ONCOIMMUNOLOGY
2016, VOL. 5, NO. 3, e1066062 (11 pages)
http://dx.doi.org/10.1080/2162402X.2015.1066062

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ORIGINAL RESEARCH

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Dexamethasone co-medication in cancer patients undergoing chemotherapy causes substantial immunomodulatory effects with implications for chemo-immunotherapy strategies

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ABSTRACT

The glucocorticoid (GC) steroid dexamethasone (Dex) is used as a supportive care co-medication for cancer patients undergoing standard care pemetrexed/platinum doublet chemotherapy. As trials for new cancer immunotherapy treatments increase in prevalence, it is important to track the immunological changes induced by co-medications commonly used in the clinic, but not specifically included in trial design or in pre-clinical models. Here, we document a number of Dex -induced immunological effects, including a large-scale lymphodepletive effect particularly affecting $CD4^+$ T cells but also $CD8^+$ T cells. The proportion of regulatory T cells within the $CD4^+$ compartment did not change after Dex was administered, however a significant increase in proliferation and activation of regulatory T cells was observed. We also noted Dex -induced proportional changes in dendritic cell (DC) subtypes. We discuss these immunological effects in the context of chemoimmunotherapy strategies, and suggest a number of considerations to be taken into account when designing future studies where Dex and other GCs may be in use.

ARTICLE HISTORY
Received 11 May 2015
Revised 18 June 2015
Accepted 19 June 2015

KEYWORDS
Immunotherapy; glucocorticoid; lymphodepletion; mesothelioma; dendritic cell; T cell; flow cytometry; treg

Introduction

The rapidly developing field of cancer immunotherapy aims to boost or direct a patient's immune system in a manner that can aid recognition of, and/or reactivity against, tumors. We and others have demonstrated that some chemotherapies can be immunogenic——inducing tumor cell death in such a way that tumor neoantigens are "unmasked" and become visible to the host immune system.[1] "Chemoimmunotherapy" combines the properties of immunogenic chemotherapies with any one of a number of immunotherapeutic treatments currently under development. Immunotherapies are generally directed at specific rate-limiting steps in the development of an antitumor immune response——whether that be an enhancement of immune activation, or conversely the removal of immunological suppression.[2] These finely tuned interventions undergo initial development in animal model systems; however it is commonplace for co-medications that accompany chemotherapy as part of standard clinical care to be omitted from pre-clinical models.

During retrospective analysis of data from two recent chemoimmunotherapy clinical trials in patients with malignant pleural mesothelioma (MPM), we observed large-scale lymphodepletion occurring between the first and second of two "immunological baseline" blood samples taken from each patient prior to receiving chemotherapy. This effect coincided with patients taking the GC steroid Dex. In standard care treatment of MPM (a highly aggressive, incurable, asbestos-induced cancer) Dex is given prior to and for three to five d after pemetrexed/platinum chemotherapy in order to control skin rash, emesis, and inflammatory side effects.[3]

Dex is routinely prescribed to patients with advanced cancer in a wide range of doses (0.5 mg up to 16 mg daily) for a variety of additional reasons: fatigue, stimulation of appetite, night sweats, and to combat the side effects of some chemotherapies (including platinum agents and taxanes) both as an antiemetic and to prevent hypersensitivity or allergic reactions.[4] It is also used specifically to decrease oedema associated with primary and secondary tumors of the central nervous system, for brain metastases in advanced melanoma, or as a single agent therapy for leukemia, lymphoma, and multiple myeloma.

GCs induce immunosuppressive and anti-inflammatory effects predominantly through binding to the glucocorticoid receptor (GCR), of which there are several splice variants, and subsequent direct or indirect genomic interactions.[5] In addition a number of non-genomic interactions have been described, including direct interaction with ion channels in the cell or mitochondrial membranes, and also the release of heat shock proteins and chaperonins.[5] It has long been known that Dex and other GCs can modulate the immune system in a wide variety of ways, with varying mechanisms of action in different cell types (for recent comprehensive reviews, see refs. 6 and 7).[6,7] However, the precise nature and scope of immunological changes induced in response to the particular regimen of Dex used in pemetrexed/platinum premedication has not been studied in detail——nor more generally in patients with cancer. We hypothesized that

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additional phenotypic and functional changes of importance to current chemoimmunotherapy strategies had occurred in response to Dex in this setting, and therefore further interrogated our data to highlight what these might be.

Understanding the immunological changes induced by Dex is important both in the context of combining immunotherapy with chemotherapy, and given the broadening indications and study settings for single agent or combination immunotherapy. The data presented here can inform future planning of combined chemoimmunotherapy modalities, timing of baseline immunological investigations, and suggest the importance of studying the effects of Dex in animal models of chemoimmunotherapy.

Results

We examined PBMC samples from 35 patients with MPM who had registered consent for either of two chemoimmunotherapy clinical trials. Criteria for acceptance onto either study were identical. Patients received three 4 mg doses of Dex in the 24 h prior to undergoing standard care pemetrexed + platinum (carboplatin or cisplatin) chemotherapy (Fig. 1A). Peripheral blood samples were collected on the day the patient consented to participation in the study, and again immediately prior to the first cycle of chemotherapy treatment−−with a mean time between samples of 9.6 d (± 5.8 d). Immunological parameters were assessed by flow cytometry (see materials and methods). The patients were a typical cohort of advanced MPM, with median overall survival (OS) of 17.8 mo (Fig. 1B).

The data for all parameters were collated, together with the percentage increase or decrease in that parameter between pre-Dex and post-Dex timepoints, and the corresponding p value resulting from paired t-test analysis (Table 1).

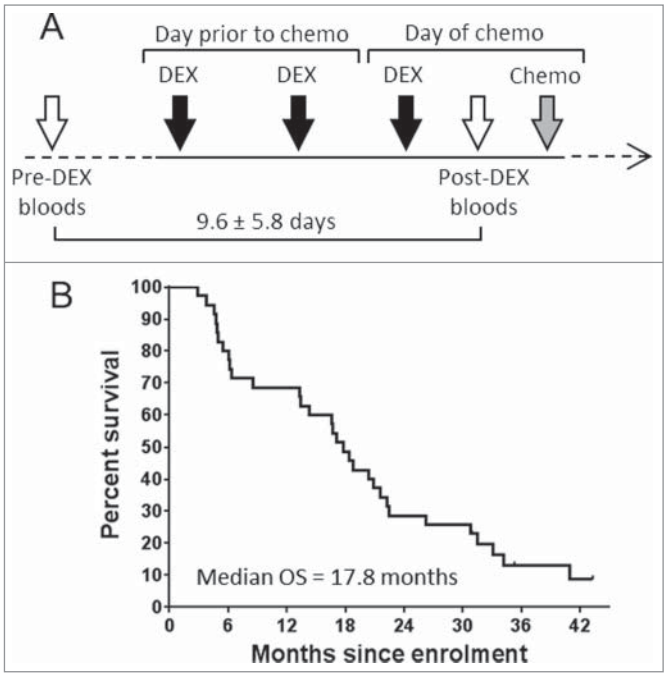

Figure 1. Study group treatment schedule and overall survival. Timeline of patient treatment schedule (A). Patient blood samples were drawn on the day of study enrolment, and again immediately prior to receiving chemotherapy. 3 × 4 mg doses of Dex were given in the lead up to chemotherapy as shown. Open arrows represent study blood collections, black arrows represent oral administration of 4 mg Dex, gray arrow represents infusion of chemotherapy. (B) Kaplan−Meier plot showing overall survival of all patients involved in this study.

