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# Cyclophosphamide Promotes Chronic Inflammation–Dependent Immunosuppression and Prevents Antitumor Response in Melanoma

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Low-dose cyclophosphamide (CP) therapy induces immunogenic tumor cell death and decreases regulatory T cell (Treg) numbers in mice with transplantable tumors. Using the *ret* transgenic murine melanoma model that resembles human melanoma, we detected no beneficial antitumor effects with such treatment, despite a decrease in Tregs. On the contrary, low-dose CP enhanced the production of chronic inflammatory mediators in melanoma lesions associated with increased accumulation of $Gr1^+CD11b^+$ myeloid-derived suppressor cells (MDSCs), which exhibit elevated suppressive activity and nitric oxide (NO) production as well as inhibition of T-cell proliferation. Moreover, the frequencies of $CD8^+$ T cells in the tumors and their ability to produce perforin were decreased. To study whether the observed CP-induced MDSC expansion and activation also occurs under chronic inflammatory tumor-free conditions, mice exhibiting chronic inflammation were treated with CP. Similar to tumor-bearing mice, CP-treated inflamed mice displayed elevated levels of MDSCs with enhanced production of NO, reactive oxygen species, and a suppressed *in vivo* natural killer (NK) cell cytotoxic activity indicating CP effects on the host immune system independent of the tumor. We suggest that melanoma therapy with low-dose CP could be efficient only when combined with the neutralization of MDSC immunosuppressive function and chronic inflammatory microenvironment.

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## INTRODUCTION

Human malignant melanoma, known for its poor response to therapeutics, is one of the fastest growing cancers (MacKie *et al.*, 2009). Chemotherapy belongs to the standard melanoma treatment, although its efficacy is low. Moreover, chemotherapeutics in maximum tolerated doses can induce immunosuppression in tumor-bearing hosts (Gerber and Steinberg, 1976; Athanasiadou *et al.*, 2002; Weiner and Cohen, 2002). In contrast, low-dose chemotherapy stimulated antitumor immunity (Obeid *et al.*, 2007; Zitvogel *et al.*, 2008). In particular, anthracyclins increased the tumor cell immunogenicity by inducing immunogenic cell death through efferocytosis (Obeid *et al.*, 2007). In contrast to classical apoptosis, which is considered as tolerogenic and anti-inflammatory (Zitvogel *et al.*, 2008), efferocytosis mediated by calreticulin (CRT) and LRP/91/Rac-1 pathway showed its immunostimulatory potential (Gardai *et al.*, 2006).

Tumor progression is driven by chronic inflammation developed in the tumor microenvironment and represented by infiltrating leukocytes and soluble mediators (Baniyash, 2006; Gabrilovich and Nagaraj, 2009; Ostrand-Rosenberg and Sinha, 2009; Rook and Dalgleish, 2011). These mediators support tumor development by stimulating pro-tumor mutations, resistance to apoptosis, and angiogenesis (Tan and Coussens, 2007; Mantovani *et al.*, 2008). Moreover, some of these factors induce the expansion and activation of immunosuppressive cells, such as myeloid-derived suppressor cells (MDSCs) and M2 macrophages, in tumor lesions and in the periphery (Baniyash, 2006; Serafini *et al.*, 2006; Gabrilovich and Nagaraj, 2009; Ostrand-Rosenberg, 2010).

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*Abbreviations: BCG, Bacillus Calmette–Guérin; BM, bone marrow; CP, cyclophosphamide; CRT, calreticulin; DC, dendritic cell; LN, lymph node; MDSC, myeloid-derived suppressor cell; NK cell, natural killer cell; NO, nitric oxide; ROS, reactive oxygen species; Treg, regulatory T cell*

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Cyclophosphamide (CP) at low doses has been recently applied in melanoma treatment combined with various immunotherapies (Koike *et al.*, 2008; Quan *et al.*, 2008). In animal models, low-dose CP showed an antiangiogenic activity by killing circulating endothelial progenitors (Bertolini *et al.*, 2003) and decreased nitric oxide (NO) concentrations in serum (Loeffler *et al.*, 2005). Moreover, CP reduced numbers and suppressed the function of regulatory T cells (Tregs) in tumor-bearing hosts (Lutsiak *et al.*, 2005; Motoyoshi *et al.*, 2006). Furthermore, CP administration to tumor-bearing mice not only stimulated the mobilization of bone marrow (BM) dendritic cells (DCs) (Salem *et al.*, 2009), but also activated their antigen-processing machinery by inducing pre-apoptotic surface translocation of CRT on tumor cells (Obeid *et al.*, 2007; Sistigu *et al.*, 2011). However, the CP effects on chronic inflammatory environment that drives the tumor progression are poorly investigated.

In this study, we used the *ret* transgenic mouse model that, in contrast to transplantation models, shows similarity to human melanoma (Kato *et al.*, 1998; Umansky *et al.*, 2008). Tumor-bearing mice developed metastases in lymph nodes (LNs), lungs, liver, brain, and BM (Umansky *et al.*, 2008). This metastatic profile resembles that of human melanoma (Houghton and Polsky, 2002; Patnana *et al.*, 2011). We found that although low-dose CP induced pre-apoptotic surface translocation of CRT on Ret melanoma cells *in vitro* and decreased Treg numbers in transgenic tumor-bearing mice *in vivo*, it could not delay the melanoma progression. CP stimulated the production of chronic inflammatory mediators associated with an elevation of MDSC frequencies and immunosuppressive activity in melanoma lesions. Mice with chronic inflammation induced by Bacillus Calmette–Guérin (BCG) (Vaknin *et al.*, 2008) displayed a similar enhancement of MDSC frequencies and functions. Our data suggest that an enhancement of antitumor T-cell reactivity because of Treg depletion by low-dose CP can be efficiently blocked by activated MDSCs induced by chronic inflammation enhanced with CP treatment.

