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Richmond, VA 23298

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14. ABSTRACT
We investigated how autophagy plays a role in accelerating or delaying recurrence of neu-overexpressing mouse mammary carcinoma (MMC) following adriamycin (ADR) treatment, and in affecting response to immunotherapy. We explored two strategies: 1) transient blockade of autophagy with chloroquine (CQ), which blocks fusion of autophagosomes and lysosomes during ADR treatment, and 2) permanent inhibition of autophagy by a stable knockdown of ATG5 ($ATG5_{KD}$), which inhibits the formation of autophagosomes in MMC during and after ADR treatment. We found that while CQ prolonged tumor dormancy, but that stable knockdown of autophagy resulted in early escape from dormancy and recurrence. Interestingly, $ATG5_{KD}$ MMC contained an increased frequency of ADR-induced polyploid-like cells and rendered MMC resistant to immunotherapy. On the other hand, a transient blockade of autophagy did not affect the sensitivity of MMC to immunotherapy. Our observations suggest that while chemotherapy-induced autophagy may facilitate tumor relapse, cell-intrinsic autophagy delays tumor relapse, in part, by inhibiting the formation of polyploid-like tumor dormancy.

15. SUBJECT TERMS
Tumor dormancy, tumor relapse, immunotherapy, immunoediting, autophagy

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1. INTRODUCTION

The objective of the proposal is to understand the role of autophagy in chemotherapy induced tumor dormancy and recurrence.

2. KEYWORDS

tumor dormancy, tumor relapse, immunotherapy, immunoediting, autophagy

3. ACCOMPLISHMENTS

- **What were the major goals of the project?**

  1) Understand the role of autophagy in chemotherapy-induced tumor dormancy (Aim 1)

  2) Understand the role of tumor IFN-gamma Ra in determining tumor recurrence under immune pressure (Aim 2)

- **What was accomplished under these goals?**

  Our observations suggest that while chemotherapy-induced autophagy may facilitate tumor relapse, cell-intrinsic autophagy delays tumor relapse, in part, by inhibiting the formation of polyploid-like tumor dormancy.

  Detailed accomplishments

  Task 1.1) In vitro assay for the induction and evaluation of immunogenic and non-immunogenic apoptosis, autophagy and senescence in MMC and SKBR3 tumor cell lines (Partnering PI)

  Task 1.2) Follow up studies for tumor relapse (Initiating PI); and Evaluation of anti-tumor immune responses, ex vivo (Initiating PI)

  Task 1.3) Tumor challenge studies in FVBN202 mice bearing MMC tumor; chemotherapy with or without blockade of autophagy in vivo (Initiating PI: evaluation of tumor relapse and neu antigen loss); and Evaluation of anti-tumor immune responses, ex vivo (Initiating PI)

  Task 2.1) Generate Atg5 knocked down MMC and perform confirmatory tumor challenge experiments in FVB mice (Partnering PI: generation of knock down tumor cells; Initiating PI: perform animal studies)

Autophagy-deficient tumor cells become less susceptible to ADR-induced apoptosis. Autophagy-deficient MMC ($ATG5^{KD}$ MMC) or autophagy-competent MMC (MMC) were treated with a single dose of ADR alone (1 uM ADR for 2 hrs). Tumor cells were analyzed by Annexin V/PI staining prior to treatment (Day 0) or three days after the treatment (Day 4). Experiments were performed in triplicates (Figure 1).

ADR-induced tumor dormancy in autophagy deficient tumor cells show an increased viability as well as an increased polyploid-like morphology compared with autophagy competent tumor cells, in vitro. MMC or ATG5^{KD} MMC tumor cells (3 million cells, Day 0) were treated with 3 daily doses of ADR (1uM for 2 hrs), and viable cells were counted at week 3 using trypan blue exclusion. Data represent triplicate experiments. Autophagy deficient tumor cells were more resistant to ADR treatment than autophagy competent tumor cells as the frequency of viable cells remained higher 4 days after ADR treatment (Figure 1A).

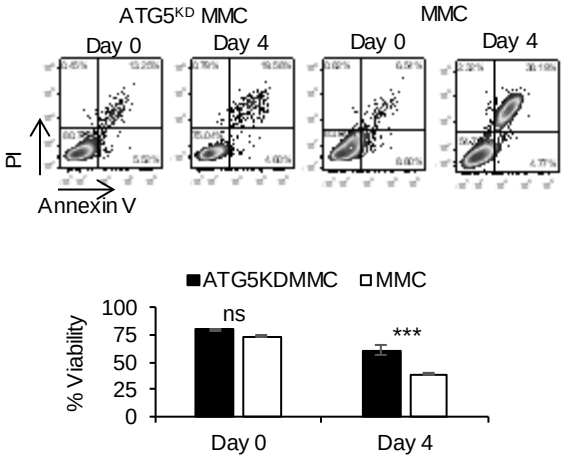
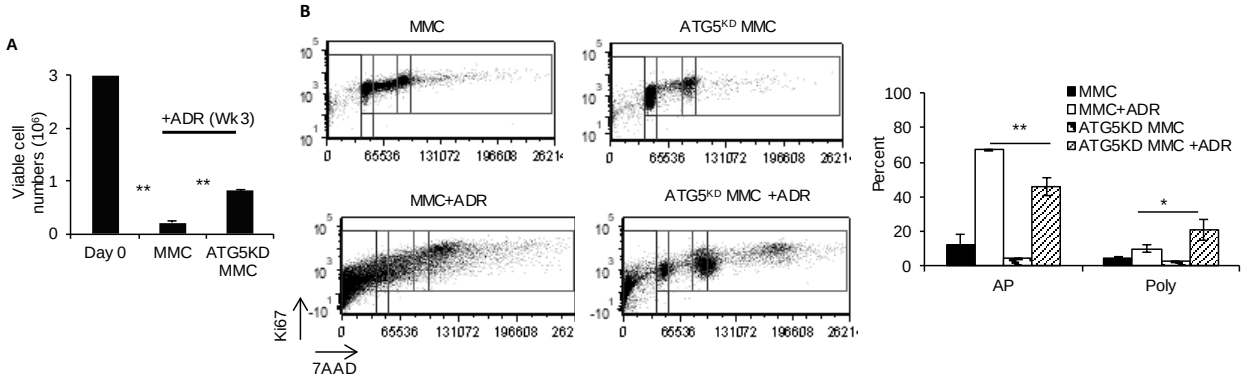


Figure 1

Figure 2

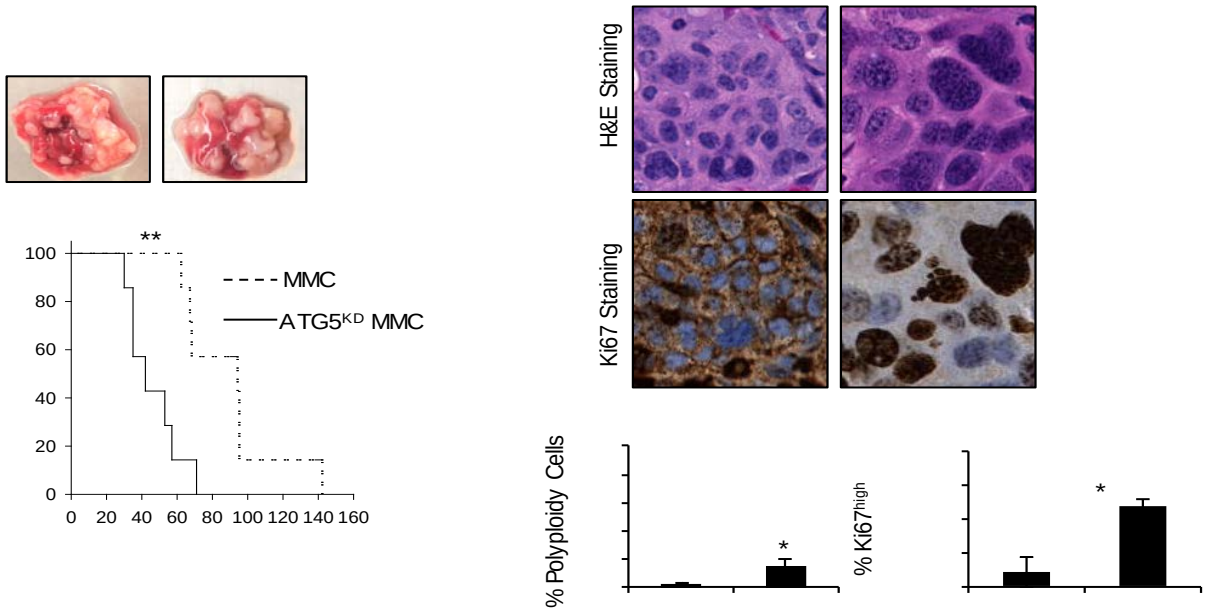


Autophagy-deficient tumor cells show earlier tumor relapse associated with increased frequency of polyploid-like cells, in vitro

In order to determine whether a higher viability of ATG5^{KD} MMC following ADR treatment (Figure 1) facilitates an earlier tumor relapse compared with wild type MMC, follow up studies were performed for three weeks after ADR treatment. As shown in Figure 2A, ATG5^{KD} MMC survived better than autophagy-competent MMC following ADR treatment showing a significantly higher number of cells by 3 weeks after the treatment. Flow cytometry analysis of tumor cells showed greater levels of apoptosis in wild type MMC compared with ATG5^{KD} MMC (Figure 2B, $p<0.001$). Interestingly, ATG5^{KD} MMC cells contained a higher number of polyploid-like cells following ADR treatment compared with autophagy-competent MMC (Figure 2B, $p<0.03$). Dot plots from each experimental group gated for cell cycle phase based upon DNA content (7-AAD) and Ki-67 expression. Events falling to the left of the G1/G0 gates are considered apoptotic cells (AP). Events falling to the far right of the G2/M gate are considered polyploid-like cells (Poly) (Figure 4B). Three independent experiments have been performed and data represent 3 replicates ± SEM (Figure 2). Similar results were obtained, in vivo (Figure 3).

Autophagy-deficient tumor cells show earlier tumor relapse associated with increased frequency of polyploid-like cells, in vivo

Figure 3



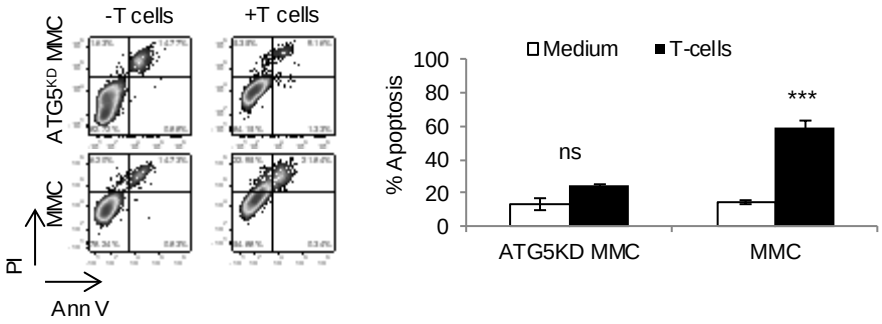
In order to determine the in vivo relevance of our in vitro findings, FVBN202 mice were used. Tumor dormancy was first established by ADR treatment in vitro; FVBN202 mice (n=7/group) were then challenged i.v. with one million viable dormant tumor cells. Animals were then sacrificed when they became moribund (lost 10% weight) as a result of massive lung metastasis. As can be seen in Figure 3A, animals that were challenged with ADR-treated ATG5^{KD} MMC developed lung metastasis significantly sooner than those that were challenged with ADR-treated MMC. Hematoxylin/eosin and immunohistochemistry analyses of tumor lesions determined a higher frequency of polyploid-like and Ki67+ tumor cells in animals that were challenged with ADR-treated ATG5^{KD} MMC (Figure 3B). To count polyploid-like tumor cells, at twenty-times magnification, three representative 0.02mm^2 areas were chosen from the H&E slides containing approximately 100 cells to measure nuclear envelope size. Cells containing a nuclear envelope equal to or greater than 16um with visible multi-nuclei were considered polyploid-like or high grade cells. The corresponding cell was then analyzed on the Ki67 stained slide to determine Ki67 expression levels.

Autophagy-deficient dormant tumor cells survive anti-tumor T cell responses

Neu overexpressing autophagy-deficient MMC (ATG5^{KD} MMC) or autophagy-competent MMC (MMC) were co-cultured with MMC-sensitized T cells and then gated CD45-Neu+ tumor cells were analyzed by Annexin V/PI staining. As shown in Figure 4, ATG5^{KD} dormant MMC were found to be resistant to T cell-induced apoptosis compared with autophagy-competent MMC

(Figure 4). These data suggest that T cell could not eliminate dormant cells, but they may inhibit tumor relapse by maintaining tumor cells in a dormant state.

Figure 4



Identification of a novel antigen presenting cells (APC) in animals that were challenged with autophagy-competent tumor cells: Gr1$^{-/low}$CD11b$^{-/low}$ cells demonstrate characteristics of professional APCs

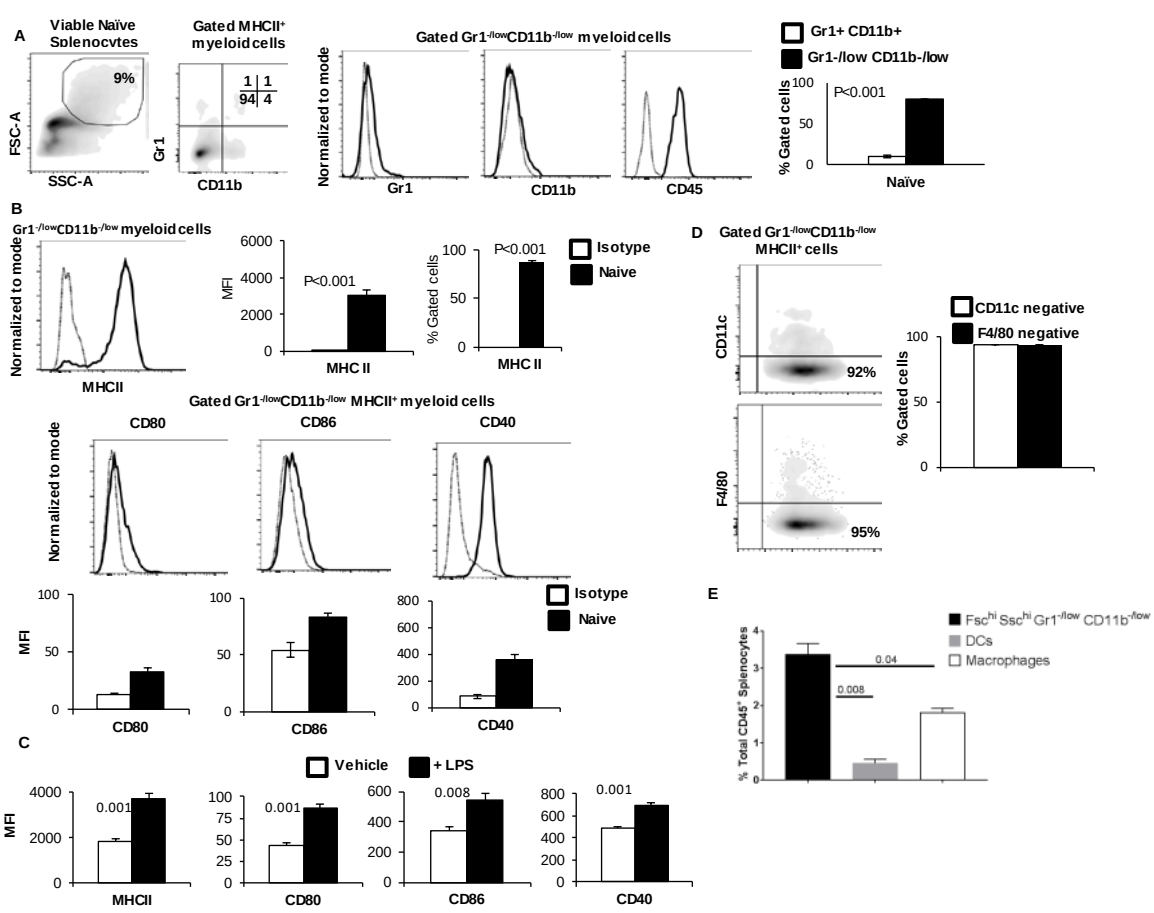
Anti-tumor immune responses are often corrupted in tumor bearing hosts due to pathological emergency myelopoiesis, which leads to the accumulation of myeloid-derived suppressor cells (MDSCs) in secondary lymphoid organs and tumor beds. However, it has been reported that lymphoid effectors, namely NKT cells, functionally alter MDSC function by promoting an immunostimulatory, rather than suppressive, phenotype in the context of anti-tumor immunity. Therefore, we sought to gain an understanding of the biology of myeloid cells under non-pathological conditions in order to appreciate their functional plasticity. First, we observed that the splenic Fsc^{hi} Ssc^{hi} myeloid cell compartment of naïve FVBN202 mice was dominated by a population of Gr1$^{-/low}$CD11b$^{-/low}$ cells (Fig. 5A, right panel; p=0.00002), which were of hematopoietic origin. Furthermore, these Gr1$^{-/low}$CD11b$^{-/low}$ cells demonstrated expression of MHCII (p=0.0002) and the co-stimulatory molecules, CD80 (p=0.001), CD86 (p=0.009), and CD40 (p=0.0003), as shown in Fig. 5B. LPS stimulation induced the maturation of Gr1$^{-/low}$CD11b$^{-/low}$ cells (Fig. 5C) by upregulating the expression of MHCII (MFI: 1851 vs. 3732, p=0.001), CD80 (MFI: 44 vs. 87, p=0.001), CD86 (MFI: 338 vs. 541, p=0.008) and CD40 (MFI: 488 vs. 800, p=0.001). Despite displaying such classical characteristics of APCs, Gr1$^{-/low}$CD11b$^{-/low}$ cells did not express pan markers of DCs, CD11c, or macrophages, F4/80 (Fig. 5D). The total frequency of Fsc^{hi} Ssc^{hi} Gr1$^{-/low}$CD11b$^{-/low}$ APCs was significantly higher than all DCs and macrophages in the spleen (Fig. 5E, p=0.008 and p=0.04, respectively).

Gr1$^{-/low}$CD11b$^{-/low}$ APCs exhibit immune stimulatory function

In order to determine if Gr1$^{-/low}$CD11b$^{-/low}$ APCs possess immune stimulatory function during tumor burden with autophagy-competent MMC and/or following adoptive immunotherapy (AIT) of FVBN202, splenic lymphocytes from the AIT and the control group were independently cultured with autophagy-competent MMC tumor cells in the presence or absence of sorted autologous Gr1$^{-/low}$CD11b$^{-/low}$ cells. As shown in Fig. 6A, lymphocytes derived from the AIT group released IFN-γ in the presence of neu^{+} MMC cells (p=0.0001). Importantly, the IFN-γ producing immune response to MMC was significantly boosted by autologous Gr1$^{-/low}$CD11b$^{-/low}$ APCs (Fig. 6A, p=0.015). On the other hand, lymphocytes derived from the control group did

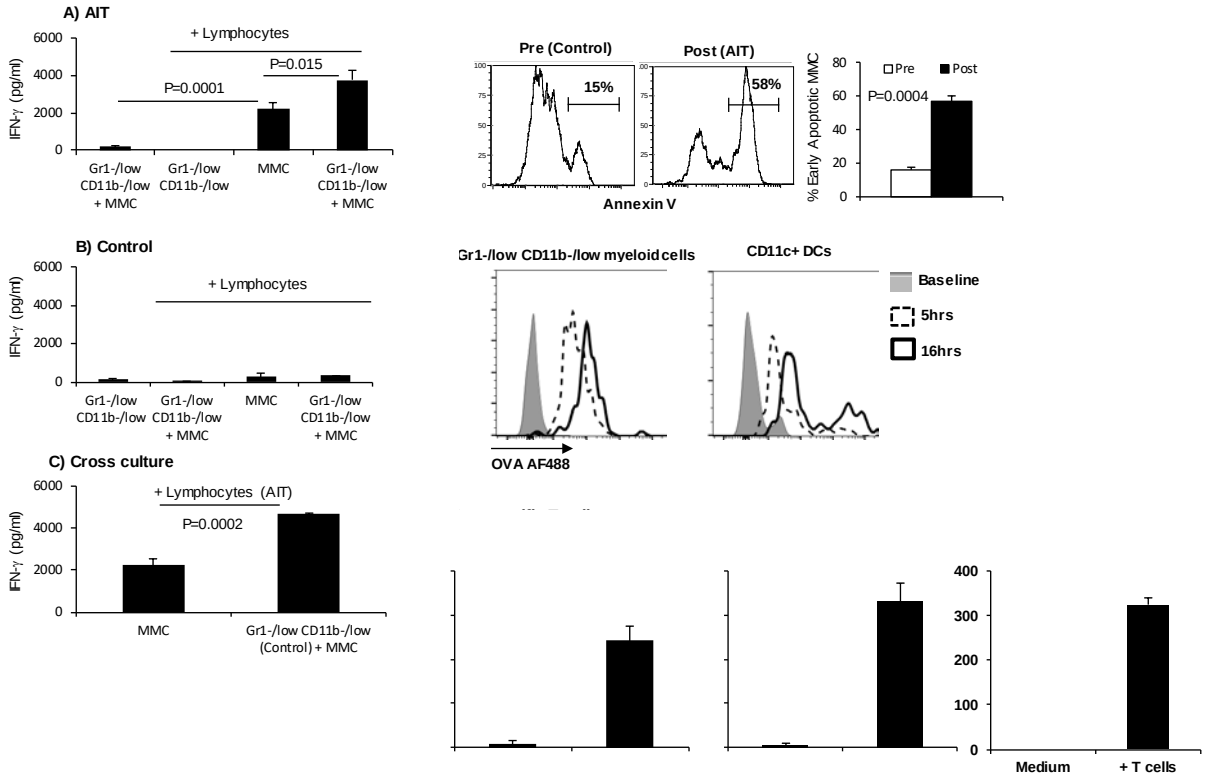
not demonstrate significant IFN-γ release in the presence of MMC; the addition of autologous $Gr1^{-/low}CD11b^{-/low}$ APCs did not enhance this response (Fig. 6B). In order to determine if $Gr1^{-/low}CD11b^{-/low}$ myeloid cells from the control group retained their immune stimulatory function, they were co-cultured with tumor-reactive T cells from the AIT group in the presence or absence of MMC. We hypothesized that T cell specific killing of MMC cells from the AIT group could facilitate cross presentation of tumor antigens by $Gr1^{-/low}CD11b^{-/low}$ APCs, resulting in the enhancement of the immune response. As shown in Fig. 6C, the presence of $Gr1^{-/low}CD11b^{-/low}$ APCs boosted tumor-reactive IFN-γ production by splenic T cells derived from the AIT group (p=0.0002). This was associated with the induction of apoptosis in MMC by reprogrammed T cells that were used for AIT compared with those of the control group (Fig. 6D, p=0.0004). To assess the possibility of $Gr1^{-/low}CD11b^{-/low}$ myeloid cells to potentially uptake and cross-present antigen to T cells, we first pulsed these cells with ovalbumin conjugated to a fluorophore. As shown in figure 6E, $Gr1^{-/low}CD11b^{-/low}$ myeloid cells demonstrated the ability to uptake this protein, with increased fluorescence intensity over time. Although it appears these cells have a reduced efficiency to uptake this antigen compared to DCs, these data suggest that $Gr1^{-/low}CD11b^{-/low}$ myeloid cells may potentially function to cross-present processed antigen to T cells.