We assessed changes in concentrations of total $CD4^+$ and $CD8^+$ T cells per μL of peripheral whole blood, by appropriate gating (Fig. 2A). Both $CD4^+$ and $CD8^+$ cell numbers approximately halved in response to Dex treatment (Fig. 2B and C). This lymphodepletive effect was significantly more pronounced in $CD4^+$ T cells, as shown by the CD4:CD8 ratio (Fig. 2D).

Within the overall $CD4^+$ T cell subset, we focused on regulatory T cells (Treg). The absolute concentration of $CD4^+CD25^+$ $CD127^{lo}Foxp3^+$ Treg cells (see Fig. 3A for a description of gating strategy) in peripheral blood was also seen to decrease in response to Dex, with the overall Treg proportion of total $CD4^+$ T cells remaining constant (Fig. 3B). However, the proliferating proportion of Tregs (determined by intracellular staining for Ki67, a nuclear protein expressed in dividing and recently-divided cells, but not in resting or naïve lymphocytes)[8] rose by around 80% in comparison to the approximately 50% increase seen in the non-Treg $CD4^+$ population (Fig. 3C and D). The inducible co-stimulator molecule (ICOS), a member of the CD28 family of co-stimulatory molecules, is expressed on activated T cells, and has been described as an indicator of antigen-specific activation.[9,10] An increasing proportion of Tregs were seen to express ICOS in response to Dex, whereas the ICOS-expressing proportion of non-Treg $CD4^+$ T cells remained low (Fig. 3E and F).

We examined the $CD8^+$ T cell compartment in more detail, focusing on the "effector" $CD38^{hi}HLA\text{-}DR^{hi}$ population (see Fig. 4A for gating strategy). The number of effector cells ($CD38^{hi}HLA\text{-}DR^{hi}$ cells as a proportion of $CD3^+CD8^+$ cells) underwent a small but significant increase in response to Dex (Fig. 4B). This population consists of predominantly proliferating ($Ki67^+$) cells with low expression of Bcl^-2 (a constitutively expressed anti-apoptotic protein that becomes downregulated in T cells upon their activation in response to antigen).[11,12] The proportion of Ki67-expressing cells within this effector population increased by ~10% in response to Dex; however expression of ICOS did not change. Although the overall proportion of proliferating ($Ki67^+$) CD8 T cells was small−−as expected−−there was a significant increase in response to Dex (Fig. 4C). In terms of activation, however, the cell-surface expression of ICOS remained constant (Fig. 4D).

All T cell subsets underwent substantial depletion from blood, however we wanted to examine whether the balance between $CD8^+$ T cells and Tregs had changed as this might alter the stoichiometric potential between immune suppression versus an antitumor immune response. The ratio of Tregs to $CD8^+$ T cells was calculated here as follows: total $CD4^+$ and $CD8^+$ T cell concentrations were obtained from whole blood cell count experiments; Treg concentrations were subsequently calculated using the $CD25^{hi}CD127^{lo}Foxp3^+$ percentage of total $CD4^+$ T cells from PBMC staining as described above. The number of $CD8^+$ T cells per Treg showed approximately a 65% increase in favor of $CD8^+$ T cells (Fig. 4E).

The effect of Dex on DC subpopulations of patient PBMC was also examined. PBMC from patients recruited to only one of the two clinical trials were analyzed for DC markers by flow cytometry, in line with the outcomes targeted in that particular study (n = 15). The gating strategy identified DCs−−classically defined as $HLA\text{-}DR^+lin^-$−− with subsets identified as

Table 1. Results of flow cytometry analysis.

	Pre-Dex			Post-Dex				
Parameter measured	Mean	StDev	n =	Mean	StDev	n =	% change	*p* value
CD3 T cells per μL	848	401	30	370	207	32	−56.3	<0.001
CD4 T cells per μL	551	199	31	236	149	33	−57.2	<0.001
CD4 % of CD3 T cells	65.4	10.8	32	57.6	12.8	35	−11.9	<0.001
CD8 T cells per μL	246	212	30	124	103	31	−49.8	<0.001
CD8 % of CD3 T cells	27.1	10.3	32	33.7	13.4	34	24.4	<0.001
CD4 to CD8 T cell ratio	3.0	1.9	32	2.3	1.7	34	−25.3	<0.001
Tregs per μL	24.0	11.9	31	9.7	6.5	33	−59.3	<0.001
Treg % of $CD4^+$ T cells	4.2	1.3	35	4.2	1.4	35	−1.2	0.755
$Ki67^+$ % of Tregs	21.3	6.9	35	38.3	13.0	35	79.5	<0.001
$Ki67^+$ % of non-Treg CD4 T cells	3.1	1.4	35	4.8	2.4	35	53.1	<0.001
$ICOS^+$ % of Tregs	35.2	15.3	35	46.7	15.7	35	32.4	<0.001
$ICOS^+$ % of non-Treg CD4 T cells	6.5	5.0	35	7.0	5.2	35	8.4	0.121
effector ($CD38^{hi}HLA\text{-}DR^{hi}$) CD8 T cells per μL	4.4	3.9	30	2.3	1.3	31	−46.5	0.003
effector % of CD8 T cells	1.9	1.5	35	2.5	1.9	35	33.4	<0.001
$Bcl2^{lo}$ % of effector CD8 T cells	66.5	17.0	35	64.1	18.1	35	−3.6	0.293
$Ki67^+$ % of effector CD8 T cells	63.3	17.2	35	69.7	17.1	35	10.0	0.009
$ICOS^+$ % of effector CD8 T cells	27.9	14.2	35	28.8	13.7	35	3.3	0.608
$Bcl2^{lo}$ % of CD8 T cells	3.8	2.2	35	3.6	2.6	35	−5.9	0.527
$Ki67^+$ % of CD8 T cells	2.0	1.5	35	2.9	2.0	35	45.7	0.001
$ICOS^+$ % of CD8 T cells	2.1	3.8	35	1.7	1.9	35	−20.7	0.353
CD8 T cells to Treg ratio	10.7	6.3	30	17.0	13.5	31	59.2	<0.001
DC ($HLA\text{-}DR^+$ Lin^-) % of PBMC	0.4	0.2	15	0.3	0.2	15	−31.4	0.035
$HLA\text{-}DR^+$ lin^- $BDCA1^+$ % of total PBMC	0.159	0.120	15	0.085	0.102	15	−46.3	0.024
$HLA\text{-}DR^+$ lin^- $BDCA2^+$ % of total PBMC	0.147	0.095	15	0.048	0.053	15	−67.2	0.001
$HLA\text{-}DR^+$ lin^- $BDCA3^+$ % of total PBMC	0.028	0.019	15	0.022	0.015	15	−21.9	0.279
$BDCA1^+$ % of DC	35.6	19.8	15	21.8	18.8	15	−38.8	0.023
$BDCA2^+$ % of DC	32.6	13.7	15	17.2	14.4	15	−47.1	0.008
$BDCA3^+$ % of DC	5.6	2.4	15	12.0	8.4	15	116.2	0.003
$BDCA1^+$ Dc to $BDCA^{2+}$ DC ratio	2.1	2.6	15	2.2	2.3	15	5.3	0.884
$BDCA1^+$ DC CD40 MFI	43.0	32.3	15	75.6	58.3	15	75.7	0.005
$BDCA1^+$ DC CD83 MFI	78.7	34.1	15	64.8	27.8	15	−17.7	0.188
$BDCA1^+$ DC CD80 MFI	110	42	15	135	69	15	22.7	0.036
$BDCA2^+$ DC CD40 MFI	27.0	24.1	15	17.0	19.3	15	−37.0	0.138
$BDCA2^+$ DC CD83 MFI	34.7	13.8	15	35.8	12.7	15	2.9	0.831
$BDCA2^+$ DC CD80 MFI	129	25	15	241	440	15	86.4	0.344
$BDCA3^+$ DC CD40 MFI	29.5	70.9	15	25.2	84.3	15	−14.5	0.744
$BDCA3^+$ DC CD83 MFI	46.6	58.2	15	37.5	30.2	15	−19.5	0.408
$BDCA3^+$ DC CD80 MFI	198	45	15	229	96	15	15.8	0.118