## RESULTS

### Low-dose CP induces immunogenic melanoma cell death leading to increased DC maturation *in vitro*

Ret melanoma cells established from skin melanomas of *ret* transgenic mice were treated *in vitro* with CP at low concentrations (5 or 10 mM). Cytotoxic effects were estimated by the staining with Annexin V and propidium iodide (PI) (Figure 1). CP at both concentrations induced a significant increase in the numbers of dead melanoma cells at day 2 after treatment (Figure 1b). Analysis of immunogenic cell death marker, CRT, revealed its appearance on some nonapoptotic AnnexinV$^-$PI$^-$ Ret cells (i.e., before phosphatidylserine translocation) at day 2 of coincubation with 5 mM but not 10 mM CP (Figure 1c). Moreover, these cells displayed a significantly

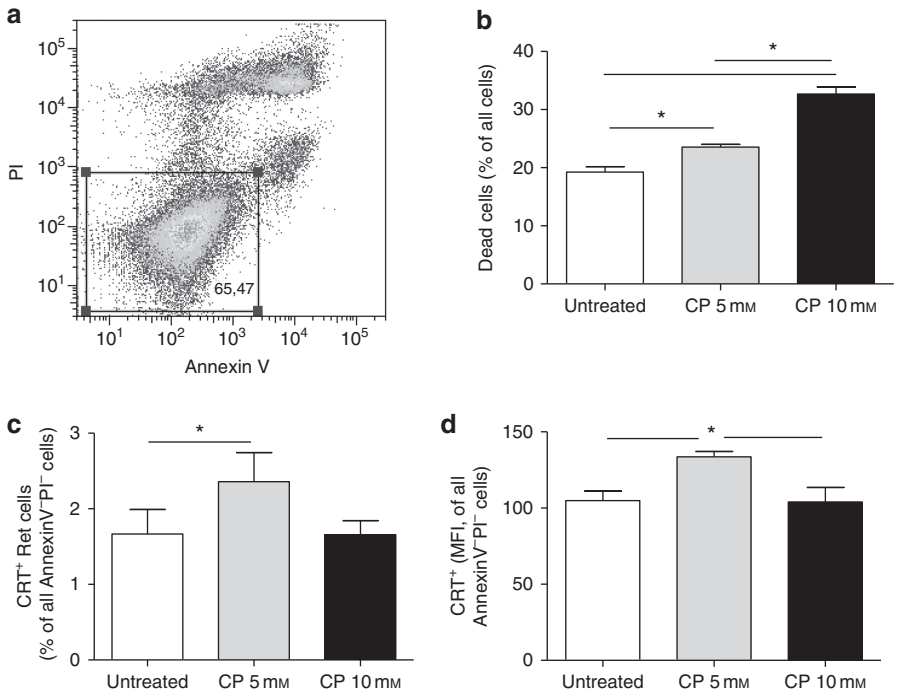


**Figure 1. Low-dose cyclophosphamide (CP) induces the calreticulin (CRT) expression on Ret melanoma cells.** Tumor cells were treated with CP for 48 hours followed by flow cytometry. (**a**) A representative dot plot of cells treated with 10 mM CP is shown. (**b**) Cumulative data for dead cells are expressed as the percentage of apoptotic and necrotic cells (AnnexinV$^+$PI$^+$) among the total treated population. PI, propidium iodide. (**c**) Cumulative data for the surface CRT expression on live cells (AnnV$^-$PI$^-$) are shown as the percentage of CRT$^+$ cells within total live cells. AnnV, Annexin V. (**d**) The level of CRT expression on live cells is presented as mean fluorescence intensity (MFI). The results from **b** to **d** are representative of three independent experiments conducted in triplicate with similar data. Error bars represent SE. *$P<0.05$.

higher level of CRT expression (Figure 1d). Therefore, an increase in CRT expression on melanoma cells upon coculture with CP was detected before the beginning of apoptosis, suggesting an induction of immunogenic cell death.

To test directly the increased immunogenicity of Ret cells upon treatment with 5 mM CP, we cocultured these pretreated cells with DCs generated from normal BM precursors. The frequency of $CD11c^+$MHC class $II^+$ DCs was elevated upon coculture with pretreated melanoma cells, as compared with DCs coincubated with untreated Ret cells (Supplementary Figure S1a online). Interestingly, pretreatment of melanoma cells with 10 mM CP failed to upregulate the major histocompatibility complex class II expression on DCs (Supplementary Figure S1b online) that correlated with the failure of CP at this concentration to induce CRT on melanoma cells (Figure 1c and d). Furthermore, DCs cocultured with tumor cells pretreated with 5 mM CP displayed an increased expression of costimulatory molecules, CD80 and CD40 (Supplementary Figure S1b and c online). Another costimulatory molecule, CD86, was also upregulated under similar coculture conditions (data not shown).

### Low-dose CP downregulates Tregs *in vivo*

As low doses of CP (1–2.5 mg per mouse) can decrease Treg numbers and suppressive activity in tumor-bearing mice (Lutsiak *et al.*, 2005; Motoyoshi *et al.*, 2006), we tested such CP effect on *ret* transgenic mice with skin melanoma. At day 7 after the therapy onset, Treg frequencies in skin tumors were decreased in a dose-dependent manner, as compared with those in untreated animals (Figure 2a). A significant reduction of Treg frequencies was also detected in the spleen of these mice (Figure 2b). As melanoma microenvironment is characterized by chronic inflammation, we applied a murine model of BCG-induced chronic inflammation (Vaknin *et al.*, 2008) to investigate CP-mediated Treg alterations in the absence of tumors. Similar to tumor-bearing animals, CP injection at both the low doses significantly reduced Treg frequencies in the peripheral blood (Figure 2c) and spleen (Figure 2d) of inflamed mice, as compared with CP-untreated animals.

### CP therapy provides no antitumor effects and enhances chronic inflammatory mediators

Next, we studied the effects of low-dose CP (1 or 2.5 mg per mouse) on melanoma progression in transgenic mice. Despite the potential induction of tumor cell immunogenicity and the decrease in Treg frequencies, the survival of CP-treated tumor-bearing mice was not significantly longer than that of untreated mice (Figure 3a). Furthermore, we found no delay in melanoma progression if low-dose CP therapy was combined with the vaccination by a peptide derived from the melanoma-associated antigen tyrosinase-related protein-2 (Figure 3a).

