypes (bottom panel; results, mean ± SD, n=3 per genotype). (B) p53 null and wt p53 BMAPC express the receptor for pro-migratory chemokines, CCR7. BMDC from wt p53 and p53 null mice were activated with CpG (1 μg/mL) or left untreated. Cells were harvested and stained with antibodies toward CD11c and CCR7. The percentage of CD11c cells positive for CCR7 in activated cells is compared with that in untreated (UA), and cells stained with the isotype control using flow cytometry (Top panel). Mean fluorescence intensity of CCR7 expression is compared between p53 genotypes with and without CpG activation (bottom panel, results mean ± SD, n=3 per genotype; p < 0.05). (C) The migration of BMAPC to draining lymph nodes is increased in p53 null BMAPC. BMAPC from wt p53 and p53 null mice were labeled with different concentrations of CFSE (wt p53 BMDC were labeled with a higher CFSE concentration compared to p53 null BMDC). Draining lymph nodes were harvested 8–72 h after injection and the percentage of CFSE positive cells measured by flow cytometry and compared between genotypes. Results, mean ± sd, n=3 per genotype; p < 0.05; **p < 0.01; ***p < 0.001.
mediates the early production of IL-12 necessary for the generation of cytotoxicity.

**Discussion**

We hypothesized that the apoptosis of activated DC, necessary to avoid detrimental effects on the immune response, was mediated by p53. Our data not only support this but also show that p53 expression by activated BMAPC, containing DC, is required for the production of sufficient IL-12 to drive the differentiation of cytotoxic effector cells necessary for the destruction of tumors. It is worth noting that we found low p53 expression following BMAPC activation was sufficient to affect the function of the cells; indeed, high expression may have been detrimental leading to premature apoptosis.

We generated BMAPC from bone marrow precursors for our investigations because these have been successfully used in antitumor DC vaccination regimens. In fact, BMAPC generated in the presence of GM-CSF (originally termed DC) and activated with TLR ligands, have been shown to be more effective than those produced in the alternative culture strategy using Flt3L. Their *in vivo* equivalent has not yet been established though previous work has suggested that they resemble a transitory subset of DC, the inflammatory DC, which develop *in vivo* in response to inflammatory stimuli and then disappear. Interestingly, these cells have now been shown to be a heterogeneous population mainly comprising DC derived from common DC precursors and macrophages derived from monocyte precursors as well as a poorly defined CD11c+ MHCIIC+ component. These may each contribute different functions following activation. We have not dissected out the roles of these components but have simply asked whether p53 affects antigen presentation, cytotoxic T cell effector cell generation and vaccine efficacy associated with GM-CSF-generated BMAPC using well-established approaches.

Although relatively large numbers of DC are used in immunotherapeutic strategies, it has been known for some time that only a low percentage of those administered *in vivo* actually arrive at the draining lymph nodes. Our evidence now suggests an explanation: the high degree of cell death is brought

![Image](https://example.com/image.png)

**Figure 4.** T cell proliferation is p53 independent, however, for an effective cytotoxic T cell response p53 is required. (A) p53 null BMAPC-induced T cell proliferation similar to wt p53 BMAPC. OTI CD8+ T cells labeled with CFSE were adoptively transferred to wt p53 recipient mice. One day later, BMAPC from wt p53 and p53 null mice pulsed with SIINFEKL peptide were injected intra-dermally into the recipient mice on opposite flanks. 72 h later, draining lymph nodes were removed and CFSE labeled cells measured using flow cytometry and compared between genotypes (the results from eight recipient mice (1–8) are shown). (B) wt p53 BMAPC induces a cytotoxic T cell response more effectively. wt p53 recipient mice were vaccinated with BMAPC from wt p53 or p53 null mice pulsed with ovalbumin (OVA), SIINFEKL peptide, or were left untreated. One-week later, mice were injected with wt p53 splenocytes pulsed with SIINFEKL peptide and labeled with a high concentration of CFSE at a 1:1 ratio with splenocytes not pulsed with antigen and labeled with a low concentration of CFSE. Blood and spleen of recipient mice were collected and CFSE high and low dose labeled cells identified by flow cytometry. The percentage of specific lysis was calculated (material and methods) and compared between genotypes (results, mean ±SEM, n = 3 per genotype). *p < 0.05; **p < 0.01; ***p < 0.0001.
about by p53-mediated apoptosis in response to damage to the cells during transfer and this results in low numbers trafficking to the draining nodes. Interestingly CCR7, the chemokine receptor required for DC to migrate to the nodes, which is normally only upregulated after activation,16 was associated with p53 expression since this marker was elevated in p53 null BMAPC even before activation. This may also contribute to the survival of the cells since the upregulation and binding of CCR7 to CCL19/21 has been shown to inhibit apoptosis of activated DC.24