List of leukocyte parameters retrospectively examined for changes in response to Dex. For timing of blood sample withdrawal and Dex administration, see Figure 1A. "% change" refers to difference in mean values between "pre-Dex" and "post-Dex" timepoints. *P* values were calculated using the paired t-test.

"myeloid" or "plasmacytoid" through use of BDCA antibodies (Fig. 5A; for recent reviews on DC nomenclature and subtypes, see refs. 38 and 39).[13,14] These subsets are often reported as a percentage of total PBMC (Fig. 5B). However, due to the substantial depletive effects of Dex on T cells, the proportion of PBMCs identified as DCs would change whether or not DCs were actually affected by Dex. Therefore, we also assessed DC subsets as a proportion of the $HLA\text{-}DR^+lin^-$ DC population (Fig. 5C). Notably, the BDCA-1^+ ($CD1c^+$, major myeloid) and BDCA-2^+ ($CD303^+$, plasmacytoid) proportion of both total PBMC and $HLA\text{-}DR^+lin^-$ cells reduced significantly in response to Dex. The BDCA-3^+ ($CD141^+$ myeloid) DC proportion of total PBMC did not change, and indeed was seen to increase as a proportion of $HLA\text{-}DR^+lin^-$ cells. Relative expression of the CD80 (B7.1) co-stimulatory molecule, and the maturational markers CD40 and CD83, were analyzed by mean fluorescence intensity (MFI) on all three of the DC subsets mentioned above. No significant changes were observed in response to Dex treatment with the exception of significant (but highly heterogeneous) increases in the MFI of CD40 and, to a lesser extent, CD80 on the $BDCA1^+$ DC subset.

Discussion

Understanding the immunological changes induced by Dex is likely to be an important consideration in the context of combined immunotherapy and chemotherapy, and also given the broadening indications and study settings for single agent or combination immunotherapy. While most current clinical trials of anti-CTLA4 and anti-PD-pathway "checkpoint blockade" agents exclude patients requiring Dex treatment, or limit the dose of Dex, this is empirical and there is little data upon which to base a recommendation. Furthermore, some studies are now enrolling participants who may be Dex-dependent, such as those with intracranial primary or metastatic disease, without a clear understanding of how this may affect treatment efficacy.

Here, we describe a large-scale lymphodepletive effect in response to Dex administration as a pre-chemotherapy agent, with a particular bias toward $CD4^+$ T cell loss. A similar outcome has been observed with the GC methylprednisolone in patients with multiple sclerosis, with increased numbers of apoptotic cells observed in the $CD4^+$ over the $CD8^+$ T cell populations following treatment.[15] GC-induced apoptosis of T cells