Next, we assessed the pattern of different chronic inflammatory mediators in tumor lesions. A significant increase in concentrations of GM-CSF, IL-1β, IL-5, IL-10, IFN-γ, and tumor necrosis factor-α was found in lysates from skin tumors and metastatic LNs of mice treated with a single CP injection (2.5 mg per mouse), as compared with untreated tumor-bearing animals (Figure 3b and c). Such accumulation of inflammatory factors could support the MDSC recruitment and activation in tumor lesions (Baniyash, 2006; Gallina *et al.*, 2006; Gabrilovich and Nagaraj, 2009; Ostrand-Rosenberg and Sinha, 2009; Meyer *et al.*, 2011).

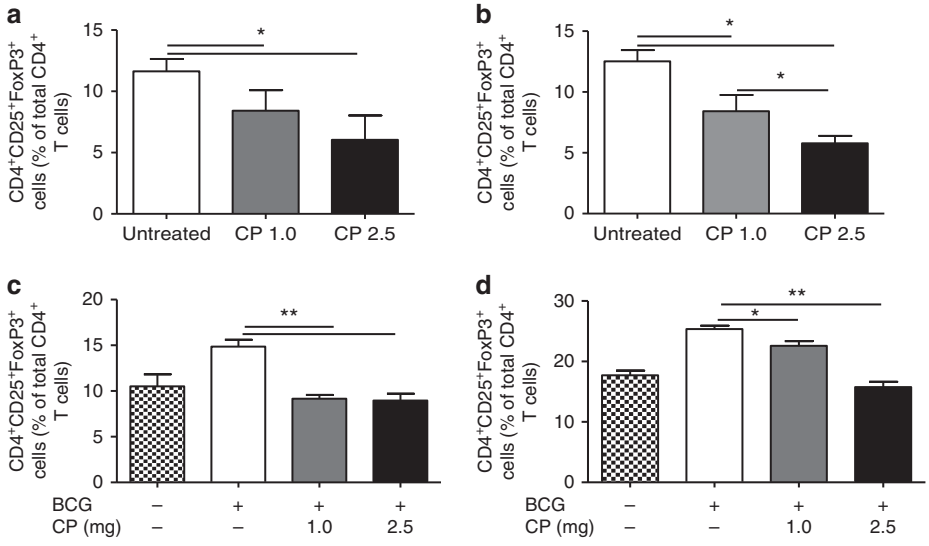


**Figure 2. Low-dose cyclophosphamide (CP) downregulates regulatory T cells (Tregs) in tumor-bearing and inflamed mice.** Mice with macroscopic tumors or with chronic inflammation induced by Bacillus Calmette–Guérin (BCG) were injected intraperitoneally (i.p.) with low-dose CP (1 or 2.5 mg per mouse). At day 7, Tregs were measured by flow cytometry in (**a**) primary tumors and (**b**) spleens of tumor-bearing mice or in the (**c**) peripheral blood and (**d**) spleen of inflamed mice. Data are shown as the percentage of $CD4^+CD25^+FoxP3^+$ Tregs within total $CD4^+$ T cells (5–10 mice per group). Three independent experiments with similar results were conducted. Error bars represent SE. $^*P<0.05$; $^{**}P<0.001$.

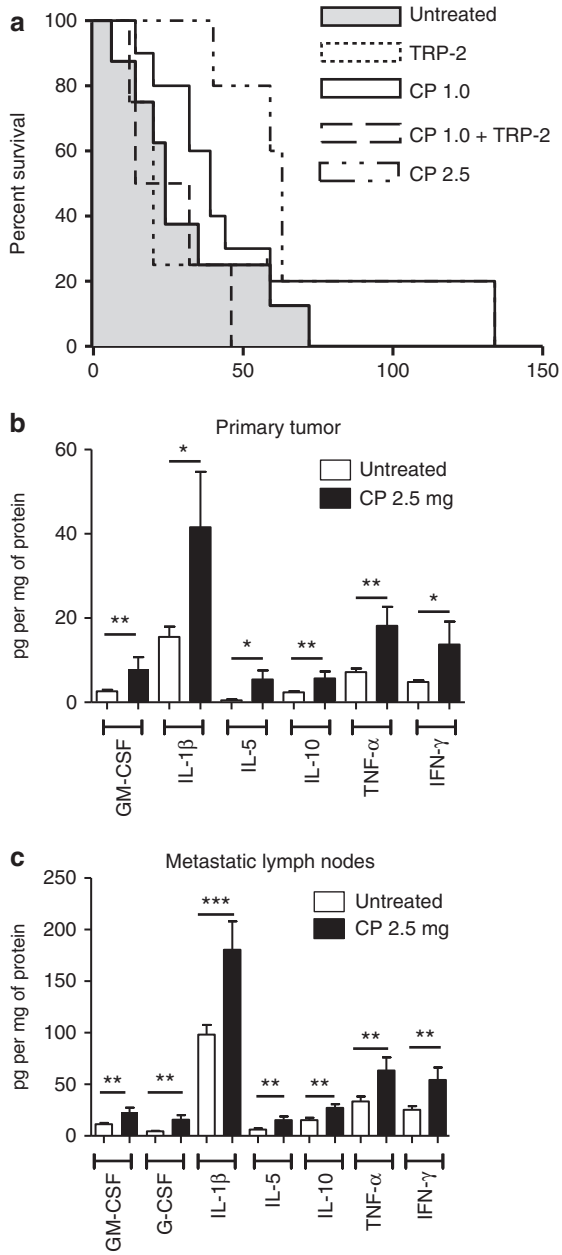


**Figure 3. Effects of low-dose cyclophosphamide (CP) therapy on melanoma progression and chronic inflammatory factors in tumor-bearing mice.** Mice with macroscopical tumors were treated with a single intraperitoneal (i.p.) injection of low-dose CP. (**a**) Survival of mice (10 animals per group) is shown as the Kaplan–Meier curve. (**b, c**) Samples were taken at day 21 upon injection of 2.5 mg CP. Levels of inflammatory mediators were detected in (**b**) primary tumors and (**c**) metastatic lymph nodes (LNs) by Bio-Plex assay (9–16 mice per group). Data are expressed as pg per mg protein. Three independent experiments were conducted with similar data. Error bars represent SE. TNF-α, tumor necrosis factor-α; TRP-2, tyrosinase-related protein-2. $*P<0.05$; $**P<0.01$; $***P<0.001$.