Figure 5



Thus, to specifically determine if $Gr1^{-/low}CD11b^{-/low}$ myeloid cells could cross-present antigen to provoke a T cell response we sorted splenic $Gr1^{-/low}CD11b^{-/low}$ myeloid cells and pulsed them with recombinant neu ECD (extracellular domain) protein, followed by a culturing period with tumor-sensitized T cells. In fact, as can be seen in Fig. 6F, $Gr1^{-/low}CD11b^{-/low}$ myeloid cells were able to induce IFN-γ production from tumor-sensitized T cells only after they were pulsed with Neu ECD, suggesting these cells possess antigen-processing and presentation functionality. Bone marrow-derived DCs were used a positive control for antigen cross presentation; irradiated MMC cells were used as a specificity control for assessing neu-reactive T cell function.

Figure 6



- **What opportunities for training and professional development has the project provided?**

  - An MD/PhD student, *Soheil Sanon*, did his rotation in the laboratory of Dr. Manjili.
  - Two undergraduate students, *Parsa Mahmoudi* and *Javid Rahseparian*, conducted their summer research program in the laboratory of Dr. Manjili.
  - One medical research resident, *Zach Benson*, continued his research training in the lab of Dr. Manjili and published a review paper.
  - A PhD student, *Hussein Aqbi*, has been working on the project and published two papers.

- **How were the results disseminated to communities of interest?**

  1) Concepts that are proposed in this project were used to formulate two graduate level lectures-advanced immunology and molecular biology of cancer- related to cancer dormancy.
  2) As an invited speaker and keynote speaker, Dr. Manjili gave lectures on tumor dormancy at 14th International Congress of Immunology and Allergy (ICIA) in April 2018 Keynote speech: "*Theoretical framework for the efficacy of cancer immunotherapy*" (April 26, 2018) Plenary speech: "*The promise and challenges of cancer immunotherapy: The adaptation model of immunity"* (April 28, 2018)
  3) One poster was presented at IMMUNOLOGY 2018TM AAI Annual Meeting, Austin, TX, May 4-8, 2018 [received 2018 AAI Laboratory Travel Grant Award]

- **What do you plan to do during the next reporting period to accomplish the goals?**

  Task 2.2) *Challenge FVBN202 mice with MMC tumor cells expressing different levels of IFN-γ Rα* (Initiating PI); *Evaluate breakage of immunological tolerance against the tumor, ex vivo* (Initiating PI); *Follow up studies for tumor escape* (Initiating PI)

  Task 2.4) *Chemotherapy combined with blockade of autophagy in FVBN202 mice challenged with MMC IFN-γ Rα* ^{low} *tumor cells; Follow up studies for tumor relapse* (Initiating PI); *Evaluation of anti-tumor immune responses, ex vivo* (Initiating PI)

4. IMPACT

- **What was the impact on the development of the principal discipline(s) of the project?**

  Received AAI Laboratory Travel Award to present an abstract in the AAI Annual Meeting in 2018

- **What was the impact on other disciplines?**

  Nothing to report.

- **What was the impact on technology transfer?**

  Nothing to report

What was the impact on society beyond science and technology?

Nothing to report

5. CHANGES/PROBLEMS

- **Changes in approach and reasons for change**

Nothing to report

- **Actual or anticipated problems or delays and actions or plans to resolve them**

  Nothing to report

- **Changes that had a significant impact on expenditures**

  Nothing to report

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

  Nothing to report

- **Significant changes in use or care of human subjects**

  Nothing to report

- **Significant changes in use or care of vertebrate animals**

  Nothing to report

- **Significant changes in use of biohazards and/or select agents**

  Nothing to report

6. PRODUCTS

- **Publications, conference papers, and presentations**

  - **Journal publications**

    1. Payne KK, Aqbi HF, Butler SE, Graham L, Keim RC, Wan W, Idowu MO, Bear HD, Wang XY, **Manjili MH**. $Gr1^{-/low}CD11b^{-/low}$MHCII+ myeloid cells boost T cell anti-tumor efficacy. *J Leukoc Biol* 2018 Jul 9. doi: 10.1002/JLB.5A0717-276RR. [Epub ahead of print] PMID: 29985529
    2. Aqbi HF, Tyutyunyk-Massey L, Keim RC, Butler SE, Thekkudan T, Joshi S, Smith TM, Bandyopadhyay D, Idowu MO, Bear HD, Payne KK, Gewirtz DA, **Manjili MH.**

Autophagy-deficient breast cancer shows early tumor recurrence and escape from dormancy. *Oncotarget* 9(31): 22113-22122, 2018 PMID: 29774126

3. Aqbi HF, Wallace M, Sappal S, Payne KK, **Manjili MH.** IFN-γ orchestrates tumor elimination, tumor dormancy, tumor escape and progression. *J Leukoc Biol* 2018 Feb 22. doi: 10.1002/JLB.5MIR0917-351R. [Epub ahead of print] PMID: 29469956
4. **Manjili MH.** A theoretical basis for the efficacy of cancer immunotherapy and immunogenic tumor dormancy: The adaptation model of immunity. *Adv Cancer Res* 137:17-36, 2018 PMID: 29405975
5. Shah SA, Zarei M, Manjili SH, Guruli G, Wang XY, **Manjili MH.** Immunotherapy of cancer: targeting cancer during active disease or during dormancy? *Immunotherapy* 9 (11): 943-949, 2017 PMID: 29338608
6. Benson Z, Manjili SH, Habibi M, Guruli G, Toor AA, Payne KK, **Manjili MH**. Conditioning neoadjuvant therapies for improved immunotherapy of cancer. *Biochem Pharmacol* 145:12-17, 2017 PMID: 28803721

- **Books or other non-periodical, one-time publications**

- **Other publications, conference papers and presentations**

  Aqbi HF, Smith TM, Idowu MO, Butler SB, Payne KK, **Manjili MH**. Autophagy-deficient breast cancer shows early escape from dormancy and recurrence following chemotherapy, IMMUNOLOGY 2018TM AAI Annual Meeting, Austin, TX, May 4-8, 2018 [received 2018 AAI Laboratory Travel Grant Award]

  Invited speaker, 14th International Congress of Immunology and Allergy (ICIA), Tehran, Iran, April 26-28, 2018. Keynote speech: "*Theoretical framework for the efficacy of cancer immunotherapy*" (April 26, 2018), Plenary speech: "*The promise and challenges of cancer immunotherapy: The adaptation model of immunity"* (April 28, 2018)

- **Website(s) or other Internet site(s)**

  VCU Institute of Molecular Medicine (VIMM) News and Views, Issue No. 13, January 2018. Tumor dormancy: a natural byproduct of evolutionary survival mechanism.

- **Technologies or techniques**

  Nothing to report

- **Inventions, patent applications, and/or licenses**

  Nothing to report

- **Other Products**

Nothing to report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

- **What individuals have worked on the project?**

Name:	Savannah Butler
Project Role:	Lab Specialist
Researcher Identifier:	
Nearest person month worked:	2
Contribution to Project:	Ms. Butler has performed in vitro and in vivo studies of chemotherapy-induced tumor dormancy
Funding Support:	DoD

Name:	Timothy Smith, PhD
Project Role:	Graduate student
Researcher Identifier:	
Nearest person month worked:	8
Contribution to Project:	Mr. Smith has performed in vitro studies of chemotherapyinduced tumor dormancy.
Funding Support:	DoD

Name:	Hussein Aqbi
Project Role:	Graduate student
Researcher Identifier:	
Nearest person month worked:	12
Contribution to Project:	Mr. Aqbi has performed in vivo studies associated with chemotherapy-induced tumor dormancy, and immune response studies.
Funding Support:	DoD, PhD scholarship

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Nothing to report

- **What other organizations were involved as partners?**

Nothing to report

8. SPECIAL REPORTING REQUIREMENTS

- **COLLABORATIVE AWARDS**

  N/A

- **QUAD CHARTS**

  N/A

9. APPENDICES

Documents attached.

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JLB JOURNAL OF LEUKOCYTE BIOLOGY

ARTICLE

Gr1$^{-/low}$CD11b$^{-/low}$MHCII^{+} myeloid cells boost T cell anti-tumor efficacy

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Abstract

Conventional APCs that express MHC class II (MHCII) and co-stimulatory molecules include dendritic cells (DCs) and macrophages. Beyond these conventional APCs, immune stimulatory cells have been more recently shown to extend to a class of atypical APCs, composed of mast cells, basophils, and eosinophils. Here, we describe a unique type of APC, Gr1$^{-/low}$CD11b$^{-/low}$ cells with a granularity and size characteristic of myeloid cells and with the ability to present Ag for crosspresentation. These cells constitutively express MHCII and the costimulatory molecules, CD80, CD86, and CD40. They do not express pan markers of myeloid DCs (CD11c), plasmacytoid DCs (Ly6C), or macrophages (F4/80), and their frequency is inversely correlated with myeloid-derived suppressor cells (MDSCs) in tumor-bearing mice. Among splenocytes, they are more abundant than DCs and macrophages, and they exhibit antitumor immune stimulatory function at a steady state without further activation, ex vivo. They are also found within the tumor bed where they retain their immune stimulatory function. Our findings suggest the use of these novel APCs in additional preclinical studies to further investigate their utility in APC-based cancer immunotherapies.

KEYWORDS

adoptive immunotherapy, Ag presenting cells, breast cancer, cancer vaccine, myeloid-derived suppressor cells

1 | INTRODUCTION

Dendritic cells (DCs) play a central role in inducing immune responses against infectious diseases and cancer. However, their efficacy as a cell-based vaccine is limited despite continued optimization of various vaccination parameters. This is in part due to the host-derived immune suppressive cells such as myeloid-derived suppressor cells (MDSCs). The accumulation of MDSCs hinders protective immune responses to cancer and infectious diseases such as tuberculosis,[1,2] AIDS,[3–5] hepatitis C,[6,7] hepatitis B,[8,9] pneumonia,[10,11] and *Staphylococcus aurous* infection.[12] Importantly, an elevation of MDSCs is associated with a reduced efficacy of vaccines.[13,14] In addition, the generation of monocyte-derived DCs or bone marrow-derived DCs requires extensive ex vivo culturing, conceivably hampering

Abbreviations: AIT, adoptive immunotherapy; DC, Dendritic cell; ILC, innate lymphoid cell; MDSC, myeloid-derived suppressor cell

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the immunogenicity of the vaccine. Recent studies, therefore, have focused on vaccines that make use of primary DCs.[15] For instance, Sipuleucel-T is the only FDA-approved therapeutic vaccine for metastatic prostate cancer.[16] The vaccine uses readily isolated circulating DCs cultured with prostate tumor Ag and GM-CSF. However, circulating DCs are very rare and tumor-induced immune suppressive cells, such as MDSCs, limit their efficacy in inducing a sustained antitumor immune response. Therefore, there is an urgent need to identify a new class of APC that are highly efficient in orchestrating profound antitumor immunity to facilitate the development of a new class of cell-based cancer vaccines.

In recent years, there has been a rapid increase in our understanding of the biology of cells with APC characteristics, namely the ability to activate T cells. For instance, mouse neutrophils can induce Th1 and Th17 responses[17,18] and tumor-associated neutrophils have been demonstrated to stimulate T cell responses in early-stage human lung cancer.[19] A recent review discusses a number of atypical APCs including mast cells, basophils, eosinophils, and innate lymphoid cells (ILC).[20,21] However, these APCs are rare in the circulation and their maintenance of effective antitumor immune responses is likely to be inhibited due to high frequencies of MDSCs in locations of T cell priming. Very recently, it was reported that activated NKT cells decrease the frequency and immunosuppressive activity of MDSCs in tumor-bearing mice.[22] In an animal model, activated NKT cells converted MDSCs into immunogenic APCs.[23] Using peripheral blood mononuclear cells (PBMC) of patients with early stage breast cancer, we also demonstrated that conversion of MDSCs to $CD33^{+}CD11b^{-/low}HLA\text{-}DR^{+}$ APCs, in vitro, was associated with an increased frequency of CD25+ NKT cells in reprogrammed immune cells.[24]

In an effort to understand this MDSC-APC axis during the application of adoptive immunotherapy (AIT) to treat breast cancer, we identified a class of $Gr1^{-/low}CD11b^{-/low}$ MHCII+ APCs. These cells retain their immune stimulatory function during tumor progression and are inversely correlated to the frequency of splenic and tumor-infiltrating MDSCs. Importantly, we identified the presence of these cells in nonpathological conditions, whereupon we confirmed their ability to cross-present Ag to stimulate T cells. Therefore, these APCs offer a potentially novel APC-based vaccine for cancer therapy.

2 | MATERIALS AND METHODS

2.1 | Mouse model

FVBN202 transgenic female mice (The Jackson Laboratory; Bar Harbor, ME) were used between 8 and 12 weeks of age throughout these experiments. These mice overexpress a nonmutated, nonactivated rat neu transgene under the regulation of the mouse mammary tumor virus promoter.[25] These mice develop premalignant mammary hyperplasia similar to ductal carcinoma in situ prior to the development of spontaneous carcinoma.[26] Premalignant events in FVBN202 mice include the accumulation of endogenous MDSCs.[26] These studies have been reviewed and approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

2.2 | Tumor cell lines

The neu overexpressing mouse mammary carcinoma (MMC) cell line was established from a spontaneous mammary tumor harvested from FVBN202 mice. Tumor cells were maintained in RPMI 1640 supplemented with 10% FBS.

2.3 | Ex vivo reprogramming and expansion of splenocytes

Reprogramming of tumor-sensitized immune cells was performed as previously described by our group.[5] Briefly, FVBN202 transgenic mice were inoculated in the mammary fat pad with 3×10^6 MMC cells. Tumor growth was monitored by digital caliper, and tumor volumes were calculated by volume (v) = (L [length] $\times$ W [width]^2)/2. As previously described,[11] splenocytes were harvested 21–25 days after tumor challenge, when the tumor had reached $\geq$ 1000 mm.^{3} Splenocytes were then cultured in complete medium (RPMI 1640 supplemented with 10 % FBS, L-glutamine (2 mM), 100 U/ml penicillin, and 100 μg/ml Streptomycin) and were stimulated with Bryostatin 1 (2 nM; Sigma, Saint Louis, MO), Ionomycin (1 μM; Calbiochem, San Diego, CA), and 80 U/ml/10^6 cells of IL-2 (Peprotech) for 16–18 h.[24,27] Lymphocytes were then washed thrice and cultured at 10^6 cells/ml in complete medium with IL-7 and IL-15 (20 ng/ml of each cytokine, Peprotech, Rocky Hill, NJ). After 24 h, 20 U/ml of IL-2 was added to the complete medium. The following day, the cells were washed and cultured at 10^6 cells/ml in complete medium with 40 U/ml of IL-2. After 48 h, cells were washed and cultured at 10^6 cells/ml in complete medium with 40 U/ml of IL-2. After 24 h, lymphocytes were again washed and cultured at 10^6 cells/ml in complete medium with 40 U/ml of IL-2. Lymphocytes were harvested 24 h later on the sixth day and were then either used in AIT or analyzed ex vivo. Reprogramming of splenocytes consistently yielded 5-fold expansion with greater than 40% memory T cells and 35% CD25+ NKT cells.[27]

2.4 | Adoptive cellular therapy

Twenty-four hours prior to AIT, FVBN202 mice were injected i.p. with CYP (100 mg/kg) to induce lymphopenia. Approximately 18 h later FVBN202 mice were challenged i.v. with MMC cells (1×10^5). Mice then received adoptive transfer of reprogrammed splenocytes i.v. at a dose of 70×10^6/mouse later the same day (AIT), or remained untreated (Control). The study end-point and euthanasia occurred when the animals were considered moribund upon losing 10–20% of their initial body weight due to disease progression.

2.5 | Characterization of splenocytes and tumor-infiltrating leukocytes

Spleens and metastases of tumor-bearing FVBN202 mice were harvested when the animals became moribund, and were then homogenized into a single cell suspension as described previously[28] and below; single cell suspensions were then characterized using flow cytometry. Reagents used for flow cytometry: anti-CD16/32 Ab (93); FITC-CD11b (M1/70); PE-GR-1 (RB6-8C5); PE-CD11c (N418); PE-F4/80 (BM8);

PE-CD25 (3C7); Allophycocyanin-CD49b (DX5); Allophycocyanin-Annexin V; Alexa Fluor 647-I-Aq (KH116); Alexa Fluor 700 Ly-6G (1A8); PercP/CY5.5-CD86 (GL-1); PercP/CY5.5-Rat IgG2a, k Isotype Control (RTK2758); PE-Dazzle-CD80 (16-10A1); PE-Dazzle-Armenian Hamster IgG Isotype Control (HTK888); PE/CY7-CD40 (3/23); PE/CY7-Rat IgG2a, k Isotype Control (RTK2758); Brilliant Violet 510 Ly-6C (HK1.4); Brilliant Violet 605-CD45 (30-F11); BV421-CD20 (SA275A11); BV711-Ly6C (HK1.4); BV510-CD11b (M1/70); and BV785-CD86 (GL-1), all of which were purchased from Biolegend (San Diego, CA). BD Horizon V450-Annexin V and BUV395-CD3 (SK7) were purchased from BD Biosciences (Franklin Lakes, NJ). Propidium Iodide (PI) was purchased from Sigma. (All reagents were used at the manufacturer's recommended concentration. Cellular staining was performed as previously described by our group.[24] Multicolor data acquisition was performed using a LSRFortessa X-20 (BD Biosciences) and a ImageStreamX Mark II Imaging Flow Cytometer (Millipore Sigma, Billaerica, MA). Data was analyzed using FCS Express v4.07 and v5.0 (De Novo Software; Glendale, CA).

2.6 | Sorting of myeloid cells by FACS

Splenocytes were stained for surface expression of CD11b and Gr1 as described above. Isolated cells were gated on the myeloid cell population based on their inherent light scattering properties[29] thereby excluding cells of lymphoid origin. $Gr1^{-/low}CD11b^{-/low}$ myeloid cells from the Control and AIT groups were then sorted into independent populations using a FACSAria (BD Biosciences) as previously described.[30] Purity of sorted cells was consistently greater than 90%.

2.7 | IFN-γ ELISA

Splenocytes from the Control and AIT groups were independently cultured in serum-free RPMI 1640 in order to enrich for nonadherent cells.[31] After 2 h, nonadherent lymphocytes were cultured in complete medium with irradiated MMC cells (140 Gy) at a 10:1 ratio, and with or without sorted $Gr1^{-/low}CD11b^{-/low}$ myeloid cells at a 2:1 ratio, for 20 h. Also, sorted $Gr1^{-/low}CD11b^{-/low}$ cells or bone marrow-derived DCs were pulsed with recombinant rat Neu extracellular domain (50 ug/ml) in the presence of GM-CSF (20 ng/ml) for 24 h, washed of free protein, and co-cultured with tumor-sensitized, reprogrammed T cells (1:3) for 20 h. Irradiated MMC (140 Gy) were used as positive target for tumor-sensitized reprogrammed T cells (1:10 ratio). Supernatants were then collected and stored at −80°C until assayed. IFN-γ was detected in the supernatant using a Mouse IFN-γ ELISA kit (BD Biosciences), according to the manufacturer's protocol.

2.8 | In vitro Ag uptake

Splenocytes (10^6 cells/ml) of naïve FVBN202 mice were pulsed with 50 ug/ml Alexa Fluor 488 (AF488)-conjugated ovalbumin (ThermoFisher Scientific) in RPMI1640 supplemented with 10% FBS for 5 or 16 h. Cells were then washed and stained for FVS, CD11c, CD11b, Gr1. Gated FVS- viable cells were subgated for CD11c+ DCs or $Gr1^{-/low}CD11b^{-/low}$ myeloid cells, and analyzed for Alexa Fluor 488 as a reporter of OVA internalization.

2.9 | Cytotoxicity assay

Antitumor efficacy of T cells was determined in a cytotoxicity assay, in vitro, using flow cytometry as previously described by our group[32] with minor modifications. The ex vivo expanded tumor reactive T cells were cultured in complete medium with MMC cells (10:1 E:T ratio) in the presence or absence of sorted $Gr1^{-/low}CD11b^{-/low}$ cells at a 5:1 ratio (five T cells vs. one APCs), for 48 h. Cells were collected and stained with Annexin V, PI, anti-CD45 and anti-Neu Abs immediately prior to flow cytometry acquisition.

2.10 | Isolation of tumor-infiltrating leukocytes from lung metastases

Lungs were harvested from the Control and AIT groups after animals became moribund. Metastatic lesions were individually excised from the residual lung tissue, and were minced and digested in Trypsin-EDTA (0.25%; Life Technologies) overnight at 4°C. The following day, the suspension was incubated at 37°C for 30 min, followed by gentle tissue homogenization to create a cellular suspension. The cell suspension was then washed twice with RPMI supplemented with 10% FBS. Residual red blood cells were then lysed using ACK lysing buffer, followed by an additional wash with RPMI 10% FBS. 10^6 cells of the suspension were then stained for surface molecules as described above. All analysis was performed by gating on viable leukocytes (CD45+ Annexin V^-).

2.11 | Statistical analysis

Outcomes are summarized by basic descriptive statistics such as mean and SEM; differences between groups are illustrated using graphical data presented as mean ± SEM. Statistical comparisons between groups were made using one-tailed and two-tailed Student's *t*-test per the specific hypothesis. Time to death in the in vivo survival studies was calculated from baseline to the date of death. Mice were euthanized when they had a weight loss of ≥10%. Kaplan–Meier curves and log-rank tests are used to illustrate time to death and to test the difference between each group. A *P*-value ≤ 0.05 was considered statistically significant.