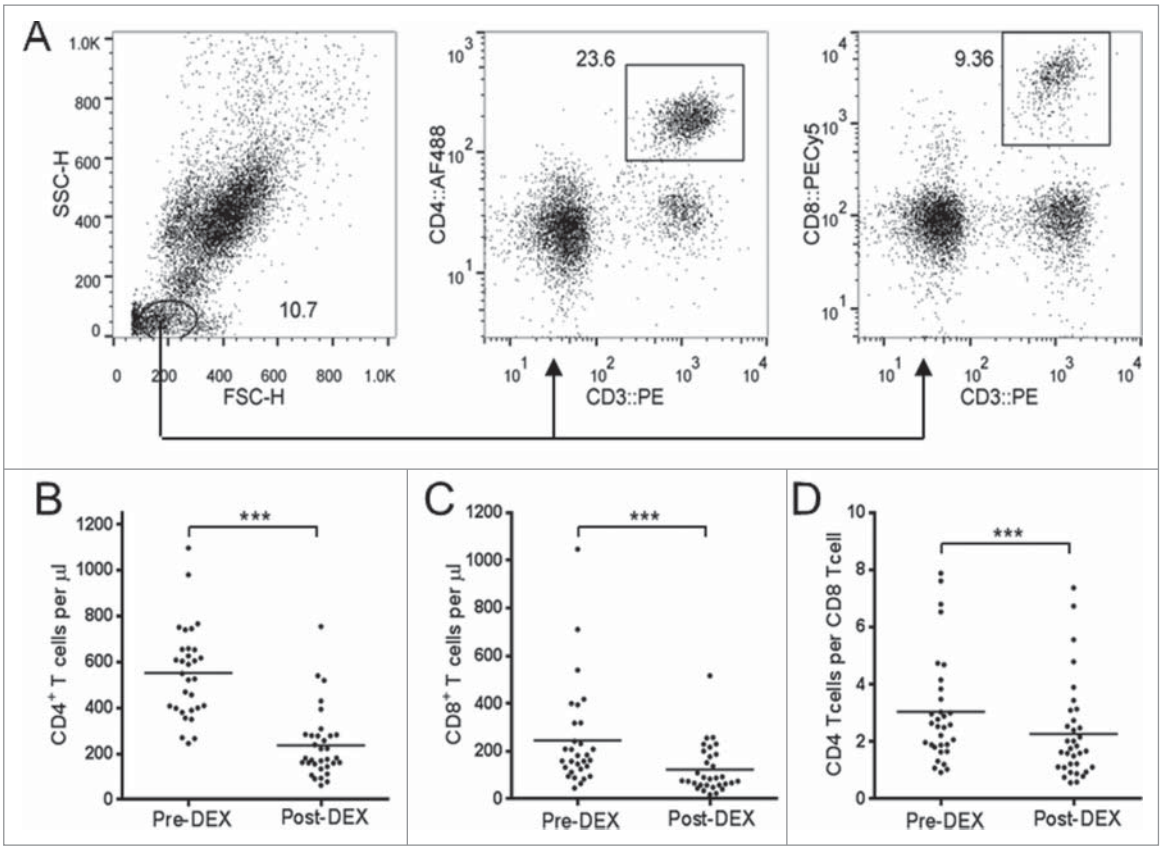

Figure 2. Dex treatment reduces the number circulating of $CD4^+$ and $CD8^+$ T cells. (A) Representative flow cytometry data showing gating strategy on whole blood samples used to obtain absolute volumetric cell count data. Lymphocytes were identified on the basis of forward scatter (FSC) vs. side scatter (SSC), with $CD4^+$ or CD8 T cells were subsequently identified as $CD3^+CD4^+$ and $CD3^+CD8^+$ respectively. (B–D) Analysis of T cell subsets in peripheral blood samples collected before (pre-Dex) and after (post-Dex) administration of dexamethasone. (B) Concentration of $CD3^+CD4^+CD8^-$ lymphocytes per μL of peripheral whole blood. (C) Concentration of $CD3^+CD4^-CD8^+$ lymphocytes per μL of whole blood. (D) The CD4:CD8 ratio, calculated by dividing the number of $CD4^+$ T cells per μL by the number of $CD8^+$ T cells per μL. Each dot represents an individual patient; significant difference between pre-Dex and post-Dex values: $^{***}p < 0.0001$, paired students t-test.

has been shown in part to be mediated by GC-GCR complex binding to the mitochondrial membrane.[16]

Regulatory T cells (Tregs) were of particular interest to us. These cells have the capacity to suppress an antitumor immune response,[17] and are typically found to be increased in the peripheral blood of patients with a number of cancers including MPM.[18-26] Infiltration of Treg into tumor tissue has been associated with a poor prognosis in several cancers including NSCLC,[27,28] ovarian cancer,[29] breast cancer,[30] gastric cancer,[31] pancreatic cancer [32] and hepatocellular carcinoma.[33,34] In particular, the intratumoral balance between $CD8^+$ tumor-infiltrating lymphocytes (TILs) and Tregs has been shown to be predictive in a number of malignancies, with a low CD8/Treg TIL ratio correlating with reduced survival in cervical and ovarian cancers for example. [35-37] GCs are known to have selective effects on Tregs over conventional T cells, although the nature of these effects remains controversial. GCs have been previously described as causing apoptosis in pro-inflammatory T cells, while aiding survival of Tregs.[7,38-40] Conversely, a recent *in vitro* study by Pandolfi and colleagues using human PBMC report significantly increased apoptosis in Tregs, and a relative increase in effector T cell frequency in response to Dex treatment——however, the authors also report that these effects can be modulated by the addition of IL-2.[41] Our findings show the proportion of Tregs within the overall $CD4^+$ T cell compartment does not change, indicating that Treg survival was not selectively affected in response to the brief but high dose exposure to Dex in our patient group. We therefore think that the *in vivo* concentration of IL-2 is likely to be sufficient to provide Tregs with a pro-survival signal in these patients. GCs have also been described to increase the proportion of $CD4^+CD25^{hi}$ "Tregs" in patients with systemic lupus erythematosus.[42]

All T cells subsets examined here were seen to proliferate in the post Dex environment; however, Tregs were more likely to be cycling and to show an activated phenotype. The increase in the turnover rate of Tregs compared to non-Treg T cells after Dex-mediated lymphodepletion is consistent with previous findings from models of irradiation and cytotoxic lymphodepletion by cyclophosphamide.[43] This higher proliferation of Tregs in the lymphopenic environment may likely be due to a greater affinity for IL-2, although TCR-mediated activation of $CD4^+$ T cells in the presence of Dex has been reported to increase the proportion of IL-10-producing cells, highlighting a possible additional mechanism whereby Tregs may be induced.[44] This view is supported by our observation that the frequency of ICOS-expressing Tregs is significantly increased following Dex treatment, suggesting antigen-specific activation of these cells. In contrast, non-Treg $CD4^+$ and the general $CD8^+$ T cell populations did not show any increase in ICOS-