### CP-induced inflammation mediates an elevation of MDSC frequencies

$Gr1^+CD11b^+$ MDSCs were quantified in skin tumors and spleens of transgenic mice treated with low-dose CP (Figure 4a–c). We demonstrated a substantial accumulation of MDSCs among leukocytes infiltrating melanomas at day 21 after the therapy with 2.5 mg CP, as compared with untreated mice (Figure 4b). Moreover, both 1 and 2.5 mg of CP upregulated MDSC frequencies in the spleen of tumor-bearing mice (Figure 4c). To investigate whether the CP-induced MDSC accumulation also occurs under tumor-independent chronic inflammatory conditions, we treated mice exhibiting BCG-induced chronic inflammation with a single injection of low-dose CP (1 or 2.5 mg per mouse). Mice challenged with BCG alone displayed an MDSC accumulation in both the peripheral blood (Figure 4d) and spleen (Figure 4e). CP treatment resulted in a further MDSC elevation in the peripheral blood and in a tendency of increase in the spleen as compared with animals injected with BCG alone (Figure 4d and e).

Next, we tested whether CP could modulate in healthy mice (in the absence of chronic inflammation) the frequency of immature $Gr1^+CD11b^+$ cells, which are considered as MDSCs in tumor-bearing animals (Gabrilovich and Nagaraj, 2009). Upon an injection of 2.5 mg CP, these cells were expanded in the spleen, peripheral blood, and LN, but not in BM, as compared with untreated mice (Figure 4f). No alterations of cytokine or growth factor levels were detected in the lymphoid organs of CP-treated healthy mice (data not shown).

### MDSCs from CP-treated mice display an enhanced suppressive activity

As NO and reactive oxygen species (ROS) are considered to be among the key factors of the MDSC-induced immunosuppression (Serafini *et al.*, 2006; Gabrilovich and Nagaraj, 2009; Ostrand-Rosenberg, 2010), their production was assessed with low-dose CP therapy in both mouse models (Figure 5). A significant increase of NO production in tumor-infiltrating MDSCs from mice treated with 2.5 mg per mouse was observed as compared with untreated animals (Figure 5b). Similar findings were obtained in mice with BCG-induced chronic inflammation upon an injection of low-dose CP. Levels of NO and ROS production in MDSCs from the peripheral blood of CP-treated mice were substantially higher than in chronically inflamed CP-untreated animals (Figure 5c and d). However, in spleen MDSCs, although the NO and ROS production was already significantly enhanced by the chronic inflammation, no further elevation of both factors was detected after the CP therapy (Figure 5e and f). In healthy mice, immature $CD11b^+Gr1^+$ cells showed no enhanced ROS production with CP treatment (data not shown).

### CP treatment aggravates chronic inflammation-induced alterations in immune effector cells

Next, we studied whether the CP-mediated stimulation of MDSCs could enhance their ability to suppress T-cell and natural killer (NK) cell functions. MDSCs from tumors of animals treated with 2.5 mg CP were cocultured with stimulated syngeneic normal splenocytes. Such MDSCs suppressed T-cell proliferation stronger than those isolated from CP-untreated tumor-bearing mice (Figure 6a and b). Analyzing tumor-infiltrating $CD8^+$ T lymphocytes in CP-treated mice (1 mg per mouse), we found a significant reduction in the