Our experiments also provide evidence that the survival of adoptively transferred BMAPC in the secondary lymphoid tissue where T cell priming occurs is p53-mediated. Activated DC normally apoptose within a few days in vivo (half-life 1.5–2.9 d25) and although multiple signaling pathways are known to induce DC apoptosis, to our knowledge the role of p53 in this process has not been investigated. Studies that have investigated the consequences of prolonged DC survival have largely focused on the development of autoimmunity where defective apoptosis has been shown to result in chronic lymphocyte activation,26 or alternatively, enhancing the antitumor effects of DC-mediated immunotherapy because higher numbers of DC persisting may stimulate greater T cell responses.27 Inhibition of mTOR in a TLR-activated DC vaccine against melanoma improved their survival as well as their capacity to reduce tumor growth28 and, similarly, the transfer of an anti-apoptotic protein gene, Bcl–X<sub>NK</sub> into DC used as a vaccine against a melanoma led to a reduction in the growth rate.29 However, tolerogenic DC that drive the development of regulatory T cells are known to survive for protracted periods in vivo (R Thomas, personal communication). These reports suggest that p53 may not only influence DC survival but it also may affect T cell priming. Indeed, a recent study reported that maintaining the expression of wt p53 in DC by inhibiting the p53/MDM2 interaction with Nutlin-3, actually enhanced T cell proliferation in vitro even though DC activation markers were not affected.30 While we could not detect any p53 dependent effect on antigen-specific T cell proliferation in our experiments, possibly attributable to our in vivo model, we have shown that the generation of antigen-specific cytotoxic effector cells in vivo is strongly influenced by p53 expression and that p53 clearly affects the efficacy of vaccination with BMAPC. BMAPC from p53 null donors pulsed with protein completely failed to impair the growth of the tumor. Some tumor protection was conferred by p53 null BMAPC when cells were pulsed with peptide and this suggested p53 might play a role in the processing of protein within BMAPC leading to reduced loading of peptide on to MHC. This is supported by our data showing lower amounts of MHC/SIINFEKL complexes on p53 null BMAPC after exposure to VLP/OVA, known to be processed effectively for cross presentation on BMAPC,15 when compared with wt p53 BMAPC. The way in which p53 contributes to antigen processing requires further investigation.