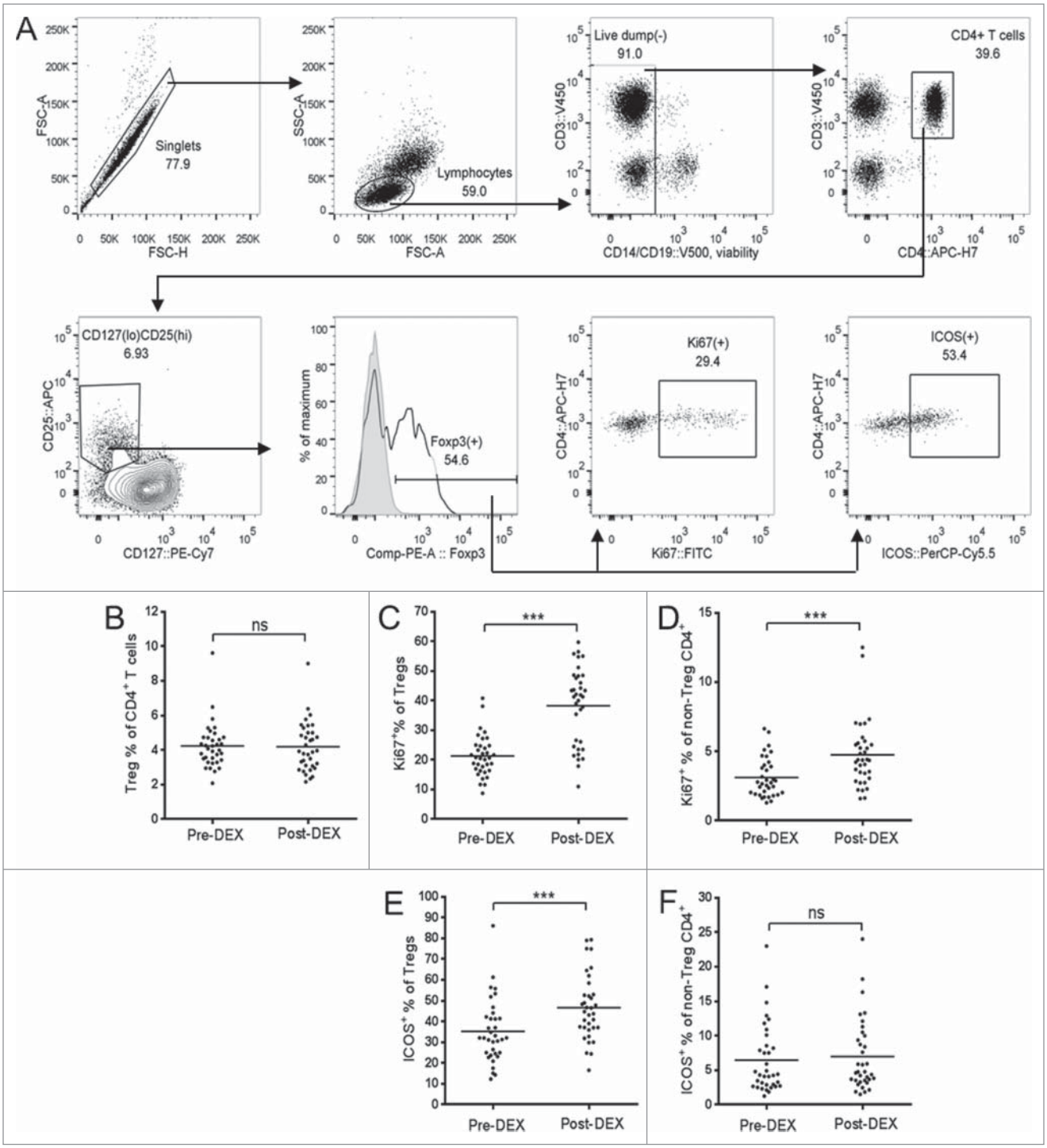

Figure 3. Dex treatment increases the proliferation and activation state of Tregs. (A) Representative flow cytometry data, demonstrating the gating strategy used for Treg identification and analysis. Forward scatter (FSC) area vs. FSC-height was used for doublet discrimination, and lymphocytes subsequently selected by FSC vs. side scatter. A "dump" channel was used to gate out dead cells (LIVE/DEAD fixable aqua viability stain), $CD14^+$, $CD56^+$, and $CD19^+$ cells. $CD4^+$ T cells were subsequently selected on the basis of CD4 vs. CD3 staining, followed by the identification of Tregs as $CD25^{hi}$, $CD127^{lo}$, and $Foxp3^+$. Tregs, or non-Treg $CD4^+$ T cells, were further gated for expression of Ki67 and ICOS. (B—F) Analysis of Tregs in patient PBMC samples collected before (pre-Dex) and after (post-Dex) administration of Dex. (B) Percentage of Tregs ($CD25^+CD127^{lo}Foxp3^+$) as a proportion of total $CD4^+$ T cells in PBMC samples. (C—D) Proportion of Tregs (C) and non-Treg $CD4^+$ lymphocytes (D) expressing the proliferation marker Ki67. (E—F) Changes in proportional expression of the activation marker ICOS, in Treg (E) and non-Treg $CD4^+$ lymphocytes (F). Each dot represents an individual patient; significant difference between pre-Dex and post-Dex values: $^{***}p < 0.0001$, paired students t-test.

positive cells, indicating that these cells were proliferating in an antigen-independent, homeostatic manner.

Previous studies have shown that high intratumoral CD8/Treg ratios are associated with better survival in a number of cancers including cervical and ovarian.[35,45] Similar observations have also been reported in peripheral blood, from dogs with osteosarcoma.[46] We were interested to see whether, in the PBMC samples we had available, the Dex-mediated depletion of T cells had the effect of altering the CD8/Treg ratio. Indeed, we observed a significant relative increase in CD8 T cells compared to Tregs. It would seem that this change simply reflects the higher susceptibility of $CD4^+$ T cells over $CD8^+$