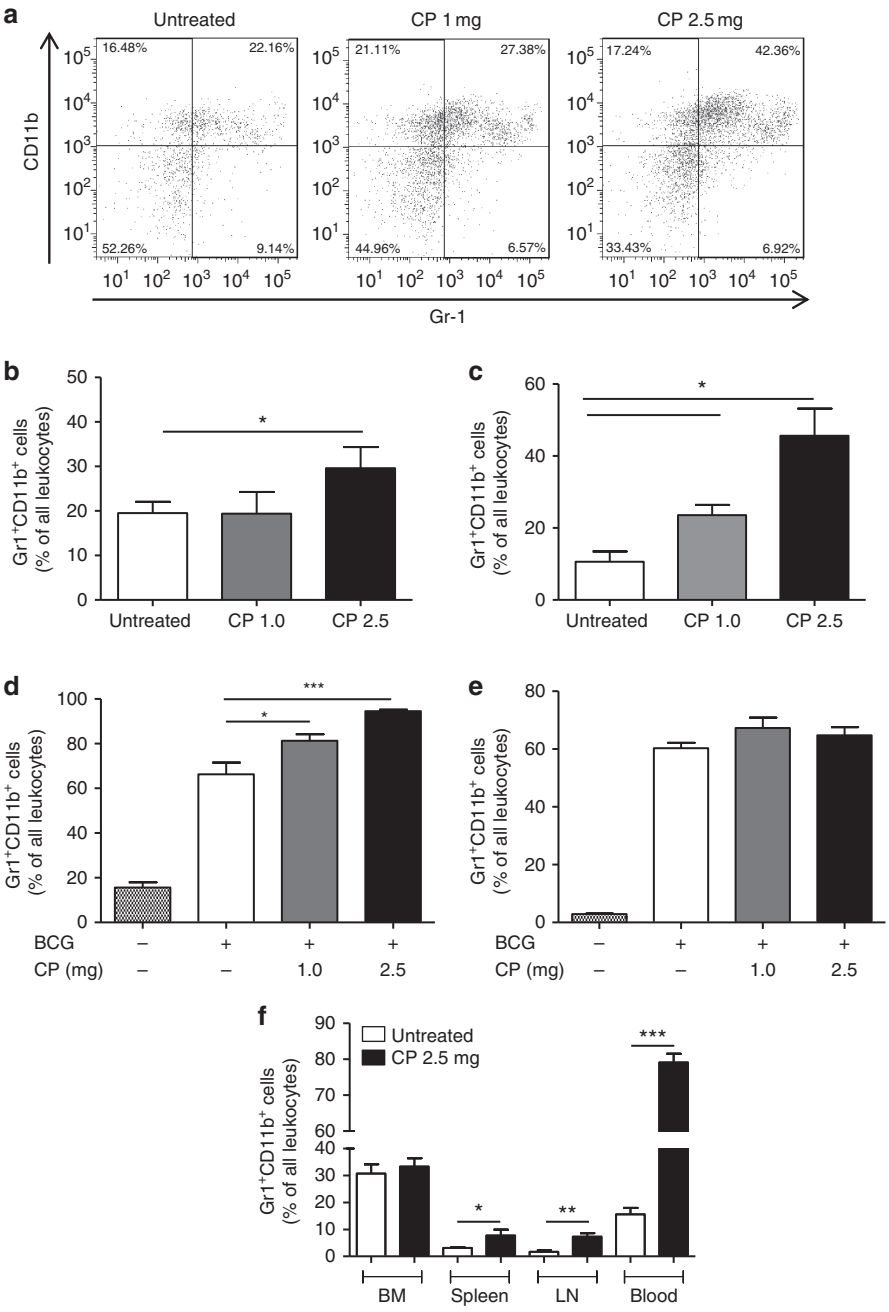


**Figure 4. Low-dose cyclophosphamide (CP) mediates an enrichment of myeloid-derived suppressor cells (MDSCs) in transgenic melanoma and chronic inflammatory models.** Tumor-bearing, inflamed, and normal mice were treated with low-dose CP. MDSCs were analyzed by flow cytometry. (**a**) Representative dot plots of primary tumor from untreated mice or treated with 1 or 2.5 mg CP. (**b, c**) Cumulative data for MDSCs in (**b**) primary tumors and (**c**) spleens from tumor-bearing mice (8–16 mice per group) or in the (**d**) peripheral blood and (**e**) spleens from animals with chronic inflammation (4–6 mice per group) or (**f**) healthy mice (4–8 mice per group) are expressed as the percentage within leukocytes. Three independent experiments were conducted with similar results. BCG, Bacillus Calmette–Guérin; BM, bone marrow; LN, lymph node. Error bars represent SE. $*P<0.05$; $**P<0.01$; $***P<0.001$.

frequency of these cells as compared with untreated mice (Supplementary Figure S2a online). Moreover, we observed a significant downregulation of intracellular perforin concentrations in $CD8^+$ tumor-infiltrating T lymphocytes isolated from CP-treated mice (2.5 mg per mouse), suggesting their diminished cytotoxic function ($P<0.05$; Supplementary Figure S2b online). A strong tendency for a decrease in perforin levels was also detected in $CD8^+$ T cells from metastatic LNs of these mice (Supplementary Figure S2b online).

In the BCG-induced chronic inflammatory model, we focused on the modulation of NK cell functions. Splenocytes from syngeneic (C57BL/6) and allogeneic (BALB/c) mice were