3 | RESULTS

3.1 | $Gr1^{-/low}CD11b^{-/low}$ cells demonstrate characteristics of professional APCs

Antitumor immune responses are often corrupted in tumor bearing hosts due to pathological emergency myelopoiesis, which leads to the accumulation of MDSCs in secondary lymphoid organs and tumor beds.[33,34] However, it has been reported that lymphoid effectors, namely NKT cells, functionally alter MDSC function by promoting an immunostimulatory, rather than suppressive, phenotype in the context of antitumor immunity.[24,27] Therefore, we sought to gain an

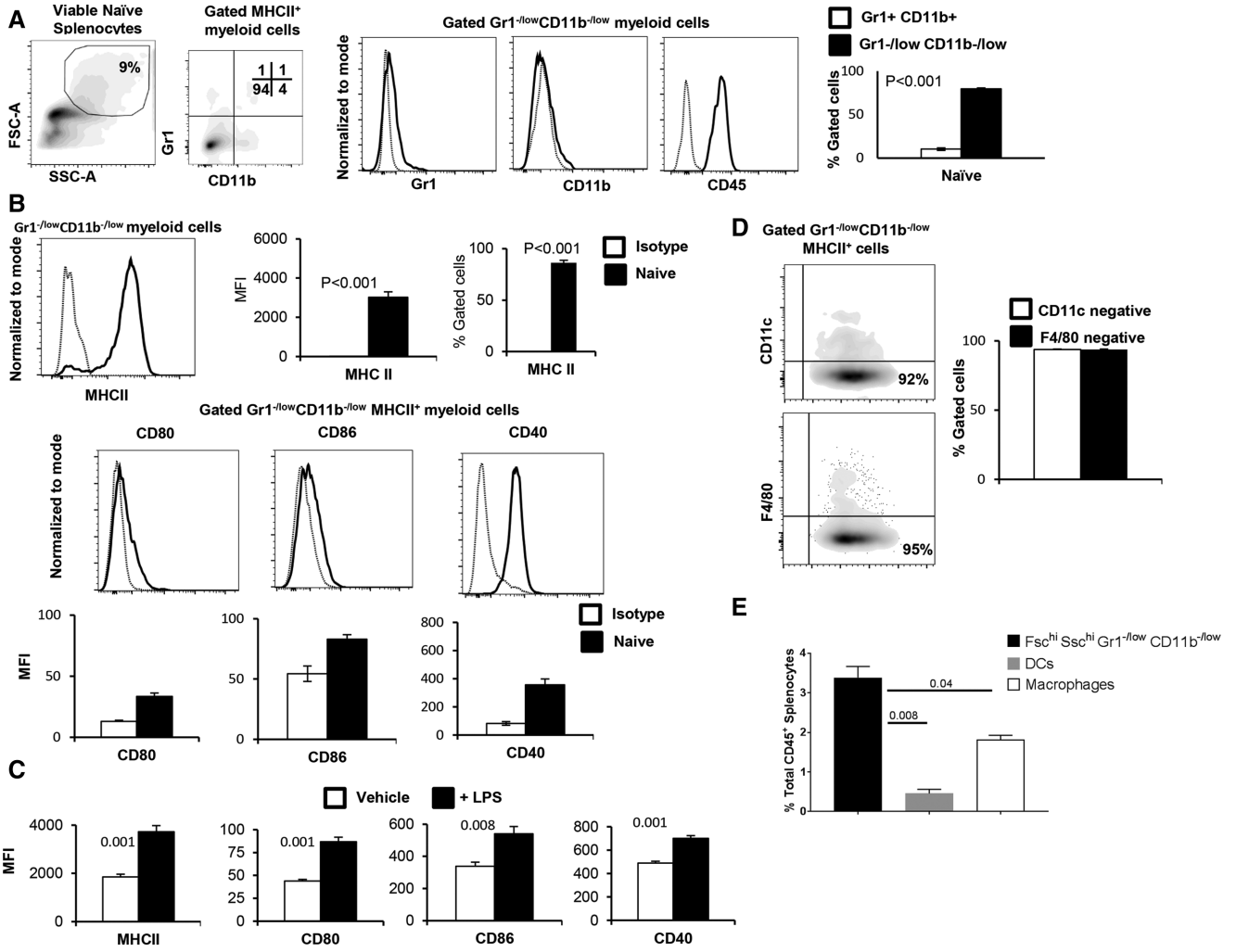

FIGURE 1 **Splenic Gr1$^{-/low}$CD11b$^{-/low}$ cells show characteristics of APCs.** (A) Splenocytes of naïve FVBN202 mice ($n = 3$) were gated within the myeloid cell region based on forward-scatter and side-scatter, and were analyzed for the expression of Gr1 and CD11b. The proportion of the splenic Gr1$^{-/low}$CD11b$^{-/low}$ myeloid cells and Gr1^{+}CD11b^{+} myeloid cells was determined. (B) Gated Gr1$^{-/low}$CD11b$^{-/low}$ cells were analyzed for the expression of MHC class II (MHCII). Gated Gr1$^{-/low}$CD11b$^{-/low}$MHCII^{+} cells were also analyzed for the expression of the co-stimulatory molecules, CD80, CD86, and CD40. Mean fluorescence intensity (MFI) of the co-stimulatory molecules showed a significant shift compared with isotype control. (C) Sorted Gr1$^{-/low}$CD11b$^{-/low}$MHCII^{+} cells were cultured in the absence (–LPS) or presence of LPS (+LPS, 1μg/ml) for 24 h. Gated Gr1$^{-/low}$CD11b$^{-/low}$MHCII^{+} cells were analyzed for the expression of MHCII as well as CD80, CD86, or CD40. MFI was calculated after the subtraction of isotype control. (D) Gated Gr1$^{-/low}$CD11b$^{-/low}$MHCII^{+} cells were analyzed for the expression of CD11c or F4/80. (E) Percent total frequency of MHCII+ Gr1$^{-/low}$CD11b$^{-/low}$, DCs and macrophages in the spleen. Data represent mean ± SEM. Data are representative of at least 3 independent experiments

understanding of the biology of myeloid cells under nonpathological conditions in order to appreciate their functional plasticity. First, we observed that the splenic Fsc^{hi} Ssc^{hi} myeloid cell compartment of naïve mice was dominated by a population of Gr1$^{-/low}$CD11b$^{-/low}$ cells (Fig. 1A, right panel; $P = 0.00002$), which were of hematopoietic origin. Furthermore, these Gr1$^{-/low}$CD11b$^{-/low}$ cells demonstrated expression of MHC class II (MHCII; $P = 0.0002$) and the co-stimulatory molecules, CD80 ($P = 0.001$), CD86 ($P = 0.009$), and CD40 ($P = 0.0003$), as shown in Fig. 1B. LPS stimulation induced the maturation of Gr1$^{-/low}$CD11b$^{-/low}$ cells (Fig. 1C) by up-regulating the expression of MHCII (MFI: 1851 vs. 3732, $P = 0.001$), CD80 (MFI: 44 vs. 87, $P = 0.001$), CD86 (MFI: 338 vs. 541, $P = 0.008$) and CD40 (MFI: 488 vs. 800, $P = 0.001$). Despite displaying such classical characteristics of APCs, Gr1$^{-/low}$CD11b$^{-/low}$ cells did not express pan markers of DCs, CD11c, or macrophages, F4/80 (Fig. 1D). Importantly, however, these Fsc^{hi} Ssc^{hi} Gr1$^{-/low}$CD11b$^{-/low}$ myeloid cells possess a similar size and granularity, and express similar levels of MHCII as well as costimulatory molecules to total splenic macrophages and dendritic cells (Supplementary Fig. 1). The total frequency of Fsc^{hi} Ssc^{hi} Gr1$^{-/low}$CD11b$^{-/low}$ APCs was significantly higher than all DCs and macrophages in the spleen (Fig. 1E, $P = 0.008$ and $P = 0.04$, respectively). Additionally, morphological studies of these cells using Diff-Quick staining demonstrated the presence of both monoblast-like (large cells), and lymphocyte-like (small cells) within the Fsc^{hi} Ssc^{hi} Gr1$^{-/low}$CD11b$^{-/low}$ gate (Supplementary Fig. 2).

Given that Diff-Quick staining revealed the presence of lymphocyte-like cells among sorted Gr1$^{-/low}$CD11b$^{-/low}$ cells from naïve mice, we sought to further determine the phenotype

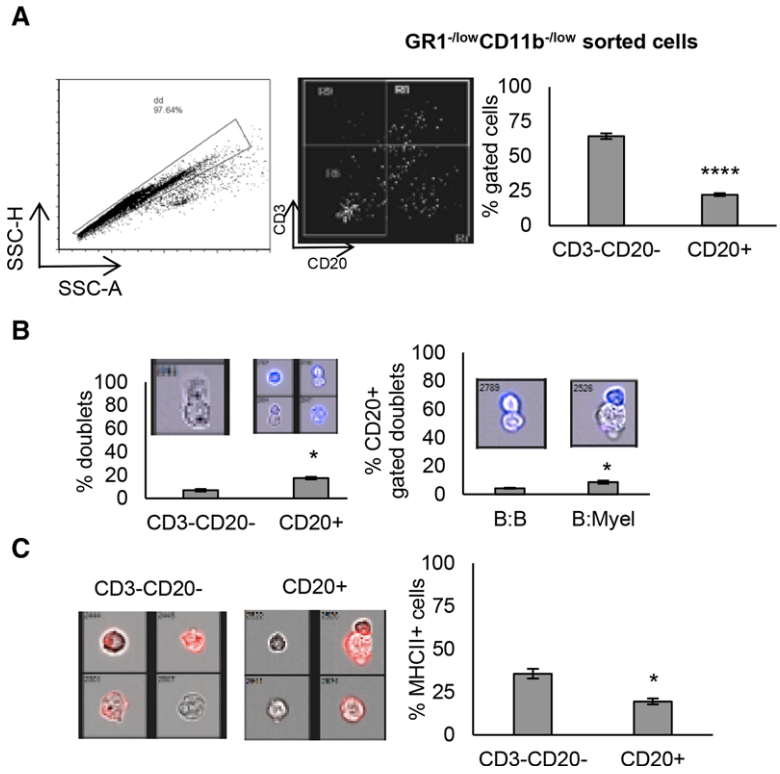

FIGURE 2 **GR1$^{-/low}$CD11b$^{-/low}$ cells contain myeloid cells and B cells.** GR1$^{-/low}$CD11b$^{-/low}$ cells within the myeloid region of the scatter plot were sorted and analyzed via Image Stream. (A) After excluding doublets, cells were analyzed for CD3 and CD20 expression to determine if T and B cells were still falling within the myeloid gate. (B) Hundred images/events from the CD3^{-}CD20^{-} and CD20^{+} populations were analyzed for doublets by inspecting each image manually. Also, doublets within CD20+ cells were analyzed based on morphology showing B cell:B cells (B:B) or B cells:Myeloid cells (B:Myel) interactions. (C) MHCII (red) expression on CD3^{-}CD20^{-} and CD20^{+} populations. Data represent mean ± SEM of triplicate experiments

and frequency of these cells within the sorted population. We found that a majority of gated Gr1$^{-/low}$CD11b$^{-/low}$ cells lacked expression of lineage markers for T or B cells (CD3^{-}CD20^{-}), although 22% of cells included CD20^{+} B cells (Fig. 2A). We then hypothesized that the presence of residual B-cells in the Fsc^{hi} Scc^{hi} myeloid region was due to cell-to-cell interactions between B cells and myeloid cells. To investigate this, ImageStreamX analysis was performed. The total events were analyzed for percentage of events that had two cells contained in one event by observing each event manually. The number of doublets containing the CD20^{+} population was significantly higher in comparison to the CD3^{-}CD20^{-} doublets (Fig. 2B, left and middle panels, 7% vs. 17.5%). Among CD20^{+} B cells in this population, the majority of cell-to-cell contacts were shown to be B cell:myeloid cell interactions (B:Myel), rather than B cell:B cell (B:B) interactions (Fig. 2B, right panel, 9% vs. 4%). We then determined the source of MHCII expression among these interacting cells. As can be seen in Fig. 2C, myeloid cells (CD20^{-}CD3^{-}) had significantly higher percent of MHCII expression compared to CD20^{+} cells. Taken together, our data suggest the presence of a unique lineage of myeloid-derived APC, which demonstrates characteristics of classical APC:B cell interactions in naïve mice.[35,36]

3.2 | Gr1$^{-/low}$CD11b$^{-/low}$ MHCII^{+} cells are heterogeneous populations that are both lineage committed and noncommitted

To further unravel the biology of Gr1$^{-/low}$CD11b$^{-/low}$ MHCII^{+} cells, we found that approximately 50% of these cells expressed Ly6G, indicative of a commitment to the granulocyte lineage, while the remainder of this population was negative for both Ly6G and Ly6C (Fig. 3A). Accordingly, the Ly6G^{+}Ly6C^{-} subset displayed a more mature phenotype than the Ly6G^{-}Ly6C^{-} subset, expressing significantly higher levels of MHCII (P = 0.001), CD80 (P = 0.03), CD86 (P = 0.0006), and CD40 (P = 0.025; Fig. 2B). As the Ly6G^{-}Ly6C^{-} subset did not demonstrate a specific myeloid-cell lineage commitment by any parameter that we tested, we hypothesized that this population would respond more robustly to activating stimuli due to a presumed lack of maturity. Indeed, as shown in Fig. 3C, the Ly6G^{-}Ly6C^{-} subset showed a stronger response to LPS stimulation when compared to vehicle treatment than the Ly6G^{+}Ly6C^{-} subset. This suggests that under non-pathological conditions there exists a population of both lineage committed and noncommitted splenic Gr1$^{-/low}$CD11b$^{-/low}$ MHCII^{+} cells, which possess the potential to perform professional Ag-presenting cellular functions.

3.3 | Adoptive immunotherapy modulates Gr1$^{-/low}$CD11b$^{-/low}$ APCs

It has been reported that activated NKT cells can convert MDSCs into immune-stimulatory APCs.[22,23] We have reported that reprogrammed lymphocytes containing CD25+ NKT cells can induce maturation of human CD33^{+}CD11b^{+}HLA-DR^{-} MDSCs into stimulatory CD33^{+}CD11b$^{-/low}$HLA-DR^{+} APCs, in vitro.[24,27] Given the inverse correlation between Gr1^{+}CD11b^{+} cells and Gr1$^{-/low}$CD11b$^{-/low}$ APCs in naïve mice (Fig. 1A), we sought to determine the impact of tumor burden as well as AIT, containing conventional tumor-specific T cells and CD25+ NKT cells, on the modulation of Gr1$^{-/low}$CD11b$^{-/low}$ APCs, in vivo. FVBN202 mice were challenged i.v. with Neu-overexpressing MMC tumor cells, and then either remained untreated (control) or were subjected to an adoptive transfer of tumor-sensitized reprogrammed T cells and NKT cells.[32] Animals were sacrificed upon disease progression culminating in metastases in the lung. As shown

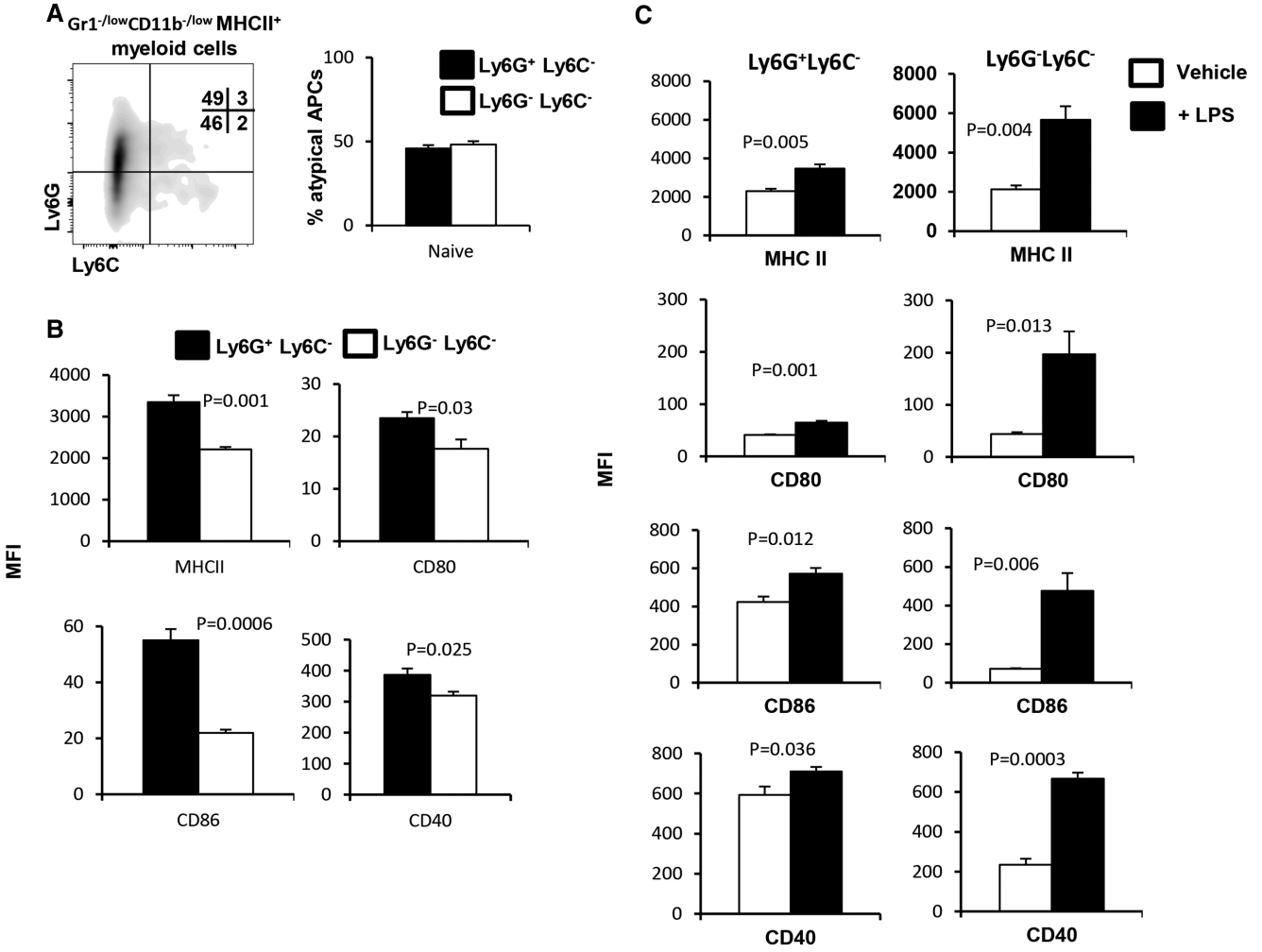

FIGURE 3 **$Gr1^{-/low}CD11b^{-/low}$ MHCII$^+$ myeloid cells contain Ly6G$^+$Ly6C$^-$ and Ly6G$^-$Ly6C$^-$ subsets.** (A) Splenocytes of naïve FVBN202 mice ($n = 3$) were gated within the myeloid cell region and expression of Ly6G and Ly6C was determined on gated $Gr1^{-/low}CD11b^{-/low}$ MHCII$^+$ cells. (B) Expression of MHCII and co-stimulatory molecules was determined on gated MHCII$^+$Ly6G$^+$Ly6C$^-$ and MHCII$^+$Ly6G$^-$Ly6C$^-$ cells. (C) Expression of MHCII and co-stimulatory molecules was determined on gated Ly6G$^+$Ly6C$^-$ or Ly6G$^-$Ly6C$^-$ subsets after 24 h stimulation in the absence (–LPS) or presence of LPS (+LPS, 1μg/ml). MFI were calculated after subtraction of isotype control. Data represent mean ± SEM. Data are representative of at least 3 independent experiments

in Fig. 4A, AIT significantly prolonged animal survival ($P = 0.015$). Such antitumor protection was associated with modulation of the myeloid cell compartment, resulting in a significantly increased frequency of $Gr1^{-/low}CD11b^{-/low}$ APCs (Fig. 4B, 56% vs. 38%); the frequency of these cells dominated $Gr1^+CD11b^+$ MDSCs in the AIT group compared to the control group (Fig. 4B, 56% vs. 33%), even at equally advanced stages of tumor progression. Unlike naive mice and AIT recipients, the myeloid cellular compartment of the untreated control group mainly consisted of MDSCs (Fig. 4B, $P = 0.03$). The emergence of $Gr1^{-/low}CD11b^{-/low}$ APCs in the animals treated with AIT was associated with a significantly increased frequency of splenic CD25$^+$ NKT cells compared with the control group (Supplementary Fig. 3, $P = 0.037$). Further analyses showed similar levels of MHCII expression (MFI and % gated) in both groups, though those treated with AIT had a significantly higher frequency of $Gr1^{-/low}CD11b^{-/low}$MHCII$^+$ APCs among all splenocytes (Fig. 4C, $P = 0.001$). AIT also resulted in the up-regulation of CD86 (Fig. 4D, MFI: 32 vs. 66, $P = 0.01$) and down-regulation of CD40 (Fig. 4D, 616 vs. 278, $P = 0.001$) on $Gr1^{-/low}CD11b^{-/low}$ cells. In fact, AIT restored the frequency of $Gr1^{-/low}CD11b^{-/low}$MHCII$^+$ APCs and the expression of CD40 to the levels similar to those in naive mice, though CD86 expression was uniquely up-regulated following AIT (Supplementary Fig. 4A). AIT also resulted in a significantly increased frequency of splenic CD11c$^+$ DCs and F4/80$^+$ macrophages (Supplementary Fig. 4B, $P = 0.001$ and $P = 0.018$, respectively). In order to determine whether $Gr1^{-/low}CD11b^{-/low}$ APCs of the control and AIT groups had the capacity to respond to inflammatory stimuli and undergo maturation, LPS stimulation was performed in vitro. While LPS stimulation resulted in similar trends for both groups, as shown in Fig. 4E, tumor burden with or without AIT resulted in a unique pattern of maturation; we observed that $Gr1^{-/low}CD11b^{-/low}$ cells of the AIT group increased the expression of CD86 (MFI: 360 vs. 667, $P = 0.022$) and CD40 (MFI: 662 vs. 902, $P = 0.023$) whereas those of the control group increased the expression of MHCII (MFI: 2200 vs. 5647,