Our data suggest that the absence of p53 expression in BMAPC results in a defect in the signaling during the BMAPC/CD8<sup>+</sup> T cell interaction needed to drive the differentiation of CD8<sup>+</sup> T cells into cytotoxic effector cells. This process is known to require a third signal and we hypothesized that it is timely, sustained, p53-dependent production of IL-12 by BMAPC that plays a major role in the effectiveness of wt BMAPC antitumor vaccines. This cytokine was selected because it can act as a third signal leading to the generation of cytotoxic T lymphocytes.18 Our results strongly suggest that the crucial production of IL-12 is dependent on p53 expression. This finding is supported by a report showing that Nutlin-3, sustaining p53 expression, increases the production of IL-12p40 by cultured macrophages.21 Importantly, we have also provided strong evidence that p53 controls the production of this cytokine by demonstrating that this transcription factor binds to the IL-12p40 promoter. The in vivo cytotoxicity assay used in our experiments implicated CD8<sup>+</sup> effector cells but other IL-12-responsive cytotoxic cells, NK and NKT cells, that play an important role in the antitumor protective response could also have been detrimentally affected by non-functional p53.31-33
Figure 6. IL-12 production is higher and transcription occurs earlier in wt p53 activated BMAPC. (A, B) IL-12p40/70 is increased in wt p53 BMAPC following CpG activation. BMAPC from wt p53 and p53 null mice were activated with CpG (1 μg/mL) or left untreated. Cells were harvested and stained with antibodies toward CD11c and IL12p40/70. Positive cells were identified by flow cytometry (upper panel) and the percentage of IL-12p40/70 positive cells compared between genotypes (lower panel: results, mean ± SD n = 3 per genotype). *p < 0.05; **p < 0.001. (C) IL-12 is increased in BMAPC supernatants from wt p53 mice. wt p53 and p53 null BMDC were activated with LPS (1 μg/mL) or left untreated (UT), cultured for 24–120 h, and IL-12 measured in supernatants by ELISA (results, mean ± SD n = 4 per genotype) *p < 0.05; **p < 0.01. (D) Il12b is expressed earlier in wt p53 BMAPC following CpG activation. BMAPC from wt p53 and p53 null mice were activated with CpG (1 μg/mL) or left untreated. Cells were harvested 30 min to 2 h post activation and the quantity of the Il12b transcript measured by real time PCR and expressed as the fold difference compared with three housekeeping genes measured. (E) p53 binds to the IL-12p40 promoter. Chromatin immunoprecipitation assay on wt p53 (+/+ IP) and null MEFS with (−/− IP) pre-immune cleared with normal rabbit serum and Protein G beads (negative); (+/+ Input; −/− Input = 5% of lysate subjected to IP).
There is a very large literature on p53 in cancer biology but except for an emerging interest in p53 in inflammation (reviewed in34), some recent work on the effect of p53 on T cell responses35 and a report implicating DC-derived inflammatory TNF-α in the increased invasiveness of mutant p53 cancer cells,36 comparatively little research has been carried out on the effects of this protein on the functions of the immune system. This area warrants more work particularly in the light of the very promising recent developments in immunotherapy for the treatment of cancer.37,38 DC are crucial to the initiation of an immune response against a tumor and we have shown that p53 is an important regulator of their function using BMAPC derived in vitro in which DC comprise a major component. However, the collective term 'DC' belies the complexity of DC subsets in vivo which are present in virtually every tissue in different microenvironments fulfilling a range of different functions (reviewed in39). One of these subsets, the CD8αα DC, is pre-dominantly associated with IL-12 production40 and this 'cross-presenting' subset41 important in alerting the immune system to the presence of a tumor, is likely to be the most vulnerable if p53 is rendered non-functional. Although there do not appear to be any reports of DC expressing p53 mutations and DC neoplasms that might result from this are rare,42 this may be simply because these cells have not been scrutinized for p53 mutations. In this context, Li Fraumeni patients with a germline p53 deficiency due to missense mutations in p53 (reviewed in43), could provide a very valuable source of cells suitable for analysis. It may be that non-functional p53 in DC resulting in a compromised immune response, contributes to the high rate of tumors observed in these individuals.

In summary, our work suggests that DC within a GM-CSF-derived BMAPC population in which p53 has been rendered nonfunctional via mutation, may be impaired in their capacity to initiate immune responses against tumors and this could be particularly relevant where these cells are used as antitumor vaccines.

Methods

Animals and cell lines

Specific pathogen free (C57BL/6) mice were obtained from the Animal Resource Center, Western Australia and housed in the animal facility within the Department of Microbiology and Immunology, University of Otago and p53 null mice were obtained from the Children’s Medical Research Institute Animal Facility, University of Sydney. OTI mice, originally from W Heath, University of Melbourne were obtained from Sarah Hook, School of Pharmacy, University of Otago. All experimental protocols were approved by the University of Otago Animal Ethics Committee.