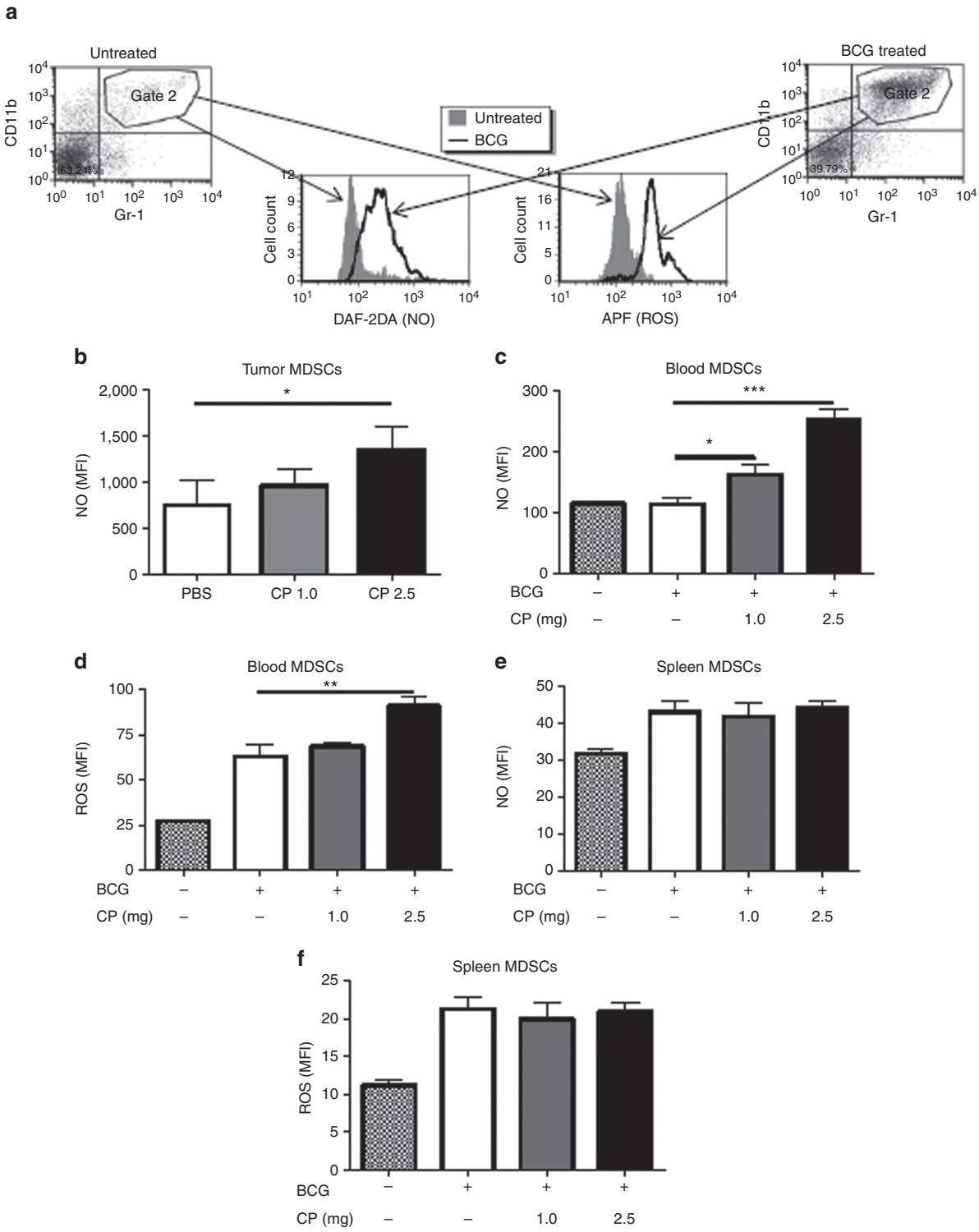


**Figure 5. Low-dose cyclophosphamide (CP) stimulates nitric oxide (NO) and reactive oxygen species (ROS) production by myeloid-derived suppressor cells (MDSCs).** Mice with tumors or Bacillus Calmette–Guérin (BCG)-induced chronic inflammation were treated intraperitoneally (i.p.) with low-dose CP. NO and ROS production was measured in MDSCs by flow cytometry. (**a**) Representative dot plots and histograms showing the gating strategy for NO and ROS measurements in the peripheral blood of inflamed mice. (**b**) Levels of NO production in tumor-infiltrating MDSCs are expressed as mean fluorescence intensity (MFI; 4–6 mice per group). PBS, phosphate-buffered saline. Levels of NO and ROS in MDSCs from the (**c, d**) peripheral blood and (**e, f**) spleen of chronically inflamed mice are presented as MFI (4–6 mice per group). Three independent experiments were conducted with similar data. Error bars represent SE. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

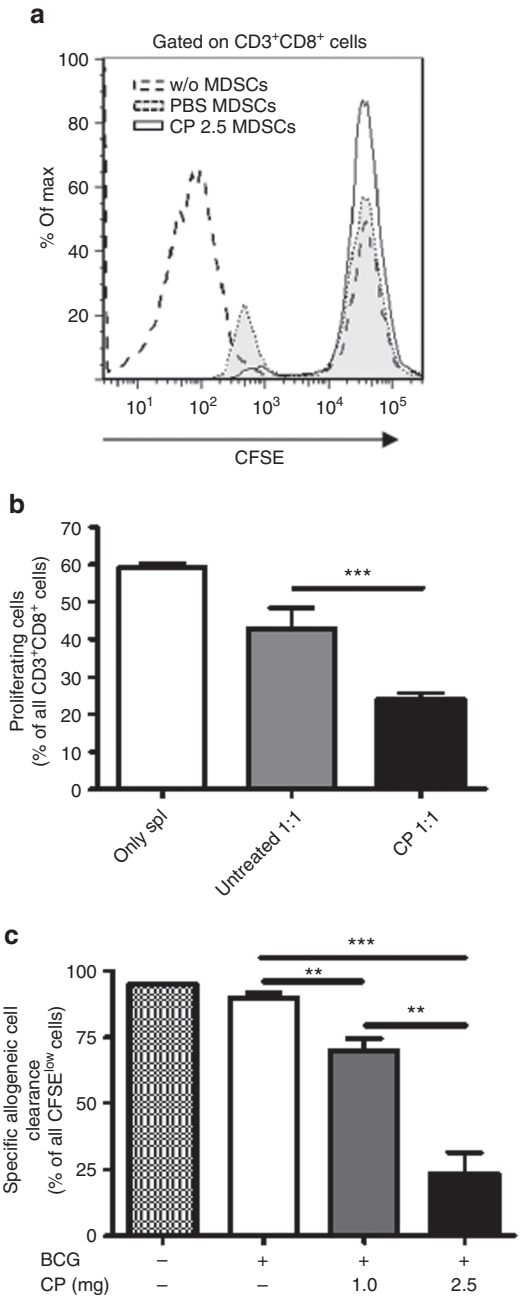


**Figure 6. Low-dose cyclophosphamide (CP) stimulates myeloid-derived suppressor cell (MDSC) immunosuppressive activity and downregulates effector immune cells.** (**a, b**) MDSCs from tumors of CP-treated or untreated mice were co-incubated with activated normal splenocytes. (**a**) Representative histograms for T-cell proliferation. (**b**) Data summarizing four independent experiments with similar results are presented as the percentage of proliferating T cells incubated alone (Only spl), or with MDSCs (8–13 mice per group). (**c**) CP-treated or untreated inflamed mice were injected with carboxyfluorescein succinimidyl ester (CFSE)-labeled allogeneic ($CFSE^{low}$) and syngeneic ($CFSE^{high}$) splenocytes. Natural killer (NK) cell cytotoxic activity in the peripheral blood was assessed 18 hours upon splenocyte administration. Results are shown as the percentage of allogeneic cell clearance (five mice per group). Three independent experiments were conducted with similar results. Error bars represent SE. BCG, Bacillus Calmette–Guérin; PBS, phosphate-buffered saline. $^{**}P<0.01$, $^{***}P<0.001$.

labeled with low and high carboxyfluorescein succinimidyl ester concentrations, respectively, and administered into C57BL/6 mice. The immunosuppression was verified by the impaired ability of recipients to clear allogeneic cells by cytotoxic NK cells. To test the contribution of other cells in this effect, we depleted NK cells that resulted in almost complete retention of allogeneic cells (data not shown). A substantial decline of allogeneic cell clearance was detected in the peripheral blood of chronically inflamed mice upon treatment with low-dose CP as compared with chronically inflamed CP-untreated mice (Figure 6c). A similar tendency of reduced clearance was also observed in the spleen of CP-treated mice (Supplementary Figure S3 online). Moreover, MDSCs isolated from chronically inflamed animals treated with 2.5 mg CP blocked proliferation of cocultured stimulated syngeneic normal T cells (data not shown).