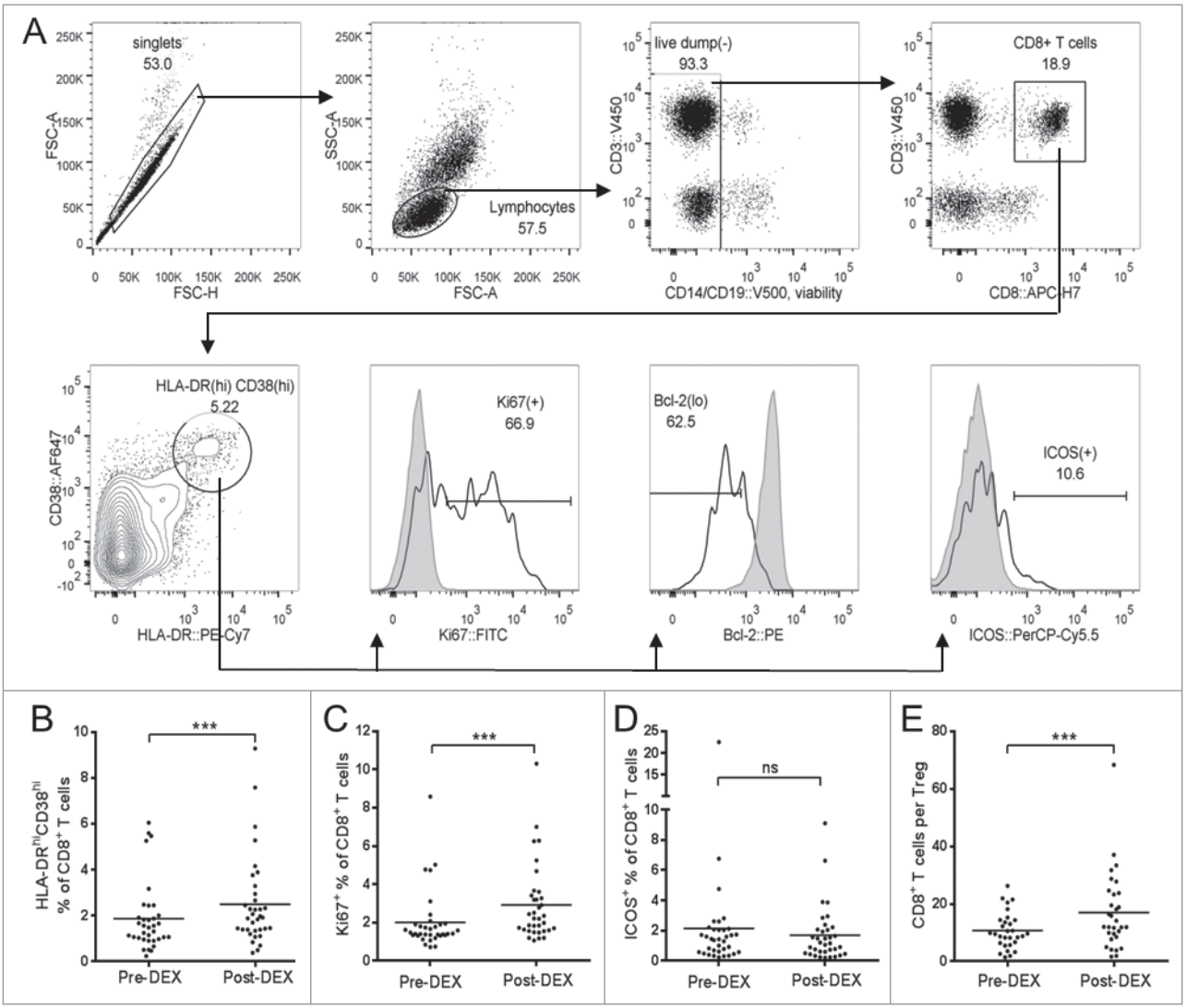

Figure 4. Dex treatment increase the proportion of $CD8^+$ T cells displaying an effector phenotype. (A) Representative flow cytometry data demonstrating the gating strategy used for $CD8^+$ T cells. Forward scatter (FSC) area vs. FSC-height was used for doublet discrimination, and lymphocytes were selected by FSC vs. side scatter. A "dump" channel was then used to gate out dead cells (LIVE/DEAD fixable aqua viability stain), monocytes/macrophages (CD14), NK cells (CD56) and B cells CD19). $CD8^+$ T cells were subsequently selected on the basis of CD8 vs. CD3 staining, followed by the identification of "effector CD8" cells as HLA-$DR^{hi}CD38^{hi}$. This population was further gated for expression of Ki67, Bcl^-2 and ICOS, using HLA-DR^-CD38^- CD8 T cells from the same sample as gating controls (filled histogram peaks, "negative" in respect to Ki67 and ICOS, and "positive" regarding Bcl^-2). (B–E) Analysis of $CD8^+$ T cells in patient PBMC samples collected before (pre-Dex) and after (post-Dex) administration of dexamethasone. (B) HLA-$DR^{hi}CD38^{hi}$ "effector" lymphocytes as a percentage of total $CD8^+$ T cells. (C–D) Proportion of CD8+ T cells expressing the proliferation marker $Ki67^+$ (C) and activation marker ICOS (D). (E) $CD8^+$ T cell to Treg ratio, calculated by dividing the $CD8^+$ percentage of total T cells by the Treg percentage of total T cells. Each dot represents an individual patient; significant difference between pre-Dex and post-Dex values: $^{***}p < 0.0001$, paired students t-test.

T cells to Dex-mediated depletion, since the overall Treg proportion of total $CD4^+$ T cells showed no significant change.

HLA-DR and CD38 are markers of antigen-specific T cell activation, previously documented through their expression during chronic viral infection.[12,47-49] This $CD8^+$ effector population is increased in MPM and non-small cell lung cancer (NSCLC) compared with healthy controls.[18] At this stage, it is not clear whether the change in the balance of suppressive Tregs vs. CD8 potential effector cells has any meaningful repercussions as far as the antitumor response is concerned; our study was not designed to, or able to test this.

Although we did not specifically examine B cells and NK cells, it has been previously published that pro-apoptotic effects on these cells are not observed in response to GC treatment.[15]

GCs have a wide range of effects on DCs, with the potential to suppress their maturation, disrupt their migration, and induce tolerogenic DC phenotypes.[6,50] GC treatment can prevent DCs from upregulating cell surface expression of MHC class II and the co-stimulatory molecules CD86 (B7.2), CD80 (B7.1), CD83 and CD40, in response to activating stimuli.[51-53] Decreased expression of mRNA for pro-inflammatory cytokines IL-1, IL-6, and IL-12 has also been reported.[54,55] In addition, GCs are known as potent inducers of apoptosis in immature DCs.[54] Although we were not able to unequivocally determine whether loss of DC subsets in response to Dex resulted from cell death or from migration of DCs out of peripheral blood and into tissue, the evidence from the literature above suggests that increased maturation and relocation of DCs was not the cause of observed changes in the frequency of myeloid (BDCA-1^+) and plasmacytoid (BDCA-2^+) DC subsets following Dex. Indeed, we saw no change in expression of maturational markers such as MHC class II, CD80, CD86, and