BMAPC preparation, flow cytometric phenotyping and cytokine analysis

Bone marrow was isolated as described previously15 and cultured in DMEM (Life Technologies, Carlsbad, CA, USA) supplemented with 5% FCS (GE Healthcare, Pittsburgh, PA, USA) and 20 ng/mL rGM-CSF (R&D Systems, Minneapolis, NE, USA). On day 4, cells were fed with fresh medium, then harvested on day 6. CpG (5μg/mL, GeneWorks, Hindmarsh, SA, Australia) was added to some cultures for the last 24 h. CD11c+BMAPC were phenotyped using flow cytometry as described previously with antibodies (BD Pharmingen, Franklin lakes, NJ, USA) to CD80 (16-10A1), CD86 (GL1), CD40 (3/23), and CCR7 (4B12).

Day 6 DC + CpG were stained to detect intracellular IL-12p40/70 and IL-6. Brieﬂy, cells were stained with CD11c antibody, fixed in PBS + 4% paraformaldehyde (Sigma-Aldrich, St Louis, MO, USA) for 1 h at 4°C, washed twice in saponin buffer (PBS + 0.1% saponin, 0.1% BSA + 10mM Hepes) incubated for 15 min in saponin buffer to permeabilize the membrane and stained with PE-labeled anti-mouse IL-12p40/70 (clone: C15.6, BD PharMingen, Franklin lakes, NJ, USA) for 30 min at room temperature. Cells were acquired using a BD FACSCalibur or BD LSR Fortessa and CellQuest or FACS Diva software. Data were analyzed using FlowJo software 9.2 or 10 (Tree Star, Ashland, OR, USA).

ELISA for detection of IL-12p40/70

The amount of IL-12p40/70 was measured by ELISA in cell culture supernatants taken daily over a 5 d period following LPS or CpG activation or untreated control of day 6 wtp53 and p53 null BMAPC. The assay was carried out per the manufacturer’s instructions. Briefly ELISA plates were coated overnight at 4°C with purified anti-mouse IL-12 p40/70 antibody (BD Pharmingen, Franklin lakes, NJ, USA), washed, blocked for 2 h at room temp and washed again. Samples or the recombinant IL-12 standard were added, the plates incubated for 2 h at 37°C and washed. Biotin anti-mouse IL-12p40/70 (BD Pharmingen, Franklin lakes, NJ, USA) was added, before further incubation and washes. Streptavidin-HRP (BD Pharmingen, Franklin lakes, NJ, USA) was added to all wells and after incubation TMB substrate (Life Technologies) was added and the reaction was stopped using 100 μL 2M H2SO4. Optical density was measured (Bio-Rad Microplate Reader, model 550) at 450 nm and the amount of IL-12 determined using Microplate Manager 5.2.1 software.

Western blot

Prior to the preparation of lysate, some BMAPC were treated with amsacrine (1 μg/mL) as a positive control to drive p53 expression. Equal amounts of each protein lysate derived from BMAPC were separated on 4–12% NuPAGE Novex Bis-Tris Mini Gels (Life Technologies, Carlsbad, CA, USA). Western blots were incubated with antibodies toward p53 (1C12, Cell Signaling Technology, Danvers, MA, USA) and β-actin (AC-15, Abcam, Cambridge, United Kingdom). Alkaline phosphatase secondary antibodies were detected using the Western-Breeze Chemiluminescent Kit detection system according to the manufacturer’s instructions (Life Technologies, Carlsbad, CA, USA), and images captured on X-ray film.
Intranuclear staining for detection of p53 in BMAPC

Immature BMAPC, sub cultured on Day 6, were cultured overnight with LPS (1 μg/mL, Sigma-Aldrich, St Louis, MO, USA) or left untreated, stained with fixable live/dead stain (Zombie NIR dye, BioLegend, San Diego, CA, USA), then CD11c antibody (BD PharMingen, Franklin lakes, NJ, USA), fixed and permeabilized using Transcription Factor Buffer Set (BD PharMingen, Franklin lakes, NJ, USA) then stained with either anti-p53 antibody (G59-12, BD PharMingen, Franklin lakes, NJ, USA) or an isotype control to detect intranuclear p53 expression. Live CD11c+ cells were gated and analyzed flow cytometrically.