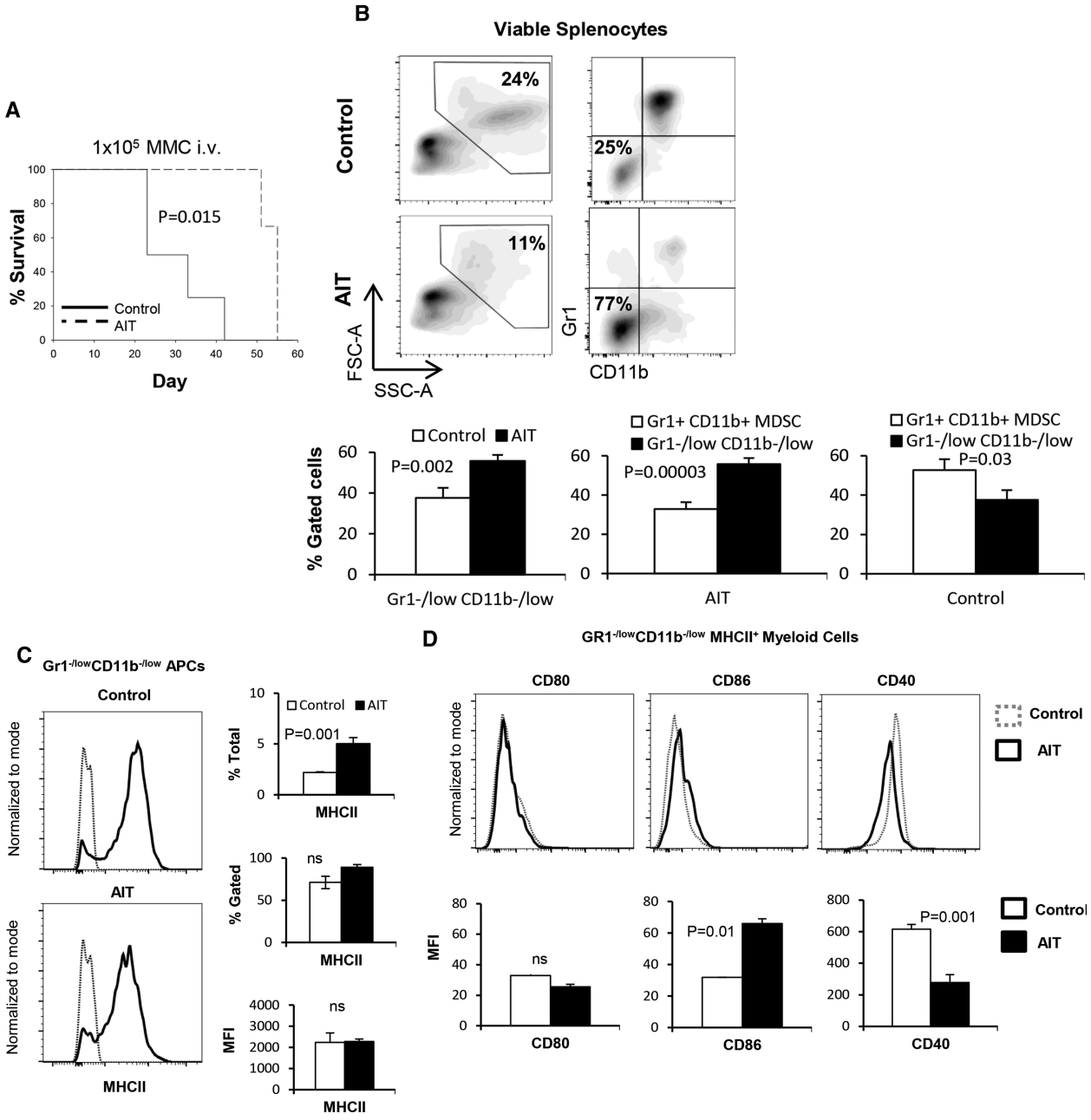

FIGURE 4 **$Gr1^{-/low}CD11b^{-/low}$ myeloid cells are modulated during tumor challenge or AIT.** (A) Kaplan–Meier analysis of survival in FVBN202 mice that were injected with 10^5 MMC cells i.v.; animals were sacrificed when they became moribund due to lung metastases. (B) Splenocytes of the control and AIT groups were analyzed by flow cytometry after staining with fluorescently labeled anti-Gr1 and anti-CD11b Abs. Data show the frequency of the splenic $Gr1^{-/low}CD11b^{-/low}$ cells and MDSCs in the control and AIT groups. (C) Frequency and expression levels of MHCII were determined on gated $Gr1^{-/low}CD11b^{-/low}MHCII^{+}$ cells of the AIT and control groups. (D) Gated Gr1–/low CD11b–/low cells were analyzed for the expression of co-stimulatory molecules in the spleens of the AIT and control groups. (E) $Gr1^{-/low}CD11b^{-/low}$ cells were sorted from the spleens of the AIT and control groups and cultured for 24 h in the presence or absence of LPS (+LPS and −LPS). Gated cells were then analyzed for the expression of MHCII and co-stimulatory molecules. Data represent mean ± SEM of triplicate experiments

$P = 0.02$), CD80 (MFI: 53 vs. 107, $P = 0.053$), and CD86 (MFI: 282 vs. 525, $P = 0.042$).

As the $Ly6G^{+}Ly6C^{-}$ subset had a higher expression of co-stimulatory molecules than the $Ly6G^{-}Ly6C^{-}$ subset in naïve mice (Fig. 3), we sought to determine whether this trend was also present during tumor burden or following AIT. Subset analysis of $Gr1^{-/low}CD11b^{-/low}$ APCs showed the emergence of a $Ly6G^{+}Ly6C^{-}$ cell population in tumor-bearing mice that received AIT when compared with the control group (Fig. 5A, 35% vs. 7%). Unlike untreated tumor-bearing mice, animals receiving AIT showed a similar trend with naïve mice in regards to the frequency of $Ly6G^{+}Ly6C^{-}$ myeloid cells (Supplementary Fig. 5). Whereas both subsets showed comparable levels of the expression of MHCII, CD80, and CD40 in the control and AIT groups, the $Ly6G^{+}Ly6C^{-}$ subset exhibited a significantly higher level of CD86 expression (Fig. 5B, Control, MFI: 17 vs. 27; AIT, MFI: 16 vs. 41). As expected, the mature $Ly6G^{+}Ly6C^{-}$ subset

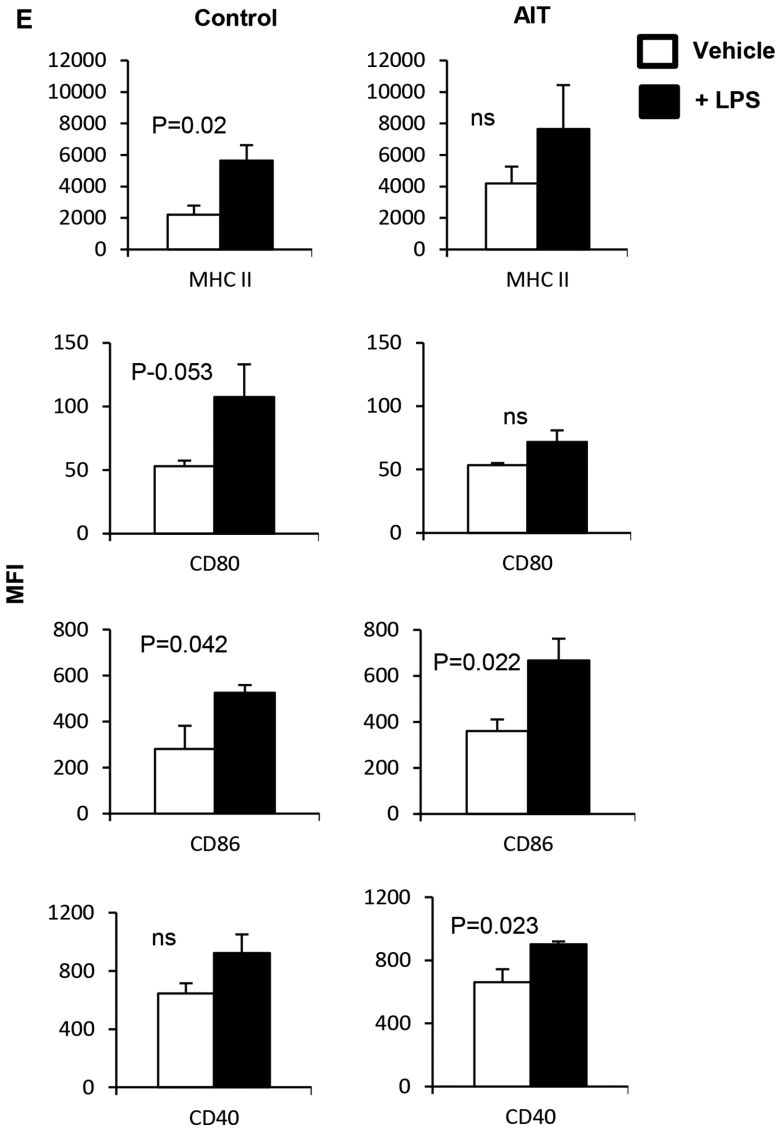

FIGURE 4 Continued

did not result in an increase in the expression of CD86 following LPS stimulation. However the Ly6G$^-$Ly6C$^-$ subset in the control group and in the AIT group did experience increases in the expression of CD80 (Fig. 4C, $P = 0.042$ and $P = 0.058$) and CD86 (Fig. 4C, $P = 0.004$ and $P = 0.058$). The Ly6G$^-$Ly6C$^-$ subset within control mice also increased the expression of MHCII ($P = 0.021$) and CD40 ($P = 0.05$) following LPS stimulation. Therefore, these data suggest that AIT rescues the myeloid compartment of tumor-bearing animals by promoting the maturation of myeloid cells to the frequency and functional potential observed in naïve mice.

3.4 | Gr1$^{-/low}$CD11b$^{-/low}$ Ly6G$^+$Ly6C$^-$ APCs are present within the tumor bed

To determine whether Gr1$^{-/low}$CD11b$^{-/low}$ APCs are present in the tumor bed, tumor lesions of both the AIT and control groups were analyzed when animals were euthanized due to tumor progression with similar tumor burden. As in the spleen, we again found that Gr1$^{-/low}$CD11b$^{-/low}$ cells dominated the tumor-infiltrating Fsc^{hi} Ssc^{hi} myeloid cell compartment within the AIT group, where they demonstrated a greater than 3-fold increase in frequency over Gr1$^+$CD11b$^+$ MDSCs (Supplementary Fig. 6A, 14% vs. 46%, p = 0.016). Such differences were, again, not observed in the control group, These Gr1$^{-/low}$CD11b$^{-/low}$ APCs had similar pattern of maturation between the AIT and control groups to that of the spleen, as shown by comparable levels of the expression of MHCII, CD80, CD86, and CD40 (Supplementary Fig. 6B). Within the tumor bed, the Ly6G$^+$Ly6C$^-$ subset was clearly dominant within the AIT group (Supplemental Fig. 6C, 63% vs. 16%; $P = 0.014$). Whereas both subsets showed comparable levels of costimulatory molecule expression, the Ly6G$^+$Ly6C$^-$ subset demonstrated more robust MHCII expression at the tumor site of the AIT group (Supplementary Fig. 6D, MFI: 11984 vs. 4739, $P = 0.026$).

3.5 | Gr1$^{-/low}$CD11b$^{-/low}$ APCs exhibit immune stimulatory function

In order to determine if Gr1$^{-/low}$CD11b$^{-/low}$ APCs possess immune stimulatory function during tumor burden and/or following AIT, splenic lymphocytes from the AIT and control group were independently cultured with MMC tumor cells in the presence or absence of

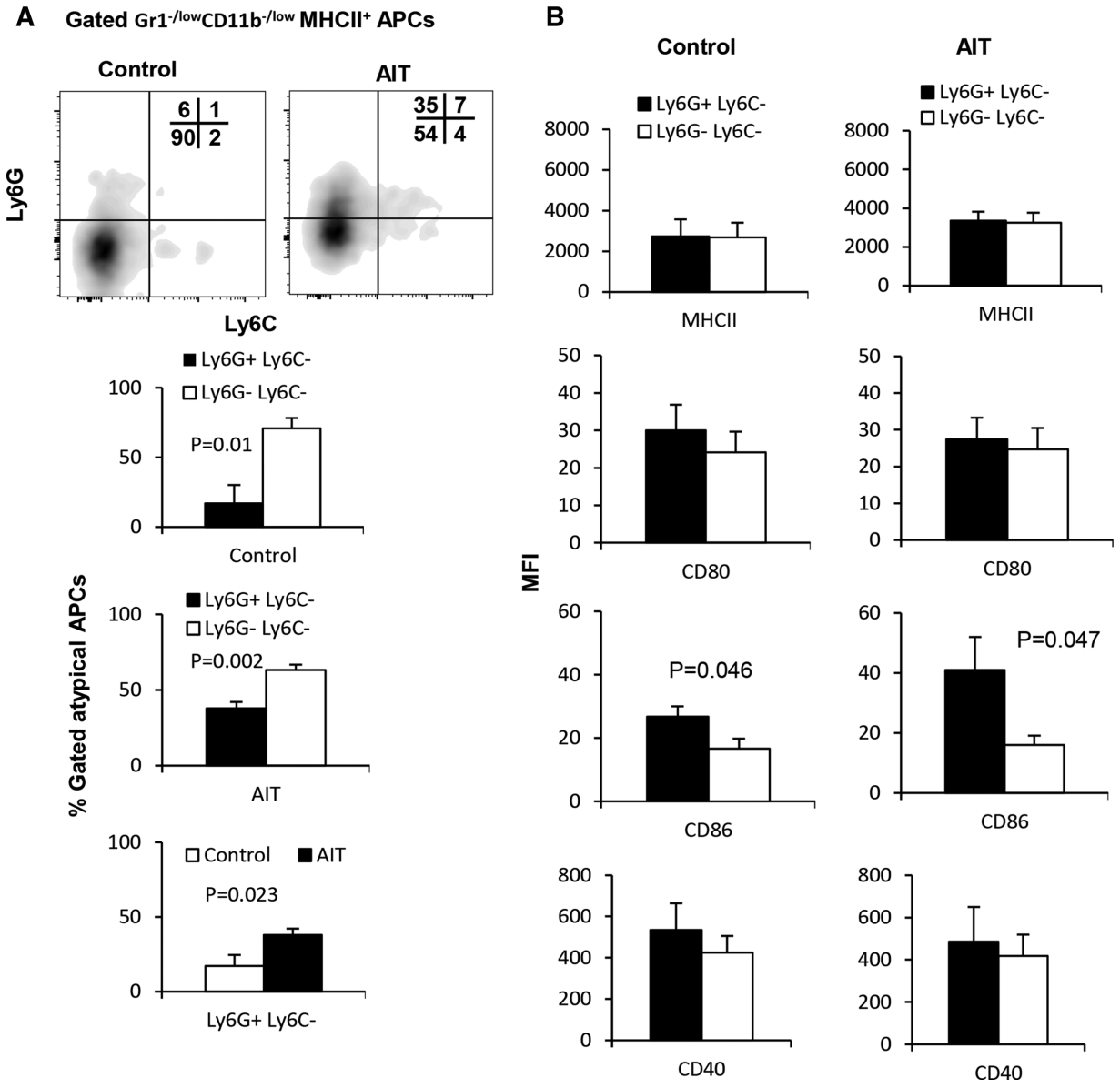

FIGURE 5 **Tumor burden or AIT modulates Gr1$^{-/low}$CD11b$^{-/low}$ myeloid cells.** (A) Splenocytes of FVBN202 mice bearing metastatic tumor in the lung without treatment (Control) or after AIT (AIT) were subjected to analysis by flow cytometry. (A) Comparative analysis of Ly6G^{+}Ly6C^{-} and Ly6G^{-}Ly6C^{-} subsets among gated APCs of control and AIT groups. (B) Expression of MHCII and co-stimulatory molecules on Ly6G^{+}Ly6C^{-} and Ly6G^{-}Ly6C^{-} subsets in gated APCs of control and AIT groups. Gated cells were then analyzed for the expression of MHCII and co-stimulatory molecules. (C) Gr1$^{-/low}$CD11b$^{-/low}$ APCs were sorted from the spleens of the AIT and control groups, and cultured for 24 h in the presence or absence of LPS (+LPS and −LPS). Data represent mean ± SEM of triplicate experiments

sorted autologous Gr1$^{-/low}$CD11b$^{-/low}$ cells. As shown in Fig. 6A, lymphocytes derived from the AIT group released IFN-γ in the presence of Neu^{+} MMC cells ($p = 0.0001$). Importantly, the IFN-γ producing immune response to MMC was significantly boosted by autologous Gr1$^{-/low}$CD11b$^{-/low}$ APCs (Fig. 6A, $p = 0.015$). On the other hand, lymphocytes derived from the control group did not demonstrate significant IFN-γ release in the presence of MMC; the addition of autologous Gr1$^{-/low}$CD11b$^{-/low}$ APCs did not enhance this response (Fig. 6B). In order to determine if Gr1$^{-/low}$CD11b$^{-/low}$ myeloid cells from the control group retained their immune stimulatory function, they were co-cultured with tumor-reactive T cells from the AIT group in the presence or absence of MMC. We hypothesized that T cell specific killing of MMC cells from the AIT group could facilitate cross presentation of tumor Ags by Gr1$^{-/low}$CD11b$^{-/low}$ APCs, resulting in the enhancement of the immune response. As shown in Fig. 6C, the presence of Gr1$^{-/low}$CD11b$^{-/low}$ APCs boosted tumor-reactive IFN-γ production by splenic T cells derived from the AIT group ($P = 0.0002$). This was associated with the induction of apoptosis in MMC by reprogrammed T cells that were used for AIT compared with those of the control group (Fig. 6D, $P = 0.0004$). To assess the possibility of Gr1$^{-/low}$CD11b$^{-/low}$ myeloid cells to potentially uptake and cross-present Ag to T cells, we first pulsed these cells with ovalbumin conjugated to a fluorophore. As shown in Fig. 6E, Gr1$^{-/low}$CD11b$^{-/low}$ myeloid cells demonstrated the ability to uptake this protein, with

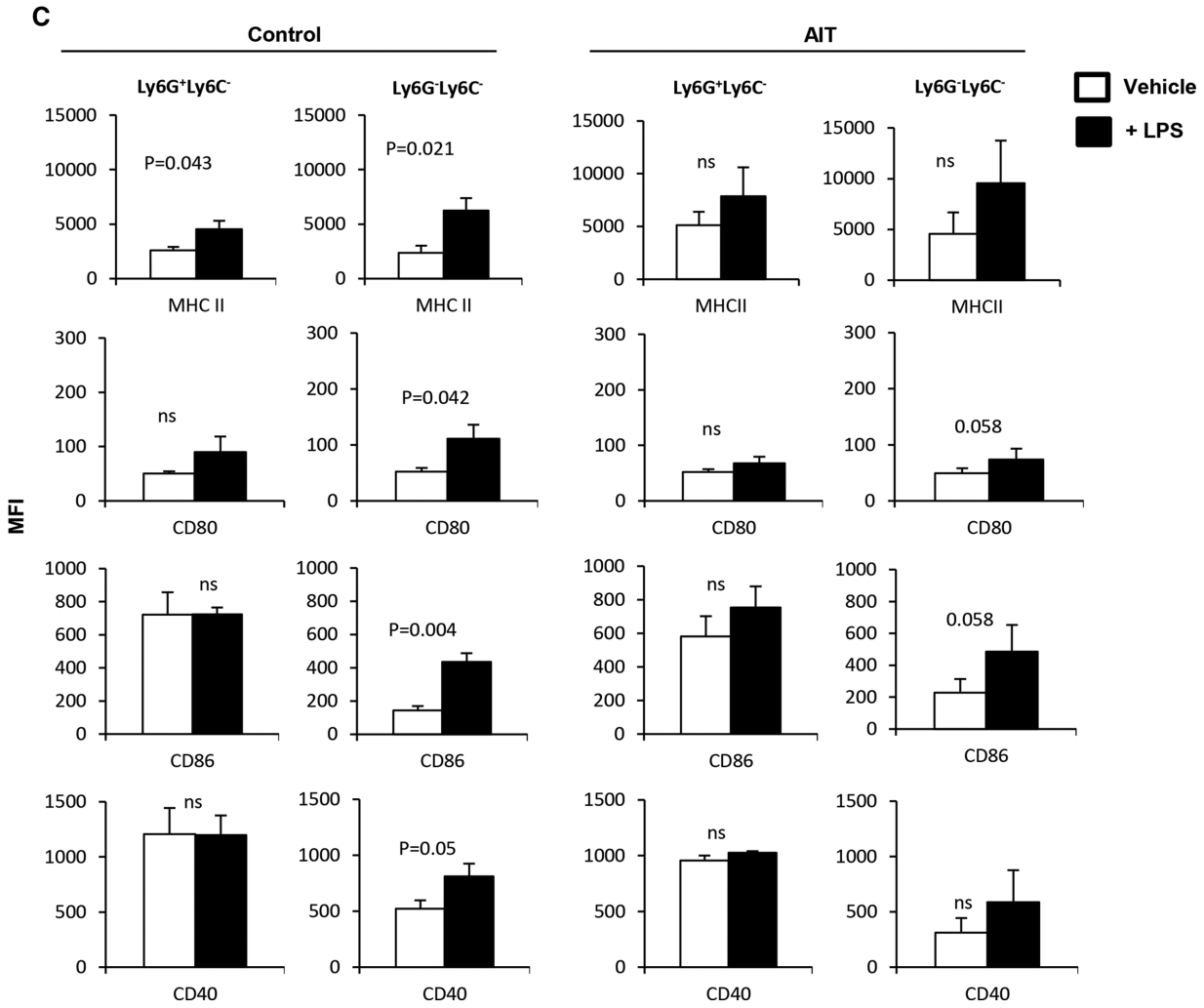

FIGURE 5 Continued

increased fluorescence intensity over time. Although it appears these cells have a reduced efficiency to uptake this Ag compared to DCs, these data suggest that Gr1$^{-/low}$CD11b$^{-/low}$ myeloid cells may potentially function to cross-present processed Ag to T cells.

Thus, to specifically determine if Gr1$^{-/low}$CD11b$^{-/low}$ myeloid cells could cross-present Ag to provoke a T cell response, we sorted splenic Gr1$^{-/low}$CD11b$^{-/low}$ myeloid cells and pulsed them with recombinant Neu ECD protein, followed by a culturing period with tumor-sensitized T cells. In fact, as can be seen in Fig. 6F, Gr1$^{-/low}$CD11b$^{-/low}$ myeloid cells were able to induce IFN-γ production from tumor-sensitized T cells only after they were pulsed with Neu ECD, suggesting these cells possess Ag-processing and presentation functionality. Bone marrow-derived DCs were used a positive control for Ag cross presentation; irradiated MMC cells were used as a specificity control for assessing Neu-reactive T cell function. We then utilized a direct cytotoxicity assay to demonstrate that sorted Gr1$^{-/low}$CD11b$^{-/low}$ myeloid cells from tumor-bearing mice boosted tumor-reactive T cell-mediated killing of MMC target cells, ex vivo (Fig. 7A, $P = 0.001$). These data suggest that although tumor burden drives the expansion of MDSCs and suppresses the expansion of mature Ly6G^{+} Ly6C^{-} APCs, these Gr1$^{-/low}$CD11b$^{-/low}$ cells retain their immune stimulatory function, but may not become fully functional in the presence of a weak antitumor immune response.