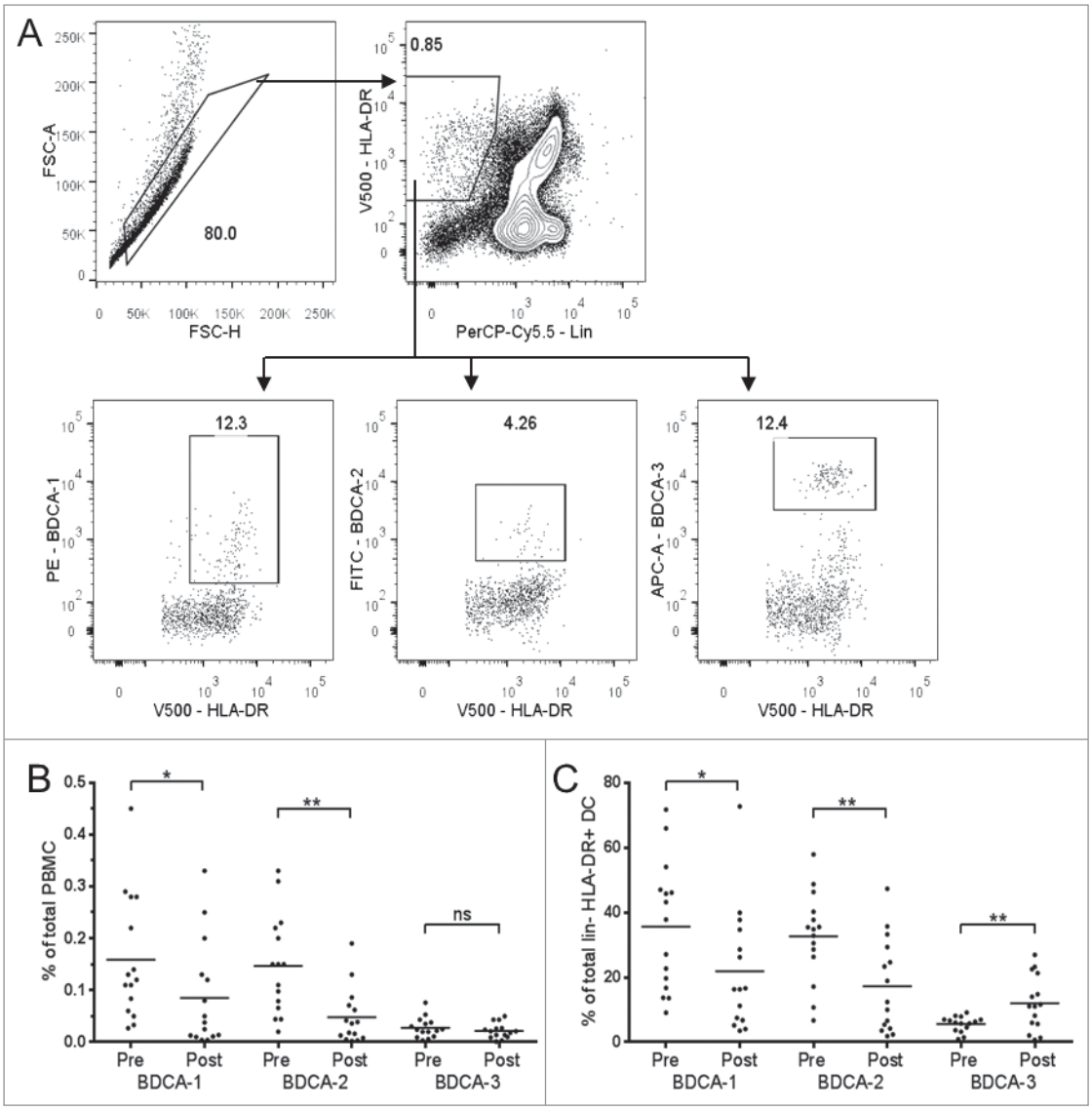

Figure 5. Effect of Dex treatment on peripheral blood DC subsets. (A) Representative flow cytometry data demonstrating the gating strategy used for dendritic cells. Forward scatter (FSC) area vs. FSC-height was used for doublet discrimination. A "dump" channel was used to gate out dead cells (LIVE/DEAD fixable red viability stain) plus those staining positively with a CD3/CD14/CD16/CD19/CD56 "lin" cocktail. DCs were identified as lin^- $HLA\text{-}DR^+$ cells, and respective DC subpopulations identified by BDCA-1, BDCA-2 or BDCA-3. (B) Compiled flow cytometric data from PBMC analysis, depicting individual patients, showing values before and after administration of dexamethasone. (B—C) DC subsets as a percentage of total PBMCs (B) and $lin^-HLA\text{-}DR^+$ cells (C) in patient samples collected before (pre-Dex) and after (post-Dex) administration of dexamethasone. Each dot represents an individual patient; significant difference between pre-Dex and post-Dex values: $^*p < 0.05$, $^{**}p < 0.001$, paired students t-test.

CD40. However, peripheral blood DC in general express low levels of these markers, with terminal differentiation occurring after migration has occurred, so at this stage a firm conclusion cannot be drawn.[56-58] The observed increase of $BDCA\text{-}3^+$ DCs most likely results from a proportional decrease in the more prevalent $BDCA\text{-}1^+$ and $BDCA\text{-}2^+$ populations.

In the context of the two chemoimmunotherapy phase I clinical trials from which the data in this paper were taken, there are effects of Dex that could potentially affect the activity of the study drug combination. Low-dose cyclophosphamide has been shown to preferentially deplete Tregs in a number of animal tumor models, with greatest efficacy at low doses,[59-65] and also in patients with metastatic melanoma and other advanced cancers.[66-68] Dex affects Treg such that the population remains proportionally constant but undergoes increased proliferation and activation. This would be counter to the desired outcome of Treg depletion by cyclophosphamide. Indeed, Dex-treated murine DC cell lines have been used to induce formation of Tregs,[55] and to subsequently protect against autoimmunity or a graft-vs.-host response.[69] The anti-CD40 study was a clinical translation of a successful pre-clinical treatment in a mouse mesothelioma model.[70] The postulated mechanism of action of this treatment is to activate DCs without the specific requirement for $CD4^+$ T cell help, thereby tipping the balance of a possible antigen-specific $CD8^+$ T cell response away from tolerogenic and toward antitumor cytotoxicity (for a recent review, see ref. 71)[71]. However, due to the modulatory effects on DCs of the particular dose and timing combination of Dex, it may be that any beneficial effects of DC activation are lessened. The potential for DC loss, and/or the prevention of increased CD40 expression on these cells, may have a negative impact on the intended target (and downstream efficacy) of the treatment. However, it should also be noted that terminally differentiated DCs are resistant to the effects of GC and continue to express these maturational markers[72] Finally, GC

treatment has been reported to inhibit TCR signaling itself——providing yet another method to subvert a potentially successful antitumor cellular immune response. [73,74] The primary aim of both of these studies was to assess safety and not efficacy, hence we are not powered to comment on survival. Since neither trial set out to look at the effects of Dex, which was administered to all patients as part of standard care chemotherapy, there are no "Dex-free" control arms with which to compare its effect on patient outcomes in the context of either study.

The data presented here can inform future planning of combined chemoimmunotherapy modalities. It may be that further work will highlight an optimal treatment schedule or dosage of Dex whereby any potentially negative impacts on the mechanisms of chemoimmunotherapy are minimised——enabling current or future treatments to realize their full potential. We recommend further in-depth analysis of the immunological effects of different dosage and timing of Dex, and to examine the duration of the effects observed here. It may be that any negative impacts of Dex on immunotherapy modalities can be minimized by adjusting dose and/or timing of GC administration. We would also recommend consideration of including Dex (or any other appropriate concomitant medications) in future pre-clinical development of chemoimmunotherapy strategies.

Patients/materials and methods

Eligibility criteria

Participants were enrolled on either one of two phase Ib chemoimmunotherapy clinical trials at Sir Charles Gairdner Hospital (Perth, WA, Australia). The first study involved patients that subsequently received treatment with metronomic low dose cyclophosphamide in combination with standard care pemetrexed/platinum doublet therapy. In the second trial, an anti-CD40 agonistic antibody was given in combination with chemotherapy. Eligible patients had a histologically or cytologically confirmed diagnosis of MPM, Eastern Co-operative Oncology Group (ECOG) performance status (PS) of 0−1, and were planned for first-line treatment with platinum and pemetrexed. All participants had measureable disease on thoracic CT scan as defined by the modified RECIST criteria.[75] All had adequate haematological parameters, renal function, and hepatic function. Patients were ineligible if they had previous therapy for MPM (including immunotherapy or investigational agents), radiotherapy to all measurable lesions, symptomatic central nervous system involvement, or a second primary malignancy within the past 10 y. Pregnant or lactating women and patients with other serious medical disorders were also ineligible. The protocols were approved by the Institutional Human Research Ethics Committee and all patients provided written informed consent. Study drug and partial funding to conduct the clinical trials were provided by Pfizer Oncology Australia, the National Health and Medical Research Council Australia, and the Cancer Council Western Australia. Clinical trial registration numbers on the Australia New Zealand Clinical Trials Registry were ACTRN12609000294257 and ACTRN12609000260224.