Comparative assay of BMAPC apoptosis, survival and antigen presentation

Aliquots of day 5 BMAPC were treated overnight with LPS (1 μg/mL) or CpG-ODN (1 μg/mL). Untreated BMDC were included as controls. Following the manufacturer's protocol, the FLICA apoptosis detection kitTM (ImmunocChemistry Technologies, Bloomington, MN, USA) was used to detect apoptosis at 96 and 120 h post treatment. Briefly, 30 min prior to the time point for apoptosis detection, 300 μL of the various cell suspensions were harvested, 10 μL of FLICA (reconstituted in DMSO) diluted 1/5 in DPBS (Sigma-Aldrich, St Louis, MO, USA) was added and then the suspensions were incubated at 37°C in 10% CO₂ for an hour. To detect BMAPC, the cells were additionally stained with CD11c-APC antibody (N418, BioLegend, San Diego, CA, USA) for 20 min. After two wash steps with FACS buffer, cells were re-suspended in 400 μL of FACS buffer and analyzed by flow cytometry.

To compare survival in vivo of p53 null and wt CpG-matured BMAPC in the skin, the skin above the inguinal lymph node (approximately 1 cm above) was shaved. BMAPC (1 × 10⁶ in 20 μL) were intra-dermally injected into the right flank of recipient mice and the same volume of the vehicle (DPBS) was injected into the left flank. Three mice per genotype were euthanized at 4 h post injection. A 1 cm cubed piece of shaved skin (from the injection site to the draining lymph node) was dissected, fixed in formalin for 24 h, cut into strips and processed into paraffin wax. Sections (4 μm) were cut onto Super Frost slides (H&C Microscope Slides, FLEX, Dako, Glostrup, Denmark) and this was subjected to heat-mediated antigen retrieval. Primary antibodies against active caspase 3 (Human/ Mouse Cleaved Caspase-3 (Asp175)), clone 269518, R&D Systems, Minneapolis, MN) that bound to injected DC in the skin were detected using the EDL (Dako, Glostrup, Denmark) and DAB methods. Active caspase 3 positive cells were detected with light microscopy (DM 2000 microscope, DFC 295 camera and Application Suite software, version 3.5.0, Leica, Solms, Germany).

To compare survival, BMAPC in the draining lymph nodes from p53 null and wt p53 mice were labeled with differential doses of CFSE, mixed in 1:1 ratio and 1 × 10⁶ in a 20 μL volume were injected intra-dermally into the right flank of recipient mice. The inguinal nodes were harvested from groups of mice at varying intervals post injection, macerated into single cell suspensions and analyzed by flow cytometry. To ensure that the dose of CFSE did not affect survival, high and low doses were used to label p53 null BMAPC in different experiments with the reciprocal in wt BMAPC.

BMAPC antigen processing

Rabbit Hemorrhagic Disease Virus VLP (5 mg/mL) and Ovalbumin (5 mg/mL) were reacted with 20-fold molar excess of NHS-azide and NHS-phosphine (Thermo Scientific), respectively. Reactions were incubated for 1 h at ambient temperature. Excess unconjugated linker was removed by dialysis through a 10 kDa MWCO membrane (Sigma). Four milligrams of VLP-azide were added to 4 mg of Ovalbumin-phosphine and incubated for 1 h at ambient temperature and then for 16 h at 4°C. The reaction was dialyzed through a 1,000 kDa MWCO membrane (Spectra/Por) to remove excess unconjugated Ovalbumin-phosphine. The conjugation reaction was confirmed by SDS-PAGE (data not shown). Day 6 BMAPC were pulsed with 100μg/mL VLP-OVA overnight and H-2Kb/SIINFEKL complexes on the cell surface were subsequently detected using PE 25 D1.16 antibody (BioLegend, San Diego, CA, USA) as previously described and analyzed by flow cytometry.