4 | DISCUSSION

Here, we describe a new class of APC, Gr1$^{-/low}$CD11b$^{-/low}$ cells that do not express pan markers of myeloid DCs (CD11c), plasmacytoid DCs (Ly6C) or macrophage (F4/80). Characterization of these cells demonstrated their expression of MHCII and the costimulatory molecules CD80, CD86, and CD40 at the steady state. Further characterization of this population revealed that while the majority of Gr1$^{-/low}$CD11b$^{-/low}$ myeloid cells do not express T or B cell lineage markers, we found that B cells interact with this myeloid APC population, a phenomenon that has classically been described to occur between DCs and B cells.[35,36] This interaction may contribute to the immune stimulatory function of these atypical APCs, as such an interaction has been reported to boost immune stimulatory function of conventional DCs.[37] The frequency of Fsc^{hi} Ssc^{hi} Gr1$^{-/low}$CD11b$^{-/low}$ APCs was significantly greater than that of DCs in the spleen. Importantly, Gr1$^{-/low}$CD11b$^{-/low}$ APCs were also present at the tumor site

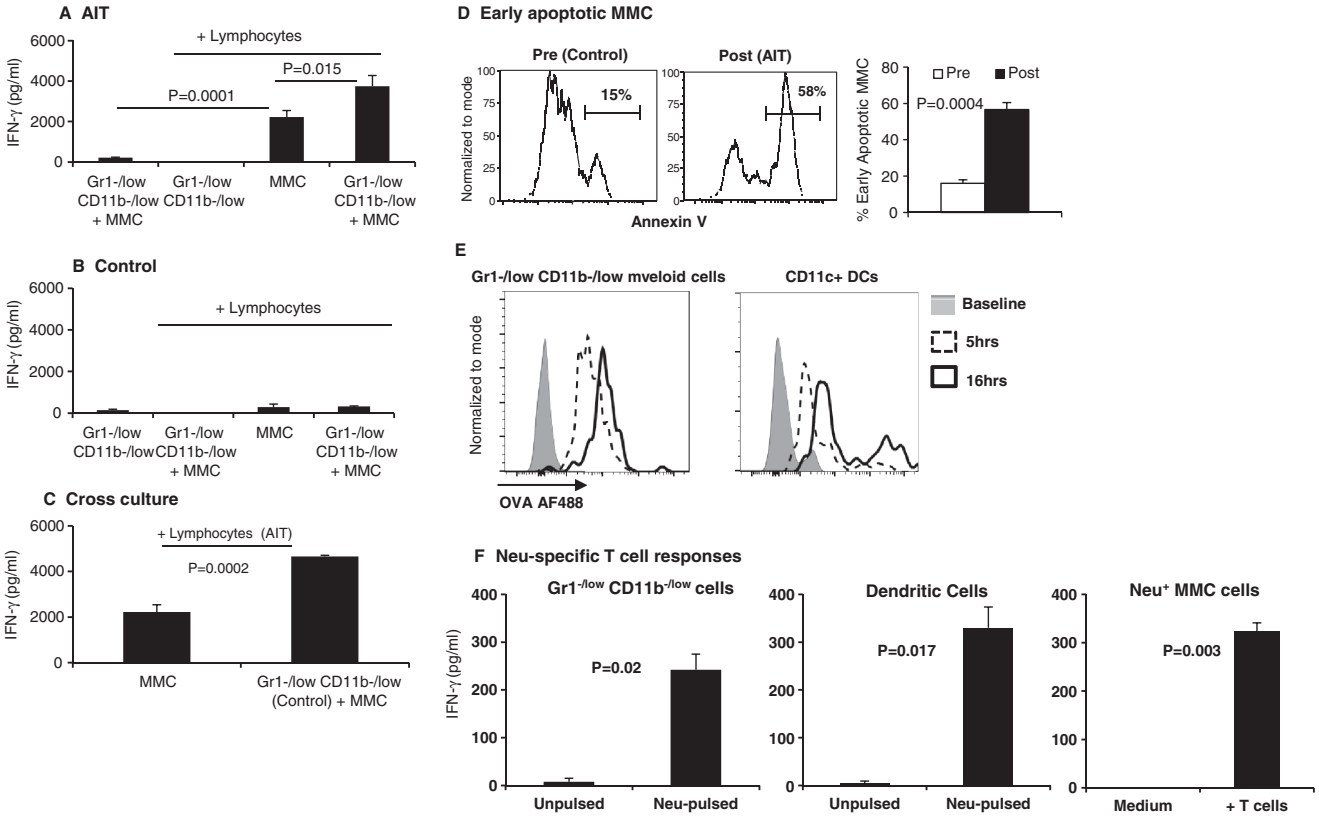

FIGURE 6 **Gr1$^{-/low}$CD11b$^{-/low}$ myeloid cells retain their immune stimulatory function during tumor burden and display characteristics of Ag-presentation.** FACS sorted splenic Gr1$^{-/low}$CD11b$^{-/low}$ cells from (A) AIT recipients or (B) Control mice were co-cultured without or with MMC (5:1) and without or with endogenous splenic lymphocytes (1:2) for 20 h; supernatant IFN-γ concentration was determined by ELISA. (C) Lymphocytes of the AIT group were cultured with MMC in the presence or absence of sorted Gr1$^{-/low}$CD11b$^{-/low}$ cells of the control group. Data represent mean ± SEM after subtracting background signal from control conditions. (D) Quantification of Annexin V+ early apoptotic MMC cells after culture with freshly isolated lymphocytes of tumor-bearing control mice prior to the ex vivo re-programming (Pre) or with re-programmed lymphocytes used for AIT (Post). Data represent quadruplicate experiments. (E) Splenocytes (10^6 cells/ml) of naïve FVBN202 mice were pulsed with 50 ug/ml Alexa Fluor 488 (AF488)-conjugated ovalbumin in RPMI1640 supplemented with 10% FBS for 5 or 16 h. Unpulsed cells were used as control (Baseline). Gated FVS– viable cells were subgated for CD11c+ DCs or Gr1$^{-/low}$CD11b$^{-/low}$ cells, and analyzed for intensity of Alexa Fluor 488 as a marker of ovalbumin internalization. (F) Sorted Gr1$^{-/low}$CD11b$^{-/low}$ splenic cells or bone marrow-derived CD11c+ DCs were pulsed with Neu ECD and cultured with tumor-sensitized T cells. Irradiated MMC target cells were used as a positive control

of animals bearing lung metastases at a frequency that was inversely proportional to that of MDSCs. Interestingly AIT drove the accumulation of Gr1$^{-/low}$CD11b$^{-/low}$ APCs while concomitantly reducing the frequency of Gr1^{+}CD11b^{+} MDSCs both in the spleen and within the tumor bed; this was associated with an improved survival of tumor-bearing animals.

Gr1$^{-/low}$CD11b$^{-/low}$ APCs were abundant in the steady state in naïve mice in vivo and had antitumor immune stimulatory function without any need for further ex vivo activation, although stimulation by LPS suggested they maintain the potential for further activation. These data suggest that Gr1$^{-/low}$CD11b$^{-/low}$ APCs may be optimal performers in terms of Ag uptake as well as Ag presentation. In fact, Gr1$^{-/low}$CD11b$^{-/low}$ myeloid cells were capable of Ag uptake and cross-presentation with similar efficiency to CD11c+ DCs. This paradoxical property of Gr1$^{-/low}$CD11b$^{-/low}$ myeloid cells was associated with the presence of two subsets; a Ly6G^{+}Ly6C^{-} subset and a Ly6G^{-}Ly6C^{-} subset. While the Ly6G^{+}Ly6C^{-} subset showed higher basal maturity, the emergence of which was associated with prolonged survival of tumor-bearing mice, the Ly6G^{-}Ly6C^{-} subset showed less maturity and higher responsiveness to LPS stimulation.

Tumor burden altered the frequency of Gr1$^{-/low}$CD11b$^{-/low}$ myeloid cells but did not impair their immune stimulatory function; these cells, when derived from either the control group or the AIT group, were able to boost tumor-reactive T cell responses. Interestingly, AIT during tumor burden resulted in the modulation of the myeloid cell compartment, revealing an inverse relationship between Gr1$^{-/low}$CD11b$^{-/low}$ myeloid cells and MDSCs. Such modulation of Gr1$^{-/low}$CD11b$^{-/low}$ cells by AIT was associated with a significantly higher frequency of the Ly6G^{+}Ly6C^{-} subset and splenic CD25+ NKT cells, which increased survival of animals. These observations are supported by previous work from our group and others.[23,24,27,38] It has previously been shown by our group that MDSCs can be rendered immune stimulatory in the presence of CD25^{+} NKT cells. The removal of NKT cells from tumor-reactive lymphocytes resulted in the inability of AIT to modulate MDSCs to become immune stimulatory, and failed to protect animals from tumor challenge.[27] Similar observations were

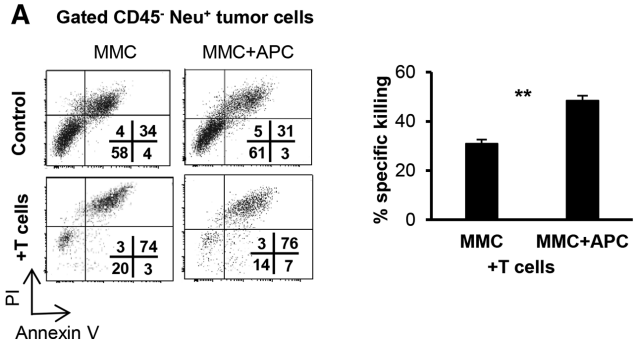

FIGURE 7 **Gr1$^{-/low}$CD11b$^{-/low}$ APCs boost antitumor function of T cells. (A)** Tumor-reactive T cells derived and expanded from FVBN202 mice were co-cultured with MMC (10:1 ratio) in the presence or absence of sorted Gr1$^{-/low}$CD11b$^{-/low}$ cells (5:1 ratio). Tumor cell cytotoxicity was determined on gated CD45-Neu+ tumor cells using control tumor cells alone, or in the presence of sorted Gr1$^{-/low}$CD11b$^{-/low}$ APCs, T cells, or sorted Gr1$^{-/low}$CD11b$^{-/low}$ APCs and T cells. Percent increased apoptosis of tumor cells by T cells in the absence (MMC) or presence of Gr1$^{-/low}$CD11b$^{-/low}$ APCs (MMC+APC) was calculated by normalizing to the respective control. Data represent mean ± SEM of triplicate experiments

made using PBMCs of patients with breast carcinoma showing that HER-2/Neu-specific T cell responses were sustained in the presence of MDSCs; these sustained T cell responses were associated with the loss of CD11b and the up-regulation of HLA-DR on MDSCs, as well as the presence of CD25+ NKT cells.[24] Therefore, our current results suggest that a sufficient frequency of activated NKT cells in secondary lymphoid organs as well as the tumor microenvironment may modulate the myeloid cell compartment in tumor bearing mice to reduce the suppressive capacity of MDSCs, while also driving the emergence of Ly6G$^+$Ly6C$^-$Gr1$^{-/low}$CD11b$^{-/low}$ immune stimulatory APCs.

The immune stimulatory function of Gr1$^{-/low}$CD11b$^{-/low}$ APCs was also associated with the induction of specific tumor cell killing by Ag-sensitized T cells. In fact, our data suggest that Gr1$^{-/low}$CD11b$^{-/low}$ myeloid cells function as APCs to process and cross-present tumor Ags to tumor-reactive T cells, resulting in the promotion of antitumor immune responses. This was further confirmed by showing a higher antitumor function of T cells in the presence of Gr1$^{-/low}$CD11b$^{-/low}$ myeloid cells, as well as the ability of these cells to uptake Ag, and to cross-present to tumor-reactive T cells. These properties of Gr1$^{-/low}$CD11b$^{-/low}$ APCs make them a potential candidate for a cell-based immunotherapy of cancer without having limitations of DC-based vaccines. Such impaired DC function is attributed to MDSCs both in vivo[39] and in vitro.[40] Similar MDSC-mediated suppressive function of macrophages has been reported in cancer patients.[41] Furthermore, DC-intrinsic immune suppressive activity has been reported in cancer patients as well as in animal models of transplanted and spontaneous carcinoma.[42–46]

In summary, we have identified Gr1$^{-/low}$CD11b$^{-/low}$ myeloid cells that possess characteristics of APCs that are unique in the following ways: (i) they are more abundant than DCs, (ii) they are heterogeneous making them highly effective in both Ag uptake and Ag presentation simultaneously, (iii) they retain their immune stimulatory function during tumor burden, and are inversely correlated with MDSCs, and (iv) their frequency is increased in the presence of CD25+ NKT cells. Moreover, human CD33$^+$CD11b$^{-/low}$HLA-DR$^+$ myeloid cells appear to have similar immune stimulatory function as murine Gr1$^{-/low}$CD11b$^{-/low}$ APCs.[24]

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DISCLOSURES

Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Department of Defense. The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

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Research Paper

Autophagy-deficient breast cancer shows early tumor recurrence and escape from dormancy

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ABSTRACT

Breast cancer patients who initially respond to cancer therapies often succumb to distant recurrence of the disease. It is not clear why people with the same type of breast cancer respond to treatments differently; some escape from dormancy and relapse earlier than others. In addition, some tumor clones respond to immunotherapy while others do not. We investigated how autophagy plays a role in accelerating or delaying recurrence of neu-overexpressing mouse mammary carcinoma (MMC) following adriamycin (ADR) treatment, and in affecting response to immunotherapy. We explored two strategies: 1) transient blockade of autophagy with chloroquine (CQ), which blocks fusion of autophagosomes and lysosomes during ADR treatment, and 2) permanent inhibition of autophagy by a stable knockdown of ATG5 ($ATG5^{KD}$), which inhibits the formation of autophagosomes in MMC during and after ADR treatment. We found that while CQ prolonged tumor dormancy, but that stable knockdown of autophagy resulted in early escape from dormancy and recurrence. Interestingly, $ATG5^{KD}$ MMC contained an increased frequency of ADR-induced polyploid-like cells and rendered MMC resistant to immunotherapy. On the other hand, a transient blockade of autophagy did not affect the sensitivity of MMC to immunotherapy. Our observations suggest that while chemotherapy-induced autophagy may facilitate tumor relapse, cell-intrinsic autophagy delays tumor relapse, in part, by inhibiting the formation of polyploid-like tumor dormancy.

INTRODUCTION

Autophagy plays a paradoxical role in the promotion and inhibition of cancer. On the one hand, autophagy has a cancer-promoting role by protecting tumor cells from chemotherapy or providing a source of energy for tumor cells to survive under hypoxic and acidic conditions despite the lack of mature vessels [1].

On the other hand, inhibition of autophagy by disruption of *Beclin 1* or deletion of *ATG5* increases the frequency of spontaneous malignancies [2] or liver tumor [3], respectively. Recently, four different mechanisms have been proposed to describe paradoxical functions of autophagy in cancer, which include cytotoxic, cytostatic, cytoprotective and non-protective autophagy [4]. There are also three major types of autophagy which include micro-autophagy involving the direct engulfment of cytosolic material by lysosomes through invagination, chaperone-mediated autophagy involving HSP70 and the lysosomal membrane associated protein 2 A (LAMP2A), and macro-autophagy which is a highly conserved pathway involving the formation of autophagosomes, which fuse with lysosomes. To this end, ATG5 is involved in the elongation of autophagosomes to engulf toxic material for degradation. A stable knockdown of ATG5 results in the inhibition of the formation of autophagosomes and progression of macro-autophagy [5]. Chloroquine (CQ), on the other hand, does not have any effects on autophagosomes but it blocks the fusion of autophagosomes and lysosomes, thereby preventing the completion of macro-autophagy. In order to investigate the role of macro-autophagy in tumor dormancy and relapse, we performed a transient inhibition of macro-autophagy by means of CQ during chemotherapy, which mainly inhibits chemotherapy-induced autophagy while cell-intrinsic autophagy will be restored after the completion of chemotherapy. We also performed a permanent inhibition of cell-intrinsic macro-autophagy by a stable knockdown of ATG5 in tumor cells. We demonstrated that cell-intrinsic, but not chemotherapy-induced, autophagy can inhibit tumor relapse.

RESULTS

Adriamycin induces autophagy in MMC

In order to determine whether ADR induces autophagy and in turn establishes tumor dormancy, MMC cells were treated with ADR in the presence or absence of CQ, a pharmacological agent used to block the final stages of autophagy, specifically the fusion of autophagosomes with lysosomes that is necessary for digestion of the cargo in the autophagosomes (frequently termed "autophagic flux"). CQ blocked this autophagic flux as evidenced by the enhanced accumulation of acidic vesicles (red signals) (Figure 1A, ADR and ADR+CQ). We further monitored degradation of the p62/SQSTM1 protein as a marker of autophagic flux, and LC.3B expression as a marker of autophagosomes formation (since LC3 is a component of the autophagosomes). As shown in Figure 1B, ADR did not induce degradation of p62/SQSTM1 although it elevated LC.3B, suggesting that ADR induces autophagy but fails to drive autophagy to completion and p62/SQSTM1 degradation.

A transient blockade of autophagy by CQ during ADR treatment delays tumor relapse *in vitro* but not *in vivo*

Since CQ is being used to sensitize tumor cells susceptible to chemotherapy [6], we sought to determine whether blockade of autophagy by CQ during ADR treatment affects tumor dormancy and relapse. We showed that the presence of CQ during ADR treatment, *in vitro*, resulted in prolonging tumor dormancy such that, while ADR treated MMC resumed cell proliferation 6 weeks after the treatment, ADR+CQ treated MMC remained dormant (Figure 2A). In order to confirm tumor cell relapse after 6 weeks, flow cytometry analysis of ADR-treated MMC was performed, and indicated a shift of Ki67- non-proliferating cells to Ki67+ proliferating cells with a greater viability (Figure 2B). In fact, MMC cells remained apoptotic by producing floater dead cells following ADR treatment (Supplementary Figure 1A) which compensated for cell proliferation and maintained tumor dormancy for 3 weeks after the completion of ADR treatment. Follow up studies on floater cells showed they were all apoptotic (Supplementary Figure 1B). A transient blockade of autophagy by CQ did not affect susceptibility of tumor cells to ADR-induced apoptosis (Supplementary Figure 2). On the other hand, a transient blockade of autophagy during ADR chemotherapy, *in vivo*, did not prolong tumor dormancy in FVBN202 mice (Supplementary Figure 3).

A transient blockade of autophagy by CQ during ADR treatment does not change susceptibility of tumor cell to immunotherapy

In order to determine whether a transient blockade of autophagy during ADR treatment affects susceptibility of dormant MMC to immunotherapy, dormant MMC were cultured with either IFN-γ or MMC-reactive T cells three weeks after treatment with ADR or ADR+CQ. As shown in Figure 3, untreated MMC or dormant MMC treated with ADR or ADR+CQ all remained susceptible to IFN-γ treatment or T cells.

A stable knockdown of autophagy reduces susceptibility of MMC to ADR treatment

CQ only transiently blocks fusion of autophagosomes and lysosomes during ADR treatment such that after removal of CQ, accumulated autophagosomes could eventually be fused with lysosomes to complete autophagy. In order to determine the role of autophagy in tumor dormancy or relapse, we used shRNA for a stable knockdown of ATG5 ($ATG5^{KD}$) which inhibits formation of autophagosomes in MMC. Scrambled shRNA was used as control (Supplementary Figure 4A). The $ATG5^{KD}$ MMC and scrambled control MMC were irradiated to confirm that $ATG5^{KD}$ MMC cells were deficient in autophagy, using p62

and LC.3B as read outs (Supplementary Figure 4B). Tumor cells remained intact for the expression of neu antigen, as well as cell proliferation *in vitro* and *in vivo* following knockdown of autophagy (Supplementary Figure 4C–4E). Flow cytometry analysis determined a lower level of viability in MMC compared with ATG5^{KD} MMC following ADR treatment (Figure 4).

A stable knockdown of autophagy results in earlier tumor relapse associated with increased frequency of polyploid-like cells and resistance to immunotherapy

In order to determine whether a higher viability of ATG5^{KD} MMC following ADR treatment (Figure 4) facilitates an earlier tumor relapse compared with wild type MMC, follow up studies were performed for three weeks after ADR treatment. As shown in Figure 5A, ATG5^{KD} MMC survived better than autophagy-competent MMC following ADR treatment showing a significantly higher number of cells by 3 weeks after the treatment. Flow cytometry analysis of tumor cells showed greater levels of apoptosis in wild type MMC compared with ATG5^{KD} MMC (Figure 5B, $p < 0.001$). Interestingly, ATG5^{KD} MMC cells contained a higher number of polyploid-like cells following ADR treatment compared with autophagy-competent MMC (Figure 5B, $p < 0.03$).

In order to determine the *in vivo* relevance of our *in vitro* findings, FVBN202 mice were used. Tumor dormancy was first established by ADR treatment *in vitro*; FVBN202 mice (n = 7/group) were then challenged i.v. with one million viable dormant tumor cells. Animals were then sacrificed when they became moribund (lost 10% weight) as a result of massive lung metastasis. As can be seen in Figure 6A, animals that were challenged with ADR-treated ATG5^{KD} MMC developed lung metastasis significantly sooner than those that were challenged with ADR-treated MMC. Hematoxylin/eosin and immunohistochemistry analyses of tumor lesions determined a higher frequency of polyploid-like and Ki67+ tumor cells in animals that were challenged with ADR-treated ATG5^{KD} MMC (Figure 6B). Finally, ATG5^{KD} MMC were found to be resistant to T cell-induced apoptosis compared with autophagy-competent MMC (Figure 7).

DISCUSSION

Cell-intrinsic autophagy is an ongoing process, which regulates cellular metabolism and homeostasis. Autophagy is also induced by insults such as chemotherapy. Here, we studied a paradoxical role of autophagy in tumor promotion and tumor inhibition by a transient inhibition of autophagy only during chemotherapy or a stable knockdown of autophagy in MMC tumor cells. While the former transiently blocked autophagy and cell-intrinsic autophagy was restored after the completion of chemotherapy, the latter permanently blocked chemotherapy-induced autophagy and cell-intrinsic autophagy. We demonstrated that inhibition of chemotherapy-induced autophagy by CQ did not increase susceptibility of tumor cells to chemotherapy-induced apoptosis. Nevertheless, chemotherapy-induced autophagy appeared to accelerate tumor relapse such that use of CQ during chemotherapy delayed tumor relapse *in vitro*. Our observation is consistent with other reports showing that increased autophagy in residual breast cancer after neoadjuvant chemotherapy was correlated with increased risk of tumor relapse [7]. A transient blockade of autophagy during chemotherapy of tumor-bearing animals did not affect tumor relapse, perhaps, because tumor inhibitory effects of *in vivo* chemotherapy was not as effective as *in vitro* drug treatment. Also, chemotherapy-induced autophagy did not affect the sensitivity of tumor cells to apoptosis induced by IFN-γ or tumor-reactive T cells.