## DISCUSSION

In this study, we highlight the complex effects of CP treatment on the tumor and host's immune system showing that it affects not only tumor cells but displays diverse effects on immune cells. Treatment of Ret melanoma cells *in vitro* with CP at 5 and 10 mM, which were reported to be low (Wang *et al.*, 2008), induced an enhanced CRT expression on $AnnexinV^{-}PI^{-}$ live cells. CRT is involved in cell adhesion, folding of new synthesized glycoproteins, and lectin-like chaperone activity (Obeid *et al.*, 2007). However, in cancer cells, rapid CRT mobilization to the cell surface at the beginning of apoptosis (before the phosphatidylserine translocation) led to its fast recognition by DCs through CD36-$\alpha_v\beta_3$,C1qR receptor (Storkus and Falo, 2007). This phenomenon was described as an immunogenic cell death because the phagocytosis of such apoptotic tumor cells stimulated DCs, promoting antitumor T-cell responses (Obeid *et al.*, 2007). Our data on the partial restoration of DC maturation upon their coculture with pretreated Ret cells suggested a CP-mediated development of immunogenic death in these tumor cells.

The administration of CP at low doses (1–2.5 mg per mouse) (Motoyoshi *et al.*, 2006; Sistigu *et al.*, 2011) *in vivo* was found to reduce frequencies and suppress the function of Tregs in tumor-bearing hosts (Lutsiak *et al.*, 2005; Motoyoshi *et al.*, 2006; Liu *et al.*, 2010; Sistigu *et al.*, 2011). The results presented herein confirm these observations for melanoma-bearing transgenic mice. We also demonstrated a CP-mediated Treg downregulation in mice with chronic inflammation induced by BCG administration, indicating that CP-mediated reduction in Treg frequencies is tumor independent.

However, investigating the effects of low-dose CP on melanoma progression revealed no increase of mouse survival, even though immunogenic tumor cell death was induced and Treg levels were reduced. This lack of antitumor effects led us to suggest that CP modulated immunosuppressive mechanisms in tumor-bearing mice responsible for ineffective antitumor reactivity. One of the critical factors inducing immune suppression and supporting tumor progression is chronic inflammation that develops in the tumor microenvironment (Baniyash, 2006; Tan and Coussens, 2007; Mantovani *et al.*, 2008; Gabrilovich and Nagaraj, 2009;

Ostrand-Rosenberg and Sinha, 2009; Ostrand-Rosenberg, 2010; Rook and Dalgleish, 2011). Using the *ret* transgenic mouse model, we have recently demonstrated an accumulation of various chronic inflammatory factors and MDSCs in melanoma lesions (Zhao *et al.*, 2009; Meyer *et al.*, 2011). In this study, we found that low-dose CP therapy induced a significantly higher production of several key inflammatory mediators, such as GM-CSF, IL-1β, IL-5, IL-10, IFN-γ, and tumor necrosis factor-α, in skin tumors and metastatic LNs than in untreated tumor-bearing animals. As all these factors are important for MDSC expansion and activation (Baniyash, 2006; Gallina *et al.*, 2006; Tu *et al.*, 2008; Gabrilovich and Nagaraj, 2009; Greifenberg *et al.*, 2009; Ostrand-Rosenberg and Sinha, 2009; Ostrand-Rosenberg, 2010; Stathopoulos *et al.*, 2010; Meyer *et al.*, 2011) and as under CP treatment, antitumor responses were not evident, we analyzed MDSCs as being potential targets for the observed immunomodulation. In CP-treated mice, a significant increase in MDSC frequencies was observed in primary tumors, BM, and spleen, as compared with already elevated MDSC numbers in CP-untreated tumor-bearing mice. Increased circulating MDSC frequencies were previously reported in breast cancer patients and mice, with B16 melanoma injected with CP along with doxorubicin (Diaz-Montero *et al.*, 2009; Salem *et al.*, 2010). However, in contrast to our investigation, CP was used in both studies at standard high doses. Although Liu *et al.* (2010) demonstrated an expansion of tumor-infiltrating MDSCs after a single low-dose CP injection, the modulation of MDSC functions and chronic inflammatory microenvironment was not addressed. We found that the MDSC enrichment in tumor-bearing mice with low-dose CP therapy was associated with an increased immunosuppressive activity. Moreover, such treatment stimulated NO production that was detected directly in these cells, which is in contrast to findings by Loeffler *et al.* (2005), who measured reduced NO levels in the serum of tumor-bearing mice after CP treatment. These discrepancies might be because of the differences between transplantation models and the autochthonous model used in our study.

To characterize the role of chronic inflammation in CP-mediated effects on MDSCs, we applied a mouse model of BCG-induced chronic inflammation without the tumor impact (Vaknin *et al.*, 2008). Similar to tumor-generated conditions, a significant increase in MDSC frequencies and enhanced ability to produce immunosuppressive mediators (NO and ROS) with CP administration was demonstrated. Interestingly, although a CP-induced increase in the MDSC population was detected in normal mice, they failed to display an enhanced NO and ROS production. These findings suggest that chronic inflammatory conditions developing in different pathologies could be responsible for the enrichment and activation of MDSCs with low-dose CP therapy. The signaling mechanisms responsible for such CP effects are currently under investigation.