BMAPC vaccine preparation

To prepare BMAPC for in vivo administration as a prophylactic vaccine, day 5 BMAPC were pulsed overnight with OVA protein (100 μg/mL, Sigma-Aldrich, St Louis, MO, USA), then treated with CpG-ODN 1826 TCCATGACGTCTCGAGTTT MG 6363, (GeneWorks, Hindmarsh, SA, Australia) again overnight, washed and re-suspended. Alternatively, CpG treated BMAPC were pulsed with SIINFEKL peptide, Kfi257-264 (10 μM) for 2.5 h. Untreated BMAPC were retained as controls. BMAPC were re-suspended at 1 × 10⁶ cells/mL and 100 μL was injected subcutaneously into each mouse.

In vivo T cell proliferation

Splenic OT1 cell suspensions were enriched for CD8+ T cells by depleting B cells, CD4+ T cells and CD11c positive (DC) by passage through an AutoMACS column (Miltenyi Biotec, Bergisch Gladbach, Germany) and labeled with CFSE (Sigma-Aldrich, St Louis, MO, USA) diluted 1:2500, adjusted to 5.0 × 10⁶ cells/mL and 200 μL was injected intravenously into each mouse. Twenty-four hours later, CpG-matured wt or p53 null BMAPC pulsed with OVA protein (100 μg/mL), SIINFEKL peptide (10 μM) or left unpulsed were suspended at 1 × 10⁶/mL and 20 μL was injected intradermally (i.e., 2 × 10⁶ cells/mouse) into either the right (p53 deficient BMAPC) or left side (wt BMAPC). Seventy-two hours later, draining inguinal lymph nodes were harvested and single cell suspensions analyzed by flow cytometry.

In vivo cytotoxicity assay

To produce target cells for in vivo cytotoxicity, single cell suspensions were prepared from the spleens of C57BL/6 donor mice and cells were re-suspended at 2 × 10⁷ in DMEM + 5%
FCS. Cells were either pulsed with SIINFEKL peptide (10 μM) or remained unpulsed and incubated for 3 h at 37°C, 10% CO2. Cells were washed in Dulbecco’s Phosphate Buffered Saline (DPBS) three times and re-suspended at 4 x 10^5/mL. Pulsed cells were then labeled with an equal volume of CFSE diluted 1/2500 from a stock concentration of 10 mM, (CFSEhi) while the unpulsed cells were labeled with CFSE diluted 1/25,000 (CFSElo), for 8 min. An equal volume of FCS was then added to the CFSE cell suspension. Cells were washed thoroughly three times and re-suspended at 1 x 10^6 in DPBS. CFSElo and CFSEhi cells were mixed together at a ratio of 1:1, and 200 μL injected intravenously into mice vaccinated with DC pulsed with SIINFEKL peptide or OVA one week previously. Blood samples were collected and spleens harvested 48 h after target cell administration and analyzed by flow cytometry. The ratio of CFSElo to CFSEhi cells was used to determine the specific lysis:

\[
\%\text{Lysis} = \left\{ \frac{1-(\%\text{CFSEhi}/\%\text{CFSElo vaccinated})}{(\%\text{CFSEhi}/\%\text{CFSElo PBS})} \right\} \times 100
\]

**Tumor growth in vivo**

B16-OVA tumor cells cultured in serum-free medium, were passaged at least three times before administration into mice previously immunized with OVA or SIINFEKL pulsed BMDC. Unpulsed BMDC were used as a negative control vaccine. B16-OVA tumor cells were harvested using cell scrapers, and centrifuged at 250 g for 8 min at 10°C. After a single wash in 10 mL of DPBS, viable cells were counted using trypan blue. (dead cells were also enumerated). Cells were re-suspended at 2 x 10^6 cells/mL, and 50 μL were injected subcutaneously onto the contralateral side of animals vaccinated one week previously with BMDC. Unvaccinated mice were also inoculated with the tumor cells as a control. Tumors were monitored daily after they were detectable by palpation and the size of the tumor was measured with calipers and recorded as tumor area (mm²). Once the tumor grew to a maximum size of 15 mm², the animal was euthanized, and the data were recorded as the percentage of surviving animals.