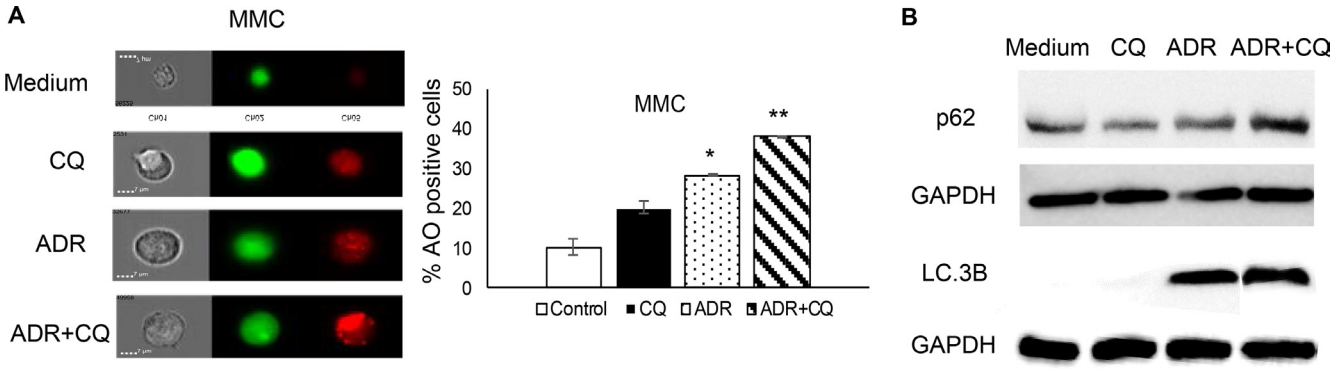

Figure 1: CQ blocks ADR-induced autophagy. MMC tumor cells received three daily doses of ADR alone (1 μM ADR for 2 hrs) (ADR) or in the presence of CQ (10 μM 3 hrs before ADR and 2 hrs during ADR treatment) (ADR+CQ), washed after each daily treatment and analyzed by acridine orange (AO) one day after the last treatment. Untreated MMC (Medium) or MMC treated with CQ (CQ) served as controls. (**A**) Acridine orange (AO) staining was analyzed for acidic vesicles (red) using image cytometry. Data represent triplicate experiments. (**B**) Levels of p62/SQSTM1 and LC.3B after treatment with ADR ± CQ indicative of autophagy induction in the absence of autophagic flux (B).

We also demonstrated that, unlike chemotherapy-induced autophagy, cell-intrinsic autophagy accelerated tumor relapse. A stable knockdown of cell-intrinsic autophagy by ATG5 shRNA resulted in a reduced sensitivity of tumor cells to chemotherapy- or T cell-induced apoptosis, and accelerated tumor relapse *in vivo*. These effects coincided with an increased frequency of multinuclear polyploid-like dormant cells. These observations suggest that chemotherapy-induced autophagy could have tumor-promoting effects and facilitate tumor relapse, whereas cell-intrinsic autophagy could synergize with cancer therapeutics and delay tumor relapse. In fact, cell-intrinsic autophagy would seem to inhibit the formation of multinuclear cells following chemotherapy, and to prevent chemotherapy-induced genetic instability associated with resistance to cancer therapeutics. Similar observations have been made in other breast tumor models by showing that CQ but not knockdown of Beclin 1 or ATG12 sensitized the tumor to chemotherapy [8]. Therefore, anti-tumor effects of autophagy inhibitors such as CQ is likely to be because of the inhibition of chemotherapy-induced autophagy while anti-tumor effects of autophagy inducers such as rapamycin may result from enhanced cell-intrinsic autophagy [9, 10]. It has been reported cancer stem cells play a role in tumor dormancy [11] and drug resistance [12], and that immunotherapeutic targeting of breast cancer stem cells inhibits growth of mammary carcinoma [13]. However, we did not detect the enrichment of CD44+CD24- cancer stem cells following ADR-induced tumor dormancy (data not shown).

Anticancer drugs and ionizing radiation tend to induce autophagy in tumor cells [14]. Treatment-induced autophagy could lead to apoptosis [15] and tumor cell dormancy [16]. We have already reported that dormant tumor cells established by ADR treatment or radiation therapy, *in vitro*, developed resistance to these treatments but remained susceptible to immunotherapy [17]. Therefore, evaluation of apoptosis or tumor growth inhibition as a single factor without evaluating

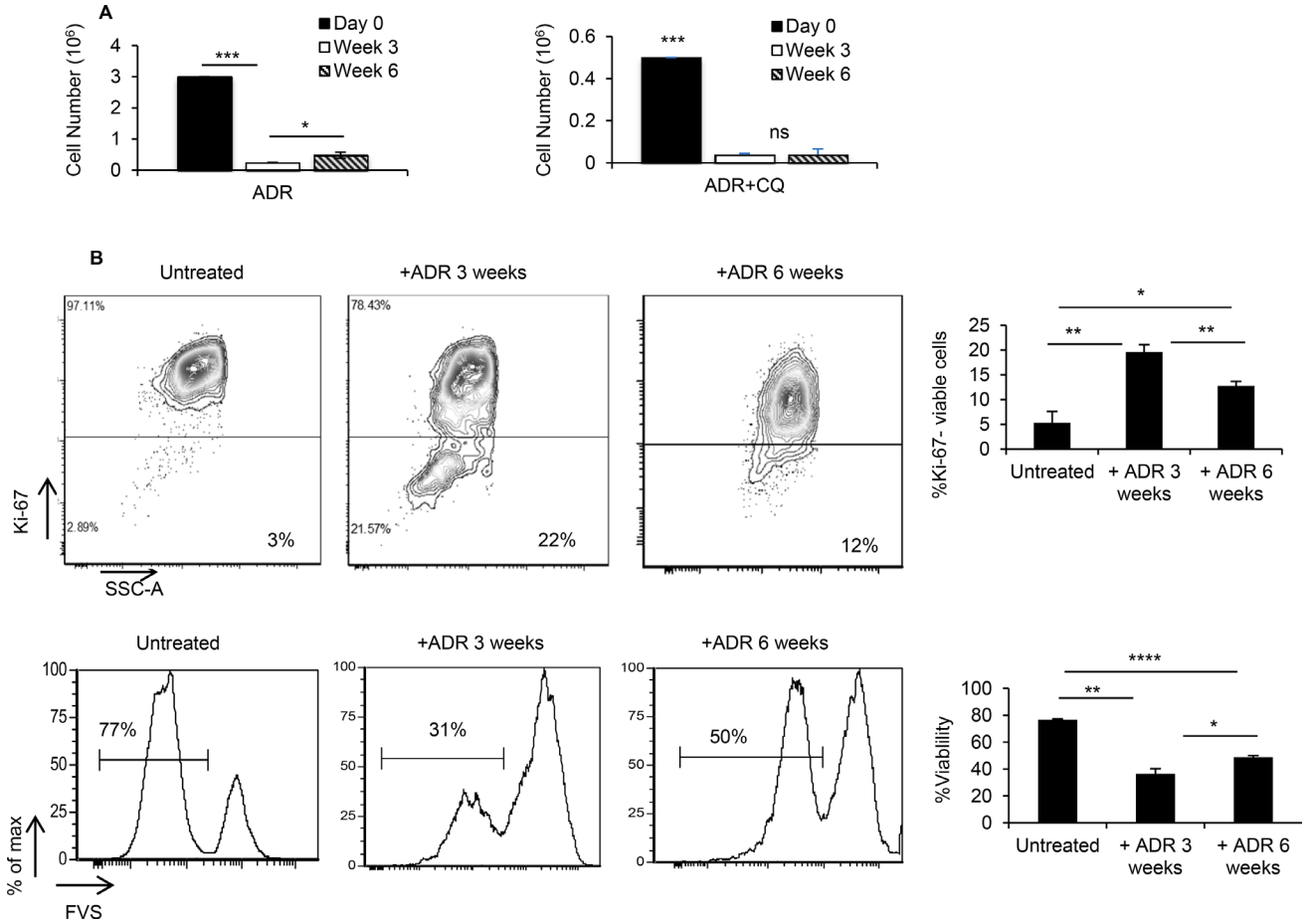

Figure 2: ADR-induced dormant tumor cells remain dormant in the presence of CQ. MMC tumor cells were treated with 3 daily doses of ADR (1 uM for 2 hrs), with one group receiving CQ (10 uM) 3 hrs prior to and during ADR treatment. Both groups remained untreated for 3 weeks and 6 weeks, *in vitro*. (**A**) Adherent viable cells were counted using trypan blue exclusion at various time points. Data represent 3 replicates ± SEM. (**B**) At weeks 3 and 6 post-treatment, Ki-67 expression (upper panel) and viability (lower panel) were quantified within the population of adherent tumor cells. Data represent 2–3 replicates ± SEM. Four independent experiments have been carried out which have shown similar results.

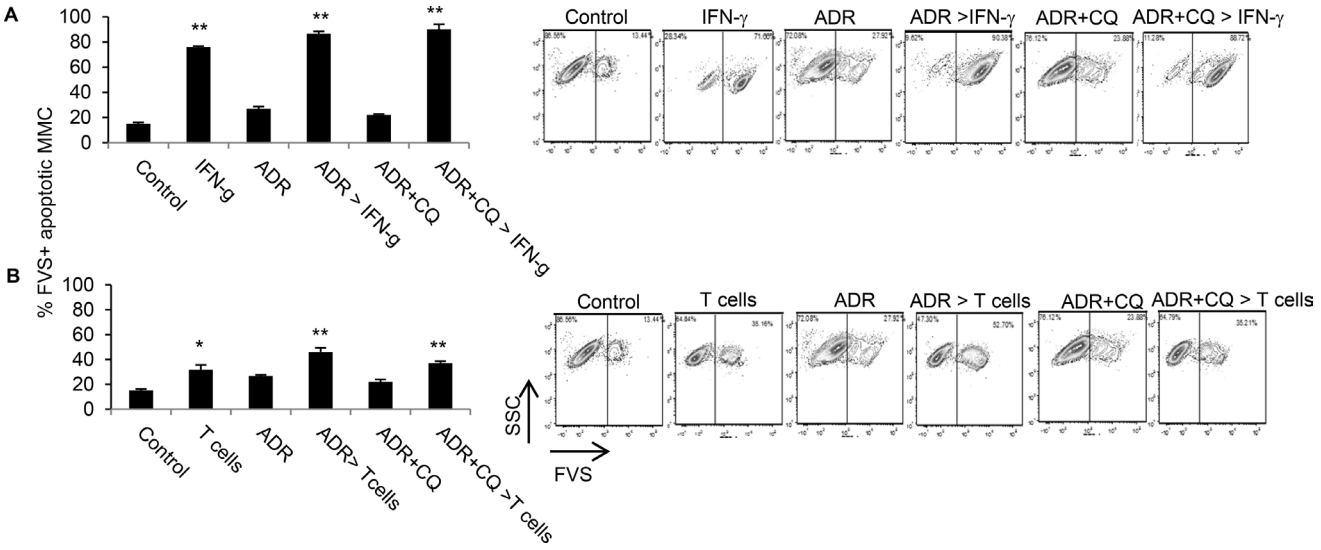

Figure 3: Dormant tumor cells established by ADR or ADR+CQ remain susceptible to immunotherapy. The *in vitro* tumor dormancy was established three weeks after three daily treatments of MMC with ADR or ADR+CQ. Untreated MMC cells were used as control. (**A**) Apoptosis was determined by FVS viability staining in MMC (control), ADR-treated dormant MMC (ADR), ADR+CQ-treated dormant MMC (ADR+CQ), as well as control MMC cultured with three daily doses of IFN-g and analyzed two days later (50 ng/ml) (IFN-g), ADR-treated dormant MMC cultured with three daily doses of IFN-g (50 ng/ml) and analyzed two days later (ADR > IFN-g), or ADR+CQ-treated dormant MMC cultured with three daily doses of IFN-g (50 ng/ml) and analyzed two days later (ADR+CQ > IFN-g). (**B**) Apoptosis was determined by FVS viability staining of MMC (control), MMC cultured with MMC-sensitized T cells for 48 hrs (T cells), ADR-treated dormant MMC (ADR), ADR-treated dormant MMC cultured with MMC-sensitized T cells for 48 hrs (ADR > T cells), ADR+CQ-treated dormant MMC (ADR+CQ), or ADR+CQ-treated dormant MMC cultured with MMC-sensitized T cells for 48 hrs (ADR+CQ > T cells). Splenic T cells were collected from MMC tumor-bearing FVBN202 mice.

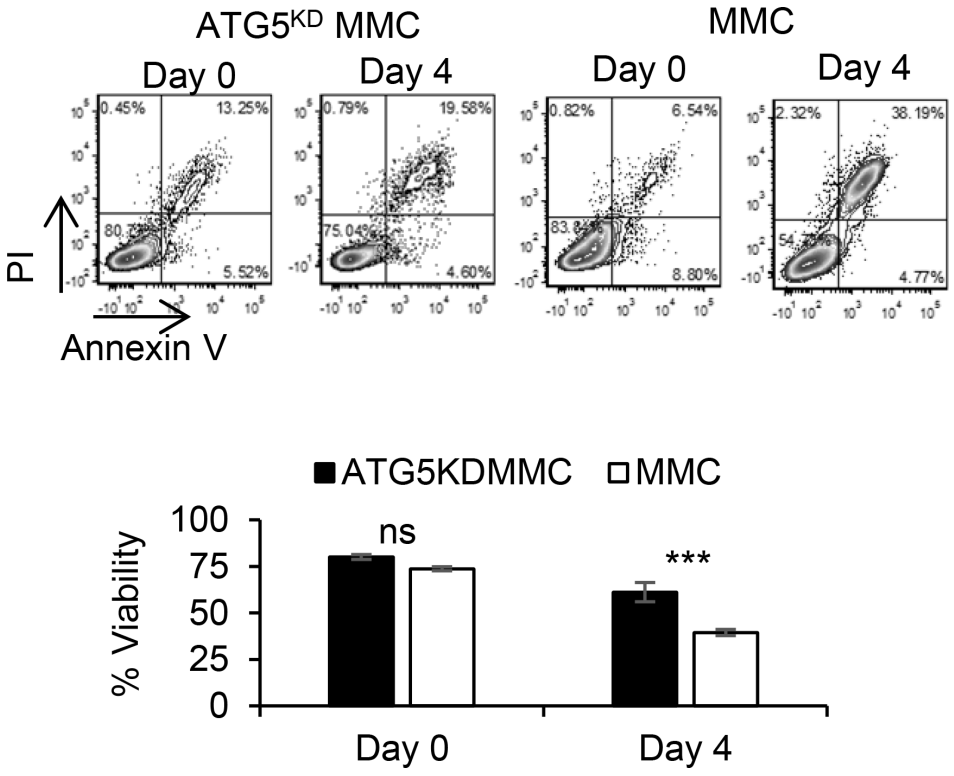

Figure 4: Autophagy knockdown tumor cells become less susceptible to ADR-induced apoptosis. Autophagy-deficient MMC (ATG5^{KD} MMC) or autophagy-competent MMC (MMC) were treated with a single dose of ADR alone (1 uM ADR for 2 hrs). Tumor cells were analyzed by Annexin V/PI staining prior to treatment (Day 0) or three days after the treatment (Day 4). Experiments were performed in triplicates.

tumor dormancy and relapse may not be sufficient for understanding anti-cancer efficacy of autophagy inhibitors such as CQ. Inhibition of autophagy by CQ during chemotherapy diminishes the expression of DNA repair proteins, resulting in tumor growth inhibition in carboplatin-resistant BRCA1 wild-type TNBC orthotopic xenografts [18]. In triple negative breast cancer, CQ sensitizes tumor cells to paclitaxel chemotherapy [19]. In several tumor models, CQ synergistically augmented sunitinib cytotoxicity on tumor cells [6]. However, the role of CQ in inhibiting tumor recurrence has yet to be determined.

Cells that are deficient in autophagy show increased levels of reactive oxygen species which result in the accumulation of DNA damage, increased double-strand breaks and polyploid nuclei [20, 21]. To this end, cell-intrinsic autophagy protects the cell from genomic instability induced by the accumulation of toxins within the cell [22]. It has been reported that Beclin1 knockout mice fail to maintain genomic integrity by increasing DNA double stranded breaks and gene amplifications [20]. A higher expression of Beclin 1 in healthy breast tissue than in breast cancer suggests a deficiency in cell-intrinsic autophagy in tumors [23], which could contribute to genomic instability during tumorigenesis. In breast cancer patients who received adjuvant chemotherapy, presence of tumor cell intrinsic autophagy contributed to reduced risk of tumor relapse [24]. Expression of ATG5 in the tumor specimens is also associated with relapse-free survival in breast cancer patients [25]. In glioma, reduced tumor cell progression and relapse by knockdown of CDGSH iron sulfur domain 2 (CISD2) was associated with the activation of Beclin 1-mediated autophagy [26].

Our observations suggest that any deficiency in tumor cell-intrinsic autophagy could result in a reduced sensitivity of breast cancer to chemotherapy or immunotherapy. Therefore, IHC analysis of tumor biopsies before and after neoadjuvant or adjuvant chemotherapy could determine cell-intrinsic and chemotherapy-induced autophagy, respectively, and in turn might predict the risk of distant recurrence of the diseases accordingly. In future studies, other murine and human breast tumor cell lines as well as other types of carcinoma cells should be used in order to determine whether our findings offer a general mechanism of autophagy-associated tumor dormancy and relapse, or it might be a cancer specific phenomenon.

MATERIALS AND METHODS

Tumor cell line

The neu overexpressing mouse mammary carcinoma (MMC) cell line was established from spontaneous mammary tumors harvested from FVBN202 mice [27]. Tumor cells were maintained in RPMI 1640 supplemented with 10% FBS.

Genetic silencing of ATG5 in MMC

Mission shRNA bacterial stocks for ATG5 and scrambled Control were purchased from Sigma Aldrich. Lentiviruses were produced in HEK 293TN cells co-transfected using Endo F ectinTM Lenti Transfection Reagent (GeneCopoeia, 1001–01) with a packaging mixture of psPAX2 and pMD2.G constructs (Addgene). Media containing the viruses was used to infect MMC cells; puromycin (1 μg/ml) was used as a selection marker to enrich for infected cells.

Antibodies

All antibodies were purchase from Biolegend (San Diego, CA, USA) unless otherwise stated. Antibodies were used as instructed by the supplier. Antibodies

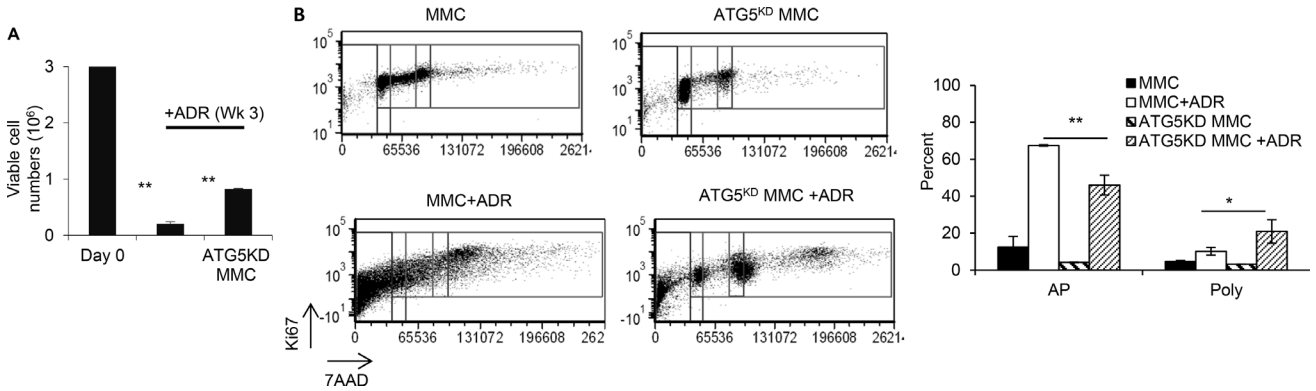

Figure 5: ADR-induced tumor dormancy in autophagy knockdown tumor cells with polyploid-like morphology compared with autophagy competent tumor cells, *in vitro*. MMC or ATG5^{KD} MMC tumor cells (3 million cells, Day 0) were treated with 3 daily doses of ADR (1uM for 2 hrs), and viable cells were counted at week 3 using trypan blue exclusion. Data represent triplicate experiments (**A**). Dot plots from each experimental group gated for cell cycle phase based upon DNA content (7-AAD) and Ki-67 expression. Events falling to the left of the G1/G0 gates are considered apoptotic cells (AP). Events falling to the far right of the G2/M gate are considered polyploid-like cells (Poly) (**B**). Three independent experiments have been performed and data represent 3 replicates ± SEM.