A remarkable MDSC stimulation in both mouse models after low-dose CP treatment was found to be associated with a marked downregulation of $CD8^+$ T-cell and NK cell functions. This could be explained by the increased MDSC capacity to produce NO and ROS, known to induce anergy of reactive T cells (Gabrilovich and Nagaraj, 2009; Ostrand-Rosenberg and Sinha, 2009). Therefore, the immunostimulatory effects of low-dose CP (e.g., a Treg depletion and induction of immunogenic tumor cell death) could be abrogated because of MDSC expansion and activation in developing melanoma. This is in agreement with a recently published report on the inability of low-dose CP to improve immunological and clinical effects of the multipeptide vaccination of melanoma patients (Slingluff *et al.*, 2011), suggesting a possible MDSC activation. Interestingly, another study demonstrated that low-dose CP-induced tumor-specific T-cell responses correlated with improved clinical outcome in breast cancer patients (Ge *et al.*, 2011). These observations raise the question of the predominant involvement of different immunoregulatory cells (MDSCs or Tregs) in the progression of various tumor types that needs further investigation.

Taken together, we found that a single injection of low-dose CP induced an immunogenic melanoma cell death and downregulated Treg frequencies in transgenic melanoma mouse model. Such therapy led to a further accumulation of chronic inflammatory mediators, inducing an enrichment and activation of MDSCs in melanoma lesions that abrogate potential CP antitumor effects. Similar changes were also induced by CP in chronic inflammatory mouse model without any tumor impact. CP-induced enhancement of immunosuppression resulted in the impairment of innate and adaptive immunity as reflected by the downregulation of $CD8^+$ T-cell and NK cell functions. We suggest that tumor immunotherapy with low-dose CP should include neutralization of chronic inflammatory factors and block MDSC immunosuppressive functions, which are enhanced with CP treatment.

## MATERIALS AND METHODS

### Mice

*Ret* transgenic mice provided by Dr I Nakashima (Chubu University, Aichi, Japan) were kept in the specific pathogen-free facility of German Cancer Research Center (Heidelberg, Germany) Experiments were conducted on female and male tumor-bearing mice that were 6–7 weeks of age. Female C57BL/6 mice, 7–8 weeks of age, were purchased from Harlan (Jerusalem, Israel) and kept at the Hebrew University specific pathogen-free facility (Jerusalem, Israel). Animal experiments were conducted in accordance to the government and institutional guidelines and regulations.

### Reagents and antibodies

Medium RPMI-1-640 was purchased from PAA (Coelbe, Germany). CP was from Sigma (Munich, Germany). Diaminofluoresciein-2 diacetate and aminophenyl fluorescein were from Cell Technology (Mountain View, CA). Rat anti-mouse directly conjugated mAbs (CD3-PerCP-Cy5.5, CD4-FITC, CD8-APC-Cy7, CD25-APC, CD45.2-PerCP-Cy5.5, CD11b-PE, Gr1-PE-Cy7, I-$A^b$-FITC, CD40-PE, CD80-PE, CD86-PE, and CD11c-APC) were from BD Biosciences (Heidelberg, Germany). FoxP3 fixation/permeabilization kit, rat anti-mouse Foxp3-PE mAbs, and AnnexinV/PI apoptosis staining kit were from eBioscience (San Diego, CA). Purified rabbit anti-mouse CRT (Santa Cruz, Heidelberg, Germany) and goat anti-rabbit IgG-AlexaFluor 488 (Invitrogen, Karlsruhe, Germany) were also used.

### Mouse model of chronic inflammation

BCG (Difco Laboratories, Detroit, MI) was administrated into C57BL/6 mice, as previously described (Vaknin *et al.*, 2008).

### CP treatment *in vitro* and *in vivo*

Ret cells established from skin melanoma of transgenic mice were incubated with 5 or 10 mM of CP for 24 hours. DCs were generated from the BM of normal mice as described (Shurin *et al*, 2009). Before co-incubation with DCs, CP-treated Ret cells were washed twice. Transgenic tumor-bearing mice received a single intraperitoneal injection of CP (1 or 2.5 mg per mouse). Mice with BCG-induced chronic inflammation were injected intraperitoneally with 1 or 2.5 mg CP 2 days after the second BCG injection. Control groups consisting of tumor-bearing transgenic mice or BCG-treated animals received phosphate-buffered saline.

### Immunization

Tumor-bearing transgenic mice were immunized with the tyrosinase-related protein-2–derived peptide (SVYDFFVWL), as described (Firat *et al*, 1999), simultaneously with the CP administration.

### Flow cytometry

Single-cell suspensions prepared from the tumor, spleen, LN, and BM were treated with Fc-block and mAbs for 30 minutes at 4 °C. Acquisition was performed by multicolor flow cytometry using FACSCanto II with FACSDiva software (both from BD Biosciences) with dead cell exclusion. FlowJo software (Tree Star, Ashland, OR) was used to analyze at least 100,000 events.

### Bio-Plex assay

Snap-frozen tumor and LN samples were disrupted and treated with lysis solution (Bio-Rad, Munich, Germany). Concentrations of inflammatory factors were measured by multiplex technology (Bio-Rad) according to the manufacturer's protocol.

### *In vitro* proliferation assay

$CD11b^+$ cells were isolated from tumors of transgenic mice using $CD11b^+$ MicroBeads isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. The proportion of $Gr1^+CD11b^+$ MDSCs in selected population was ~80%. C57BL/6 splenocytes were labeled with carboxyfluorescein succinimidyl ester (Invitrogen; final concentration of 2.5 μM), stimulated with anti-CD3 and anti-CD28 mAbs (0.5 μg ml$^{-1}$ each), and cocultured with MDSCs in a ratio of 1:1 for 96 hours. T-cell proliferation was evaluated using flow cytometry.

### *In vivo* cytotoxicity assay

Specific clearance of carboxyfluorescein succinimidyl ester–labeled allogeneic splenocytes in C57BL/6 mice was performed and evaluated as described before (Vaknin *et al.*, 2008).

### Statistical analysis

Results were assessed with Student's *t*-test and Mann–Whitney *U*-test using GraphPad Prism software (San Diego, CA). Differences in values at $P<0.05$ were considered significant.

## CONFLICT OF INTEREST

The authors state no conflict of interest.

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## SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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