Treatment administration

Dex (Aspen Pharmacare) was given as standard prophylactic medication for chemotherapy. 2 × 4 mg oral doses were given the day before, and 1 × 4 mg oral dose given the day of (but

Table 2. List of antibodies.

Antigen	Fluor	Clone	Isotype	Supplier	Catalog #	Antibody registry #	Panel	Dilution
CD4	AF488	RPA-T4	ms IgG1	BD PharMingen	557695	396804	1	1/20
CD3	PE	SK7	ms IgG1	BD PharMingen	347347	400287	1	1/50
CD8	PECy7	RPA-T8	ms IgG1	BD PharMingen	555368	395771	1	1/50
CD4	APC-H7	RPA-T4	ms IgG1	BD PharMingen	560158	1645478	2	1/40
Foxp3	PE	PCH101	rt IgG2a	eBioscience	12-4776-42	1518782	2	1/20
CD25	APC	M-A251	ms IgG1	BD PharMingen	555434	398598	2	1/5
CD127	PECy7	eBioRDR5	ms IgG1	eBioscience	25-1278-42	1659672	2	1/100
Ki67	FITC	B56	ms IgG1	BD PharMingen	556026	396302	2,3	1/10
ICOS	PerCP-Cy5.5	C398.4A	ha IgG	Biolegend	313518	10641280	2,3	1/80
CD14	V500	M5E2	ms IgG2a	BD PharMingen	561391	10611856	2,3	1/80
CD19	V500	HIB19	ms IgG1	BD PharMingen	561121	10562391	2,3	1/80
CD3	V450	UCHT1	ms IgG1	BD PharMingen	560365	1645570	2,3	1/40
CD8	APC-H7	SK1	ms IgG1	BD PharMingen	560179	1645481	3	1/40
CD38	AF647	HIT2	ms IgG1	Biolegend	303514	493090	3	1/40
HLA-DR	PECy7	L243	ms IgG2a	BD PharMingen	335795	399973	3	1/80
Bcl^{-}2	PE	Bcl^{-}2/100	ms IgG1	BD PharMingen	556535	396455	3	1/10
BDCA-1(CD1c)	PE	AD5-8E7	ms IgG1	Miltenyi	130-090-508	244316	4	1/10
BDCA-2(CD303)	FITC	AC144	ms IgG1	Miltenyi	130-090-510	244167	4	1/10
BDCA-3(CD141)	APC	AD5-14H12	ms IgG1	Miltenyi	130-090-907	244170	4	1/10
HLA-DR	V500	G46.6	ms IgG2a	BD PharMingen	561224	10563765	4	1/100
CD3	PerCP-Cy5.5	SK7	ms IgG1	Biolegend	344808	10640736	4	1/100
CD14	PerCP-Cy5.5	HCD14	ms IgG1	Biolegend	325622	893250	4	1/100
CD16	PerCP-Cy5.5	3G8	ms IgG1	Biolegend	302028	893262	4	1/100
CD19	PerCP-Cy5.5	HIB19	ms IgG1	Biolegend	2072925	115534	4	1/100
CD56	PerCP-Cy5.5	HCD56	ms IgG1	Biolegend	318322	893389	4	1/100
CD83	PECy7	HB15e	ms IgG1	BD PharMingen	561132	10562565	4	1/20
CD80	V450	L307.4	ms IgG1	BD PharMingen	560442	1645583	4	1/20
CD40	APC-H7	5C3	ms IgG1	BD PharMingen	561211	10584325	4	1/10

List of monoclonal antibodies used for flow cytometric staining. Panels were used for absolute cell counts of whole blood (panel 1), Treg staining of PBMC (panel 2), CD8 T cell staining of PBMC (panel 3) or dendritic cell staining of PBMC (panel 4). Abbreviations: AF = AlexaFluor, ms = mouse, rt = rat, ha = hamster.

prior to), drawing of the second study blood sample and receiving chemotherapy.

Assessment of immunological parameters

"Pre-Dex" peripheral blood samples were collected at enrolment, followed within 14 d by collection of the "Post-Dex" blood sample prior to chemotherapy administration. Blood was collected into BD K2EDTA Vacutainers (BD Diagnostics). Whole blood was analyzed by flow cytometry on the day of collection to obtain absolute volumetric cell counts (cells per μL) of $CD3^+CD8^+$ and $CD3^+CD4^+$ T cells. Blood samples were stained using the "panel 1" antibodies described in Table 2, fixation and red blood cell lysis was performed using BD FACS lysing buffer, and data collected by three-color analysis using a Millipore Guava and Guava ExpressPro Software.

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque™ density gradient centrifugation following the manufacturer's instructions, then cryopreserved in 1mL aliquots of RPMI (Invitrogen, cat# 11875-119) supplemented with 10% FCS, 20mM HEPES and 10% DMSO, at 2×10^6 cells/mL. Cells were placed in a "Mr Frosty" container and frozen at −80°C for 24−48 h before transferring to storage in liquid nitrogen. PBMC from any one patient were analyzed simultaneously by flow cytometry once samples from all time points were available. Prior to analysis, PBMCs were thawed for 1 min in a 37°C water bath and washed once in RPMI, followed by two washes in PBS. Dead cells were identified using LIVE/DEAD Fixable Dead Cell Stain Kit (Molecular Probes, cat. # L34957 (Aqua) or L23102 (Red)). Antibodies were used in three further staining panels, as described in Table 2. Data was collected on a BD FACScanto II, using BD FACSDiva software (BD Biosciences), and analyzed using FlowJo software (Tree Star Inc.).

Statistical considerations

Sample sizes were calculated for each of the studies independently and the patient numbers were derived from those available; a formal power calculation was not performed for this sub-study. Results describing the mean of data at pre-Dex and post-Dex timepoints are reported ± standard deviation. *P* values reporting statistical significance were calculated using the paired t-test in Prism 6 (GraphPad Software). *p* values on figures are represented as: $^*<0.05$, $^{**}<0.01$, $^{***}<0.001$, ns>0.05 (not significant).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors acknowledge the facilities, and the scientific and technical assistance of the Australian Microscopy & Microanalysis Research Facility at the Centre for Microscopy, Characterisation & Analysis, The University of Western Australia, a facility funded by the University, State and Commonwealth Governments.

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