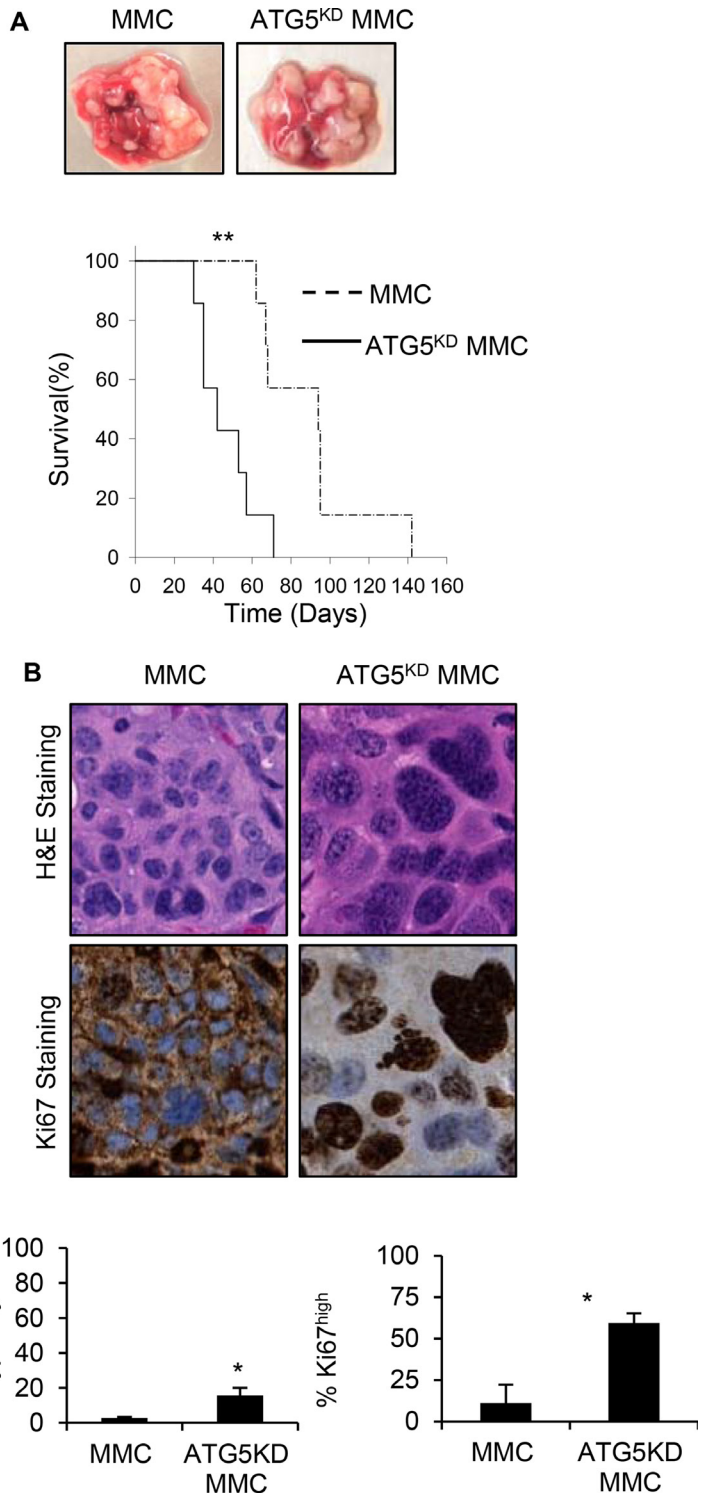

Figure 6: Earlier relapse of autophagy knockdown tumor cells with polyploid morphology compared with autophagy competent tumor cells, *in vivo*. (**A**) FVBN202 mice (n = 7) were challenged i.v. with 10^6 cells ADR-treated dormant control MMC (MMC), or ADR-treated dormant ATG5^{KD} MMC (ATG5^{KD} MMC). Animals were euthanized as soon as they became moribund. Representative tumor relapse in the lung and survival curve are shown. (**B**) Relapsed tumors were collected and immunohistochemistry slides were prepared by either staining samples with hematoxylin and eosin (H&E) or by Ki67 staining followed by subsequent digitization and analysis with NDP View software (Hamamatsu Photonics). At twenty-times magnification, three representative 0.02 mm^2 areas were chosen from the H&E slides containing approximately 100 cells to measure nuclear envelope size. Cells containing a nuclear envelope equal to or greater than 16 um with visible multi-nuclei were considered polyploid-like or high grade cells. The corresponding cell was then analyzed on the Ki67 stained slide to determine Ki67 expression levels. Data was collected from three biological samples. Significance is based on a two-tailed t-test of $p < 0.05$.

include: anti-CD16/32 (clone 93), APC-anti-mouse IgG (Poly4053), PE-Ki67 (16A8), Alexa flour 488-Ki67 (11F6), Brilliant Violet 605-CD45 (30-F11), FITC-Annexin V, APC-Annexin V, 7-AAD viability staining solution and Propidium Iodide solution (PI), mouse anti-rat neu (anti–c-Erb2/c-Neu; 7.16.4, Calbiochem, Billerica, MA, USA), FITC-FVS (BD Biosciences). All reagents were used at the manufacturer's recommended concentration.

Mice

FVBN202 transgenic female mice (The Jackson Laboratory; Bar Harbor, ME, USA) were used. These mice overexpress non-mutated, non-activated rat neu transgene under the regulation of the mouse mammary tumor virus promoter [28]. These mice develop premalignant mammary hyperplasia similar to ductal carcinoma *in situ* prior to the development of spontaneous carcinoma [29]. These studies have been reviewed and approved by the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

Experimental tumor dormancy

In vitro tumor dormancy was established by the treatment of MMC or ATG5^{KD} MMC tumor cells with 3 daily doses of ADR (Sigma-Aldrich, 1uM for 2 hrs). During ADR treatment, MMC tumor cells were cultured without or with CQ (Sigma-Aldrich, 10 uM, 3 hrs prior to and during ADR treatment). By 2 weeks after the treatment, all groups did not show any increases in the number of adherent cells, which is the characteristic of tumor dormancy. For *in vivo* induction of tumor dormancy, FVBN202 mice were challenged with ADR-treated dormant MMC or ATG5^{KD} MMC (i.v. injection of 1 million viable cells), or untreated MMC followed by 3 weekly treatments of ADR (i.v., 9 mg/kg) or with 3 weekly treatment of ADR + 60 mg/kg CQ (i.p.).

Cytotoxicity assay

Freshly isolated tumor-primed splenic T cells or *ex vivo* expanded splenic T cells were cultured with MMC at a 10:1 E:T ratio in 3 ml complete medium (RPMI-1640 supplemented with 100 U/ml of penicillin, 100 µg/ml streptomycin, 10% FBS, 10 mM L-glutamine and 5 × 10^{-5} M 2-mercaptoethanol) with 20U/ml of IL-2 (Peprotech) in 6 well culture dishes. After 48 hs cells were harvested and stained for neu (anti-c-Erb2/c–Neu, Calbiochem), Annexin V and PI according to the manufacturer's protocol (BD Pharmingen). Flow cytometry was used to analyze the viability of neu positive cells [17, 30].

IFN-γ ELISA. Reprogrammed immune cells were cultured in complete medium with irradiated (140 Gy) tumor cells, ADR-treated dormant MMC or ADR+CQ-treated dormant MMC at a 10:1 ratio for 20 hrs. Supernatants were then collected and stored at −80°C until assayed. IFN-γ was detected using a Mouse IFN-γ ELISA kit (BD Biosciences), according to the manufacturer's protocol [30].

Statistical analysis

Data are summarized as means and standard errors of the mean (SEM) with differences between groups being illustrated with graphical data presented as mean ± SEM. Statistical comparisons were made using a one-tailed or two-tailed Student *t* test and a *p*-value < 0.05 was considered significant (*: < 0.05, **: < 0.005. ***: < 0.0005, ****: < 0.00005).

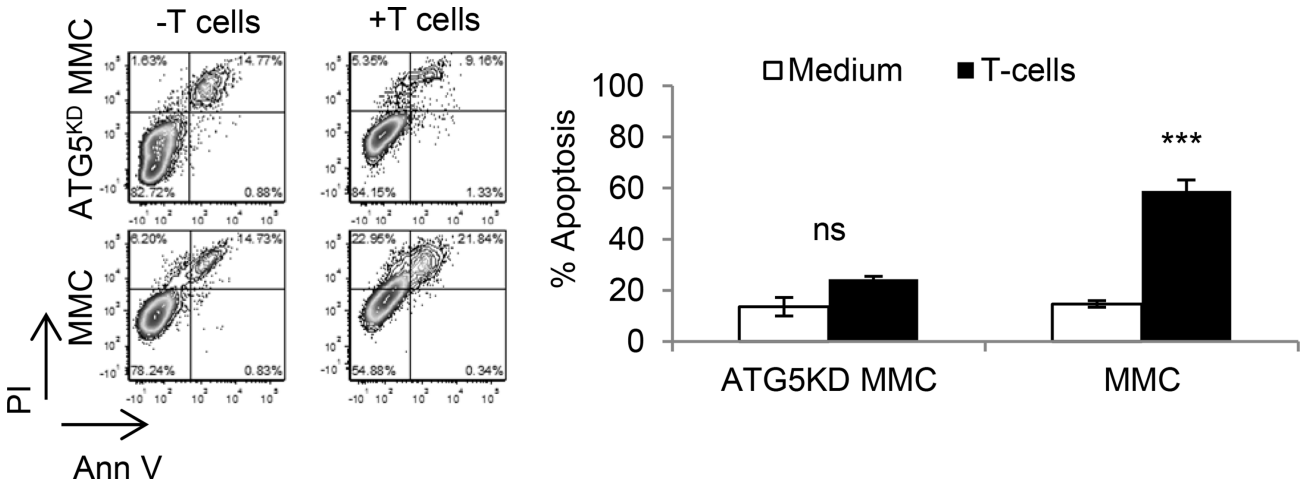

Figure 7: Autophagy knockdown tumor cells become resistant to T cell-induced apoptosis. Neu overexpressing autophagy-deficient MMC (ATG5^{KD} MMC) or autophagy-competent MMC (MMC) were co-cultured with MMC-sensitized T cells and then gated CD45-Neu+ tumor cells were analyzed by Annexin V/PI staining. Data represents triplicate experiments.

Abbreviations

ADR, Adriamycin, ATG5, Autophagy-related gene 5, BRCA1, Breast cancer gene 1, CQ, chloroquine, HSP70, Heat shock protein 70, IHC, Immunohistochemoistry LAMP2A, Lysosomal membrane associated protein 2 A, MMC, neu-overexpressing mouse mammary carcinoma, TNBC, Triple negative breast cancer.

Author contributions

M.H.M., D.A.G., K.K.P. contributed to the study's conception, design, experimental and analytical performance, and writing of the manuscript. H.F.A., L.T-M., T.T., R.C.K., S.J., S.E.B., T.M.S. contributed to the study's experimental and analytical performance and writing of the manuscript. D.B. contributed to statistical analysis and writing of the manuscript. H.D.B. and M.O.I. contributed to analytical performance and writing of the manuscript.

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CONFLICTS OF INTEREST

Authors have no potential conflicts of interest to disclose.

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REVIEW

IFN-γ orchestrates tumor elimination, tumor dormancy, tumor escape, and progression

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Abstract

Tumor immunoediting consisting of three phases of elimination, equilibrium or dormancy, and escape has been supported by preclinical and clinical data. A comprehensive understanding of the molecular mechanisms by which antitumor immune responses regulate these three phases are important for developing highly tailored immunotherapeutics that can control cancer. To this end, IFN-γ produced by Th1 cells, cytotoxic T cells, NK cells, and NKT cells is a pleiotropic cytokine that is involved in all three phases of tumor immunoediting, as well as during inflammation-mediated tumorigenesis processes. This essay presents a review of literature and suggests that overcoming tumor escape is feasible by driving tumor cells into a state of quiescent but not indolent dormancy in order for IFN-γ-producing tumor-specific T cells to prevent tumor relapse.

KEYWORDS

IFN-γ, immunotherapy, tumor dormancy, tumor immunoediting

1 | INTRODUCTION

Tumors display high levels of heterogeneity because of genetic instability, a characteristic of malignancy.[1] This results in a multitude of responses of tumor to the host immune responses or immunotherapeutics such that some tumor clones undergo apoptosis while other clones lay dormant and may later escape from the immune response and lead to distant metastasis. Antitumor immune responses utilize four major pathways to fight the tumor. Firstly, activated lymphocytes produce perforin to poke a hole in the extracellular membrane of target tumor cells as well as granzyme B to enter tumor cells and cleave caspases for the induction of apoptosis.[2] Secondly, they also express Fas-L to engage with Fas receptor on tumor cells and induce apoptosis.[2] Thirdly, they produce TNF-related apoptosis-inducing ligand (TRAIL) to engage with TRAIL receptors on tumor cells and in turn induce tumor cell apoptosis.[3] Finally, activated lymphocytes produce IFN-γ, which is a pleiotropic cytokine with a wide range of activities; IFN-γ simultaneously induces apoptosis, tumor dormancy, and immunoediting in tumor cells that could lead to tumor relapse and progression.[4–8] Paradoxically, chronic exposure of cells to IFN-γ facilitates the development of hepatocellular carcinoma (HCC),[9] colorectal carcinoma,[10] and papilloma.[11] Therefore, understanding the distinct mechanisms by which IFN-γ affects the tumor could lead to the development of highly tailored immunotherapeutics that could control the tumor without inducing tumor escape and relapse. IFN-γ is primarily produced by T cells, NK cells, and NKT cells. The receptor for IFN-γ is composed of two subunits, which include IFN-γ receptor alpha (IFN-γ Rα) and IFN-γ receptor beta (IFN-γ Rβ). Binding of IFN-γ to its cell surface receptor IFN-γ Rα induces dimerization of IFN-γ Rα, thereby forming a site for the assembly with IFN-γ Rβ. Upon heterodimerization of IFN-γ Rα/IFN-γ Rβ, their intracellular janus family kinases, JAK1 and JAK2, respectively, dimerize and become phosphorylated. This phosphorylation creates binding sites for the signal transducer and activator of transcription (STAT) proteins, primarily STAT1.[12] Phosphorylated STAT1 homodimers are then translocated into the nucleus to bind the IFN regulatory factor-1 (IRF-1) gene gamma-activated sequence sites on the promoters of downstream target genes.[13] This, in turn, activates diverse pathways in different tumor clones.

Abbreviations: BCL, B-cell lymphoma; DR5, death receptor 5; HCC, hepatocellular carcinoma; IFN-γ Rα, IFN-γ receptor alpha; IFN-γ Rβ, IFN-γ receptor beta; ROS, reactive oxygen species; RNI, reactive nitrogen intermediates; STAT, signal transducer and activator of transcription; TRAIL, TNF-related apoptosis-inducing ligand; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis

2 | IFN-γ INDUCES APOPTOSIS IN TUMOR CELLS

IFN-γ exerts its tumor killing function directly by the induction of apoptosis or by facilitating nonapoptotic cell death, as well as indirectly

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by rendering tumor cells susceptible to apoptosis-inducing function of the immune response or chemotherapies. For instance, IFN-γ induces IRF1, a tumor suppressor gene, which in turn reduces B-cell lymphoma 2 (BCL2) and increases Bak. These events facilitate the release of cytochrome c from mitochondria and activation of caspases, resulting in apoptosis.[14] Reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) at low concentrations are associated with cell proliferation. However, tumor cells that produce high amounts of RNI and ROS in response to IFN-γ tend to undergo apoptosis.[15] IFN-γ can also induce nonapoptotic cell death through the induction of autophagy in human HCC.[16] IFN-γ-induced activation of STAT1 enhances the expression of the death receptor FAS and its ligand FAS-L in hepatoma and colon adenocarcinoma cells,[17] and of TRAIL and its receptor death receptor 5 (DR5) in human tumor cell lines.[18–20] Accordingly, activated STAT1 sensitizes tumor cells to FAS or TRAIL-mediated apoptosis. Also, activation of STAT1 by IFN-γ inhibits the expression of the p53 inhibitor murine double minute 2, thereby enhancing p53-induced apoptosis by doxorubicin and cisplatin.[21]

3 | IFN-γ ARRESTS CANCER GROWTH BY DRIVING TUMOR CELLS INTO A STATE OF DORMANCY

Although the IFN-γ/STAT1 pathway induces tumor cell apoptosis, activation of STAT1 can also result in the inhibition of tumor cell growth and establishment of dormancy. In melanoma, activation of the IFN-γ/STAT1 pathway results in the down-regulation of cyclin E and cyclin A with consequent tumor cell dormancy.[22] Activated STAT1 can also interact with cyclins D1, D2, D3, and CDK4 and results in cell cycle arrest in fibrosarcoma cells.[23] Tumor inhibitory function of IFN-γ-induced STAT1 activation is also mediated by the up-regulation of the miRNA-29 family and a consequent down-regulation of CDK6 in melanoma cells.[24] IFN-γ-mediated tumor dormancy can also be induced independent from STAT1 signaling. Tumor clones that highly express indolamine 2,3-dioxygenase 1 and kynurenine-aryl hydrocarbon receptor respond to IFN-γ by upregulating the cell cycle inhibitor p27, consequently preventing STAT1 signaling and inducing tumor dormancy.[25] In fact, p21 and p27 facilitate hypophosphorylation of the tumor suppressor Rb, thereby suppressing the activity of E2F transcription factor and inhibiting the activation of genes involved in cell proliferation. In a T-antigenTag-induced multistage carcinogenesis in pancreatic islets, IFN-γ-producing $CD4^+$ T cells inhibit tumor cell proliferation and establish tumor dormancy without destroying malignant cells.[26] It was also reported that $CD8^+$ T cells maintain murine BCL1 in the state of dormancy by producing IFN-γ.[6] Radiation-induced tumor dormancy is also mediated by the production of IFN-γ in BALB/c neu transgenic mice such that neutralization of IFN-γ reversed radiation-induced tumor dormancy and resulted in tumor relapse.[27] It has been demonstrated that levels of the expression of IFN-γ Rα on mammary tumor cells determine whether IFN-γ eliminates the tumor or establishes tumor dormancy. While low expression of IFN-γ Rα in tumor cells results in tumor dormancy, high levels of IFN-γ Rα expression result in tumor elimination in the presence of IFN-γ-producing neu-specific $CD8^+$ T cell responses in FVB mice.[7] Given that STAT1 activation by IFN-γ results in the up-regulation of MHC class I molecules, which present antigens to T cells,[28] dormant tumor cells could become more susceptible to the immune surveillance.

4 | IFN-γ EDITS TUMOR CELLS AND FACILITATES TUMOR ESCAPE AND RELAPSE

In addition to apoptosis-inducing and tumor inhibitory functions, IFN-γ can also induce aberrant DNA methylation[29,30] or genetic alteration in tumor cells,[4] resulting in tumor progression and relapse. IFN-γ-induced tumor immunoediting is mediated through several mechanisms, which include the induction of tumor antigen loss,[30–34] up-regulation of PD-L1 in tumor cells,[35] recruitment of myeloid-derived suppressor cells, and tumor-associated macrophages to the tumor site.[36,37] IFN-γ-induced HER2/neu loss has been reported in FVBN202 transgenic mouse model of breast cancer,[30] and in patients with HER2/neu positive ductal carcinoma in situ or breast cancer.[32–34] Activation of STAT1 by IFN-γ results in the induction of the immune checkpoint protein PD-L1 in tumor cells.[38] In addition, chronic IFN-γ signaling in tumor cells increases resistance to immune checkpoint blockade through STAT1-related epigenetic and transcriptomic alterations, rendering melanoma resistant to radiation therapy and immune checkpoint inhibitors.[39] It was suggested that the genomic instability induced by IFN-γ during tumor progression is due to adaptation of the tumor to an immunologically hostile microenvironment.[4] This phenomenon has been predicted by the adaptation model of immunity.[40,41] Recent studies suggested that the state of tumor dormancy could determine whether IFN-γ may keep dormant cells in check or may edit dormant tumor cells and result in tumor relapse. Specifically, $Ki67^{low}$ indolent tumor cells are susceptible to immunoediting and escaping from immunotherapy, whereas $Ki67^-$ quiescent dormant cells fail to undergo immunoediting and thus remain dormant by IFN-γ-producing T cells.[8] Quiescent dormancy is due to lack of tumor cell proliferation and tumor cell arrest in G_0 phase, whereas indolent dormancy is due to a balance between tumor cell apoptosis and proliferation. As genetic and epigenetic changes take place during cell division, indolent cells remain susceptible to immunoediting and escape from immunotherapy. We have reported that IFN-γ induces the expression of PD-L1 on $Ki67^{low}$ indolent, but not on $Ki67^-$ quiescent dormant cells.[8] The detection of circulating tumor cells in breast cancer survivors even after 22 years of mastectomy without clinical evidence of disease[42] suggests the existence and maintenance of tumor dormancy in cancer survivors.

5 | CHRONIC EXPOSURE TO IFN-γ FACILITATES TUMORIGENESIS

Although IFN-γ is known for its antitumor function during antitumor immune responses, chronic exposure of normal cells to IFN-γ can also facilitate malignant transformation. In fact, IFN-γ appears to be

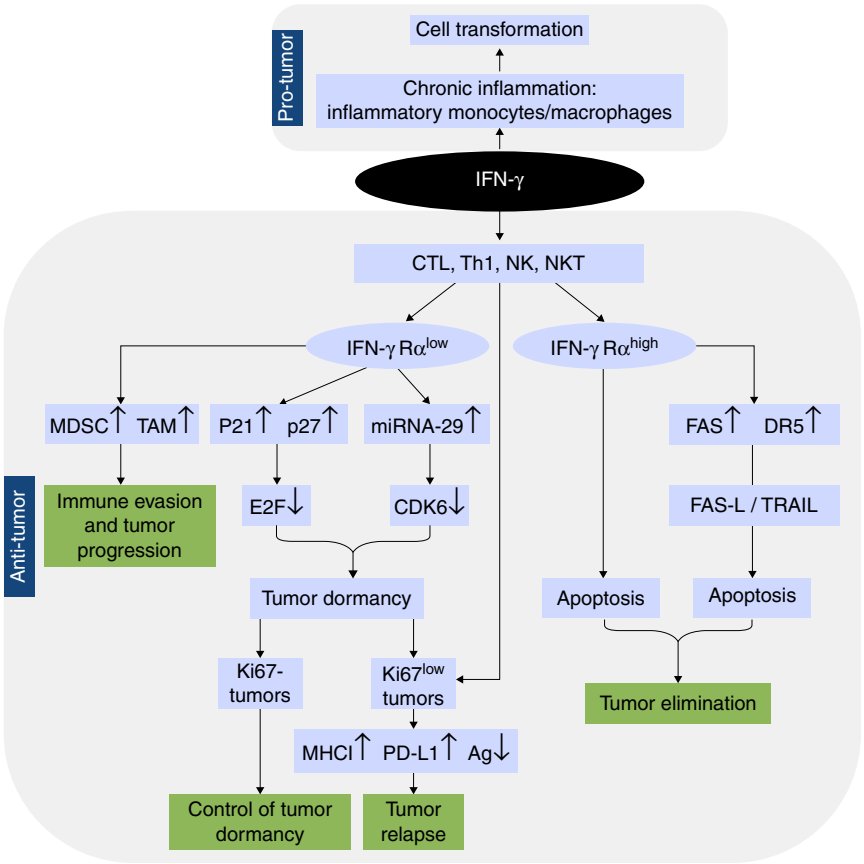

FIGURE 1 **Multifaceted role of IFN-γ in cancer.** Pro-tumor function of IFN-γ is mediated by chronic inflammation involving inflammatory monocytes and macrophages. Antitumor function of IFN-γ is mediated by cells of the adaptive immune system (CTL and Th1), NK cells, and NKT cells. The outcome of antitumor immune responses is determined by the status of the expression of IFN-γ Rα on target cells such that high levels of IFN-γ Rα render the tumor susceptible to apoptosis, while low levels of IFN-γ Rα could result in tumor immunoediting and relapse or maintenance of immunogenic tumor dormancy depending on the type of tumor dormancy being Ki67^{-} quiescent or Ki67^{low} indolent, respectively

protumorigenic early during cell transformation, whereas it manifests antitumor function against established tumors. For instance, IFN-γ has been reported to be involved in the initiation stage, but not in the promotion stage, of diethylnitrosamine-induced HCC due to its inflammatory function.[9] Suppressors of cytokine signaling-1 (SOCS1) deficient mice are not able to inhibit IFN-γ inflammatory signaling. These mice develop spontaneous colorectal carcinoma because of the IFN-γ-induced hyperactivation of STAT1, which results in the induction of carcinogenesis-related enzymes, cyclooxygenase-2, and inducible nitric oxide synthase.[10] In the 12-O-tetradecanoylphorbol-13 -acetate-induced papilloma model, IFN-γ is involved in the development of papilloma by enhancing a Th17-associated inflammatory response.[11] IFN-γ-producing macrophages were detected in 70% of human melanomas.[43] To this end, UV-induced cutaneous malignant melanoma can be abolished by systemic blockade of IFN-γ.[43] Nonalcoholic fatty liver disease (NAFLD) is also associated with the dominance of M1 macrophages that produce inflammatory cytokines, including IFN-γ.[44,45] In fact, IFN-γ-induced protein 10 is elevated in patients with progressive NAFLD.[46] Dietary saturated fatty acids are major contributors to NAFLD through the activation of NF-kB, which is a key transcription factor for M1 macrophage activation.[44,47] This, in turn, leads to inflammation-induced liver damage in nonalcoholic steatohepatitis (NASH) disease[45] and consequent progression to HCC.[48,49] Even in the absence of NF-kB signaling, IFN-γ-producing NKT cells actively participate in the pathogenesis of NASH disease.[50] Also, a higher frequency of IFN-γ-producing Th1 cells is evident as NAFLD progresses to NASH disease.[51]

6 | CONCLUDING REMARKS

IFN-γ is a pleiotropic cytokine that could manifest opposing effects on host cells ranging from cell transformation in the context of chronic inflammation, monocytes/macrophages, to antitumor effects, cytotoxic T cells (CTL), Th1, NK, NKT cells, during the immune response (Fig. 1). The antitumor function of IFN-γ also varies depending on heterogeneity of the tumor cells and tumor microenvironment. IFN-γ can induce tumor cell apoptosis, directly or indirectly by upregulating the expression of FAS and DR5 on tumor cells. This cytokine can also induce cell cycle arrest and establish tumor cell dormancy. A dual function of IFN-γ appears to be due to low expression of IFN-γ Rα in tumor cells. Depending on the type of tumor dormancy, IFN-γ-producing T cells can maintain tumor dormancy or result in tumor escape and relapse. In fact, IFN-γ could induce tumor immunoediting

in indolent dormant cells (Ki67^{low}), whereas it maintains quiescent dormant cells (Ki67^{-}) in the state of dormancy without clinical evidence of disease. To this end, CD8^{+} T cells, Th1 cells, NK cells, NKT cells could be involved in the process of tumor immunoediting. Therefore, we suggest that establishment of quiescent tumor dormancy in residual disease by novel therapeutics may render dormant cells highly responsive to immunotherapy without risk of recurrence.