**RTqPCR for detection of IL-12p40 mRNA in BMDC**

RNA was prepared from wt or p53 null BMDC, day 7 BMDC harvested, counted and re-suspended in complete Dulbecco’s Modified Eagle Medium (cDMEM)+10% FCS at 4 x 10^5 cells/mL. Cells were cultured in 24 well TC plates with 5 μg/mL CpG, 0.2 μg/mL amsacrine or left untreated. At appropriate time points, cells were harvested, washed once in PBS and cells pelleted by centrifugation and treated with TRIzol (Life Technologies, Carlsbad, CA, USA) following the manufacturer’s instructions. cDNA was made from 200 ng of RNA with Superscript III reverse transcriptase (Life Technologies, Carlsbad, CA, USA) and poly-dT as a primer. qPCR was performed using Platinum SYBR Green qPCR SuperMix-UDG (Life Technologies, Carlsbad, CA, USA) on a LightCycler480 II (Roche, Basel, Switzerland), using primers designed to bridge two separate exons of the Il12b gene and the three housekeepers, B2m, Hmbs and Hprt. Primers are available on request.

**Chromatin Immunoprecipitation (ChIP)**

Chromatin was fixed by adding 1% formaldehyde solution to 5 x 10^6 p53+/+ and p53−/− MEFs. The MEFs were then harvested and nuclei were collected by lysing cells for 5 min in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl pH 8.0, complete protease inhibitors [Roche]) and then centrifuged at 4°C for 10 min at 2300 g. The resulting chromatin was sonicated using a Bioruptor (Diagenode). Sheared chromatin was collected after centrifugation at 16,000 g for 10 min at 4°C. For immunoprecipitation of chromatin, 20 μg of rabbit p53 antibody (FL393) was incubated with the sheared chromatin in immunoprecipitation buffer (1% Triton X-100, 2 mM EDTA pH 8.0, 150 mM NaCl, 20 mM Tris-HCl pH 8.0) with protease inhibitors for 12 h at 4°C. Dynabeads protein agarose beads (Life Technologies) pre-blocked with BSA and sheared salmon sperm DNA, were then incubated with the antibody chromatin complex for 4 h at room temperature. The beads were then collected by magnetic force. The resulting beads were washed twice with wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl pH 8.0) and then chromatin eluted with elution buffer (70 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 1.5% SDS). The resulting chromatin complexes were treated with proteinaseK and purified using a PCR purification kit (Qiagen). For PCR amplification of immunoprecipitated IL-12 promoter fragments was then carried out using FastStart Taq polymerase (Roche). Primer sequences are available on request.

**Statistical analyses**

Results are expressed as the mean ± SD or SEM and are from three independent experiments unless otherwise stated. Statistical differences between two groups were determined by Student’s t-test (p < 0.05 was taken as statistically significant) using Prism 6 (GraphPad Software, La Jolla, CA, USA). BMDC yield data is expressed as the median (25th–75th percentile) from 10 independent experiments, with the statistical difference between two groups determined using the Mann–Whitney test (p < 0.05 was taken as statistically significant) using Prism 6.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

**Author contributions**

MB, TS, MW and AB initiated the study and designed the experiments; TS, MW, NF, DL and CT conducted the experiments; VW and VY prepared VLP and critiqued the manuscript; HC bred animals for the study and critiqued the manuscript; MB wrote the majority of the manuscript; TS, MW and NF analyzed the data.

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