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DISCLOSURES

Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the US Department of Defense. The authors declare no conflicts of interest.

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CHAPTER TWO

A Theoretical Basis for the Efficacy of Cancer Immunotherapy and Immunogenic Tumor Dormancy: The Adaptation Model of Immunity

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Contents

Abstract

In the past decades, a variety of strategies have been explored to cure cancer by means of immunotherapy, which is less toxic compared with chemotherapy or radiation therapy, and could establish memory for long-lasting protection against tumor recurrence. These endeavors have been successful in offering therapeutic antibodies, vaccines, or cellular immunotherapies, which resulted in prolonging survival of some cancer patients; however, complete cures have not been consistently achieved. The conception, design,

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and implementation of these promising immunotherapeutic strategies have been influenced by two schools of thought in immunology, which include the "self–nonself" (SNS) model and the "danger" model. Further progress in cancer immunotherapy to achieve consistent cancer cures requires an evolution in our understanding of how the immune system works. The purpose of this review is to revisit premises and limitations of the SNS and danger models based on the outcomes of cancer immunotherapies by suggesting that both models are two sides of the same coin describing how the immune response is induced against cancer. However, neither explains how the immune response succeeds or fails in eliminating the tumor. To this end, the adaptation model has been proposed to explain efficacy of the immune response for achieving cancer cure.

1. INTRODUCTION

The "self–nonself" (SNS) model (Janeway, 1992) and the "danger" model (Matzinger, 2002) of immunity appear to be on opposite sides of thought in describing how the immune system functions. However, growing evidence suggests that both concepts are complementary when it comes to describing how an immune response is induced against cancer rather than how it succeeds or fails to eliminate cancer. For an antitumor immune response, T cells must receive two signals. Signal I is provided by the presentation of tumor antigens to T cells in the context of major histocompatibility complex/T cell receptor (MHC/TcR) interaction, and signal II is provided by T helper cells (Bretscher & Cohn, 1970) or costimulatory molecules such as B7.1/B7.2-CD28 (Janeway, 1992). Although the original SNS model (Bretscher & Cohn, 1970) does not have an explanation for signal II, an evolved version of the SNS model suggests that signal II is also induced by foreign proteins recognized by pathogen-associated molecular patterns (PAMPs) on the immune cells (Janeway, 1992). However, PAMPs such as toll-like receptors (TLR) also recognize self-proteins or endogenous ligands (Yu, Wang, & Chen, 2010). In some classifications, cytokine signaling during T cell activation or differentiation is considered as signal III; however, the proposed classification is that signals I and II are involved in T cell activation and differentiation. Therefore, both costimulatory molecules and cytokine signaling are considered as signal II. The SNS model solely emphasizes foreignness and focuses on the affinity of T cell receptor for the antigen. This model proposes that foreign antigens usually have a stronger affinity for T cell activation because self-antigen-educated T cells develop tolerance in the thymus. The danger model emphasizes on danger signals in response to any damage being harmful to the host and which induces signal II. The

Table 1 Three Signals During Antitumor Immune Responses

Models	Signals	Molecules	Function	Outcomes
SNS	Signal I	MHC-TcR	Antigen recognition	T cell activation and differentiation
Danger	Signal II	B7.1/B7.2-CD28	T cell activation	
		Cytokines	T cell differentiation	
Adaptation	Signal III	AR-AL	T cell function	Success or failure of the immune response

danger signals include damage-associated molecular pattern; PAMP could also be considered as danger signal because of being expressed on pathogens that are harmful to humans. Without signal II, signal I induces tolerance toward antigens. In fact, the danger model is the evolution of the SNS model by theorizing the entity of signal II in the induction of the immune response regardless of the self or nonself entity of signal I, the antigen. The evolutionary relationship between the SNS model and the danger model is similar to that of tumor immunosurveillance and tumor immunoediting theories (Dunn, Bruce, Ikeda, Old, & Schreiber, 2002). Vaccines have been designed based on the inspiration from the SNS model by including highly immunogenic antigens as signal I, and from the danger model by including adjuvants, regardless of the self or nonself entity of adjuvants, to induce signal II. To understand how an antitumor immune response succeeds or fails in eliminating the tumor, a signal III has to be involved. Signal III is a communication signaling that determines whether tumor cells die, proliferate, or become dormant following vaccination or immunotherapy (Table 1). The adaptation model proposes that this communication signaling has to be orchestrated through adaptation receptors (ARs) and adaptation ligands (ALs) that are distinct from costimulation (Manjili, 2014).

2. OUTCOME OF CANCER IMMUNOTHERAPIES INSPIRED BY THE SNS AND DANGER MODELS

2.1 Targeting Tumor-Associated Antigens or Tumor-Specific Antigens?

The SNS model suggests that the sequence or nature of tumor antigens determines the strength of an antitumor immune response. Whereas

tumor-associated antigens (TAAs) are thought to be weakly immunogenic, tumor-specific antigens (TSAs) are considered to be highly immunogenic. This assumption is based on the SNS model without empirical evidence demonstrating that immunotherapeutic targeting of TSAs or foreign-like antigens is more effective than that of targeting TAAs or self-antigens. Although targeting mutant neoantigens is a viable immunotherapeutic strategy supported by the SNS model, it is not more effective than targeting TAAs. To target TAAs or TSAs in a vaccine formulation, the danger model provides a conceptual framework emphasizing the use of an adjuvant in order to induce signal II (Gallucci, Lolkema, & Matzinger, 1999). The danger model suggests that the use of an effective adjuvant and continuous vaccination is important for antitumor efficacy of a vaccine (Gallucci et al., 1999; Matzinger, 2002). Immunotherapeutics that target TAAs have been approved by the FDA based on prolonging survival of patients with carcinomas when used in a therapeutic setting. For instance, prostatic acid phosphatase is a TAA being used in sipuleucel-T (Provenge) vaccine against asymptomatic or minimally symptomatic metastatic hormone refractory prostate cancer, and extended survival of patients by a median of 4.1 months (Kantoff et al., 2010). HER2/neu is another TAA being used as a target for antibody therapy of metastatic breast cancer. Addition of anti-HER2/neu antibody therapy to chemotherapy prolonged a median survival of 5.1 months (Slamon et al., 2001). Two FDA-approved HPV and EBV vaccines containing TSAs—nonself viral antigens—have been tested in prophylactic settings for the prevention of cervical cancer and liver cancer, respectively. Importantly, the efficacy of these vaccines has more to do with their use in prophylactic settings, rather than the nature of the antigen being foreign entity or an adjuvant being a strong inducer of danger signals.

2.2 Allogeneic Cancer Vaccines

To enhance immunogenicity of cancer vaccines, an allogeneic system has been designed and tested in a randomized phase III clinical trial using Canvaxin (Kelland, 2006). The vaccine consists of allogeneic, living whole melanoma cells, as a source of foreign antigens, and BCG as adjuvant. According to the SNS model, the inclusion of foreign antigens (Bretscher & Cohn, 1970) and a foreign adjuvant (Janeway, 1992) was expected to induce robust antitumor immune responses. However, the trial was discontinued prematurely because survival benefit was unlikely to be achieved (Kelland, 2006). Another allogeneic vaccine called GVAX (Cell Genesys, Inc.) consisting of allogeneic pancreatic cancer cell lines

transfected with a human *GM-CSF* gene as adjuvant. GVAX was tested in combination with CTLA4 blockade in patients with previously treated advanced pancreatic ductal adenocarcinoma and resulted in prolonging a median overall survival of only 5.7 vs 3.6 months for CTLA4 schedule alone (Le et al., 2013). However, no complete cures were achieved. It has been suggested that the inclusion of foreign helper epitopes should be sufficient to induce an effective antitumor CD8+ T cell response (Anderson, 2014) without overloading the immune system with foreign antigens. Despite an improved efficacy, this strategy did not provide a complete protection against the tumor in animal models (Snook, Magee, Schulz, & Waldman, 2014; Steinaa, Rasmussen, Rygaard, Mouritsen, & Gautam, 2007).

2.3 Allogeneic Stem Cell Transplantation

Allogeneic stem cell transplantation (SCT) is a promising immunotherapeutic approach for the treatment of patients with hematological malignancies. This strategy is based on the SNS model, proposing that donor T cells will recognize recipient tumor cells as nonself entities and attack them. The treatment has to be performed in the setting of donor recipient being matched in major histocompatibility antigens, HLA-A, -B, -C, DR, and ideally DQ. However, mismatch in minor histocompatibility antigens could induce an alloreactive immune response, which is often associated with graft vs host disease (GVHD). Allogeneic SCT is usually given along with irradiation or chemotherapy to the recipient, which could potentially function as adjuvant depending on the immunogenic nature of some chemotherapies or radiation therapies at certain doses. The danger model proposes that signal II is readily induced in organs such as the skin and the gut because these organs are exposed to the external world, commensals and pathogens, which cause damage and induce danger (Matzinger, 2012); this could act as adjuvant or danger for allogeneic SCT and result in GVHD in these organs. However, these alarming conditions also exist in recipients of autologous SCT without causing severe GVHD. What has been less appreciated is the role of conditioning regimens in disrupting homeostatic cellular adaptation that contributes to the development of tissue-specific GVHD (Manjili & Toor, 2014). Treatment for GVHD is also inspired by the SNS model, assuming that alloreactive T cells are responsible for GVHD; therefore, immunosuppressive drugs are given as GVHD prophylaxis or as therapeutic regimens, rendering patients susceptible to infections and increasing the risk of tumor relapse. The SNS model has not been able to offer an effective therapeutic strategy for GVHD without compromising

the patient immune response. The danger model suggests that the high frequency of GVHD in the gut, the skin, and the liver is because these organs are most in contact with commensals and pathogens producing danger signals. That is why allogeneic SCT fails to induce severe GVHD in germ-free animals (Matzinger, 2012). However, similar danger signals are present in the gut, the skin, and the liver following autologous SCT without causing a severe GVHD.

2.4 Neoantigen Cancer Vaccines and Engineered TcR

The next-generation cancer vaccines that have been conceived based on the SNS and danger models contain mutant neoantigens and adjuvant. The idea is based on the understanding that cancer cells usually undergo somatic mutations resulting in the expression of mutant antigens that can be considered as nonself, because they are not expressed during central tolerance. Mutant tumor antigens have been detected in cancer patients (Assadipour et al., 2017; Verdegaal et al., 2016), though they do not induce tumor rejection. Vaccination with defined neoantigens in combination with poly I:C adjuvant has shown some efficacy in mice when combined with immune-checkpoint inhibitors (Gubin et al., 2014). Thus far, no human data are available to confirm antitumor efficacy of neoantigen vaccines. Another immunotherapeutic strategy inspired by the SNS model is enhancing affinity of T cells for target antigens by means of engineered TcR. This strategy can be combined with targeting neoantigens. A combination of two strategies by targeting KRAS-mutant neoantigens and using T cells engineered to express TcR specific for the appropriate KRAS mutations was elegantly tested in mice (Wang et al., 2016). Adoptive transfer of the KRAS-mutant-specific transduced T cells significantly reduced pancreatic tumor growth in nonobese diabetic scid gamma mice, but the treatment did not eliminate the tumors (Wang et al., 2016). Such outcomes have been attributed to the neoantigen immunoediting by T cells, and it was suggested that induction of broad neoantigen-specific T cell responses should be used to avoid tumor resistance (Verdegaal et al., 2016).

3. BEYOND THE SNS AND DANGER MODELS: TUMOR ESCAPE AND IMMUNE EVASION

Immunotherapeutic strategies that have been inspired by the SNS and danger models have shown limited efficacy against cancer. Such outcomes have been attributed to tumor escape and immune evasion, which cannot be

directly explained by either the SNS or danger models. In fact, these models can explain the induction of the immune response rather than predicting its outcome. To overcome a single tumor antigen loss, multiple tumor antigens have been used and epigenetic modulators have been tested to induce the expression of a panel of cancer testis antigens (CTAs) so as to overcome a single antigen loss during immunotherapy. A randomized phase II clinical trial of multiepitope vaccine in patients with stage IV melanoma increased median overall survival by a few months (Slingluff et al., 2013). A combination of decitabine to induce CTAs and a vaccine targeting NY-ESO1 in ovarian cancer resulted in a partial response (Odunsi et al., 2014). In patients with stage IV melanoma, a combination vaccine comprised of six HLA-DR-restricted peptides increased median overall survival of 4.1 years compared with control arm (Hu, Kim, Blackwell, & Slingluff, 2015). Immune evasion mechanisms have also been targeted by various strategies. For instance, tumor-induced immunosuppressive cells such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs) have been targeted in combination with immunotherapy, yet cancer cure has not been achieved. In patients with head and neck squamous cell carcinoma, tadalafil treatment significantly reduced both MDSCs and Tregs and increased tumor-specific immune responses, but no objective response was reported (Weed et al., 2015). In the 4T1 murine mammary tumor model, decitabine combined with adoptive immunotherapy (AIT) resulted in tumor inhibition and an increased rate of cure (Terracina et al., 2016), though its therapeutic efficacy against locally advanced tumor or established tumor metastasis has not been shown. In an animal model of HER2/neu-positive mammary carcinoma, depletion of MDSCs and induction of the expression of a panel of CTAs by decitabine, combined with AIT, resulted in prolonging survival of animals carrying metastatic breast cancer in the lung, although animals eventually succumbed to the tumor (Payne et al., 2016). In addition, targeting immune-checkpoint pathways of immune evasion by using anti-CTLA4 or anti-PD-1/PD-L1 antibody resulted in prolonging survival of cancer patients (Achkar & Tarhini, 2017), but again, a consistent and complete remission has yet to be achieved. Therefore, tackling several tumor escape pathways during immunotherapeutic regimens that were inspired by the SNS model or the danger model could improve the clinical outcome for cancer patients but could not consistently achieve a cancer cure. A continuous immunization, as suggested by Matzinger, may maintain anti-tumor immune responses, but it could not offer a cure for cancer because of tumor escape mechanisms.

4. DISCOVERY AND MODULATION OF TUMOR ADAPTATION RECEPTORS

The adaptation model of immunity was recently proposed to explain efficacy of the immune response during cancer, infectious diseases, allergy, and autoimmune diseases (Manjili, 2014). The model proposes a different theoretical perspective in tumor immunology and immunotherapy by suggesting that dysregulation of target tissues for the expression of ARs and ALs renders them susceptible or resistant to ongoing immune responses.

4.1 Central Tolerance and the Adaptation Model

Positive selection results in the maturation of CD4+CD8+ T cells into a single-positive CD4+ or CD8+ T cells via MHC class II or MHC class I restriction, respectively. During positive selection, MHC/self-peptide complex (signal I) selects and supports survival of T cells that are self-reactive. However, the affinity of these T cells for self-antigens is low due to the nature of cortical thymic epithelial cells (cTECs) expressing wobbly or private peptides that bind MHC molecules weakly. The cTECs express β5t-containing thymoproteasomes, which inefficiently cleave substrates adjacent to hydrophobic amino acids of self-peptides, and as a result create wobbly binding of β5t-derived peptides with a faster TcR off-rate (Murata et al., 2007; Ziegler, Muller, Bockmann, & Uchanska-Ziegler, 2009). On the other hand, medullary TEC or DCs express β5i-containing immunoproteasomes, which are efficient in cleaving substrates adjacent to hydrophobic amino acids and create high-affinity MHC/self-peptides for all positively selected T cells. Therefore, similar peptides can have different affinities during positive and negative selections. Medullary DCs also express costimulatory molecules such as CD40, B7-1, and B7-2 (signal II) (Klein, Hinterberger, Wirnsberger, & Kyewski, 2009). Around two-thirds of medullary DCs are $CD11c^{high}$ DCs, which contain CD8α+ thymic resident DCs, which are efficient in antigen cross-presentation, and CD8α− migratory DCs (Li, Park, Foss, & Goldschneider, 2009). Medullary DCs express a wide array of tissue-specific antigens regulated by the autoimmune regulator (*AIRE*) gene as well as *AIRE*-independent mechanisms (Derbinski, Schulte, Kyewski, & Klein, 2001; Takaba et al., 2015). Negative selection is a mystery that has not been fully understood by the SNS or the danger model. A classical explanation is that T cells die because of the high affinity for antigens, while those with a low-affinity survive. This explanation raises some questions: (i) theoretically, all positively selected T cells recognizing

β5t-derived peptides should have a higher affinity for the β5i-derived peptides in the medulla, so why do some T cells die and some survive during negative selection? (ii) Why do high-affinity T cells die upon activation in the thymus, but they survive in the periphery? T cells that were matured from double-positive into single-positive cells in the cortex should function like alloreactive T cells after activation upon recognizing high-affinity antigens. T cell activation also takes place in the medulla in the absence of any danger signals; (iii) why do surviving T cells not get activated upon receiving signal I and signal II in the medulla, but they do get activated in the periphery? The β5i-containing immunoproteasomes in the medulla increase the affinity of self-peptides for surviving T cells, while they also receive signal II, yet they do not get activated. The adaptation model (Manjili, 2014) proposes that negatively selected T cells in the medulla express ARs and thus survive upon antigen recognition, whereas defective T cells that lack ARs will be eliminated upon antigen recognition; if these T cells escape from negative selection, they would die in the periphery upon activation. Therefore, the purpose of negative selection is to eliminate faulty T cells and select functional T cells that are able to survive upon activation. Autoreactive T cells could not be the otherwise deleted T cells because thymic emigration decreases in AIRE$^{-/-}$ mice (Jin et al., 2017), suggesting that autoreactivity is not because of the escape of otherwise deleted T cells and their addition to the pool of surviving T cells. On the other hand, autoreactive T cells are perhaps those that do not die during negative selection in spite of recognizing MHC/self-antigens. In the periphery, upon engagement of ALs on DCs with ARs on T cells during activation, ARs transduce survival signals in T cells by inducing the expression of antiapoptotic proteins, such as cFLIP and Bcl-xL (Paulsen & Janssen, 2011). Lack of expression of ALs by APCs could also result in activation-induced cell death (AICD) in T cells. For instance, hepatic DCs induce apoptosis in T cells during activation, whereas splenic DCs support survival of activated T cells (Bertolino, Trescol-Biemont, & Rabourdin-Combe, 1998).

4.2 ARs and ALs: (i) The Endothelin Axis

Cancer patients often harbor preexisting antitumor immune responses that fail to protect the patients from cancer (Lu et al., 2012). Also, immunotherapy as a single agent often fails to eliminate the tumor. Similar observations were made in different diseases. For instance, healthy individuals and patients with multiple sclerosis (MS) harbor T cells that recognize myelin basic proteins (MBPs), but a pathogenic manifestation of the immune

response is evident only in MS patient (Martin, Whitaker, Rhame, Goodin, & McFarland, 1994). Similarly, preexisting anti-DNA autoantibodies were detected in healthy individuals and patients with lupus erythematosus with a pathogenic manifestation only in the latter (Martin et al., 1994). Th1 and Th17 inflammatory cells in the gut can protect the host from *Helicobacter pylori* infection without any toxicity to the tissue (Ding et al., 2013), but they become destructive during Crohn's disease. These paradoxical observations suggest that the immune response alone is not the primary factor in the pathogenesis of autoimmune diseases or inefficacy in cancer patients; rather, alterations in the expression of AR on the target cells could render them susceptible or resistant to the immune response. In fact, an altered gut microbiome profile is associated with Crohn's disease such that nutritional therapy can modulate pediatric Crohn's disease (de la Cruz-Merino et al., 2011), again suggesting that gut microbiome is an important factor in regulating the expression of ARs in the tissue. Tumor cells that arise from normal cells, perhaps, retain their ARs to survive immune surveillance. One candidate for the AR/AL is the endothelin axis, which includes the endothelin (ET) containing ET-1, ET-2, and ET-3 isoforms as ALs, and the ET receptor A (ET_A) as an AR. Activation of the ET_A AR by the ET-1 AL can lead to the induction of survival pathways, whereas activation of the ET_B, which antagonizes the ET_A, results in apoptosis (Nelson, Udan, Guruli, & Pflug, 2005). ETs are expressed by a variety of cell types including endothelial cells, macrophages, astrocytes, and neurons (Simonson, 1993). The ET_A receptor has a greater affinity for ET-1, and the ET_B receptor binds to all three ET isoforms equally (Arai, Hori, Aramori, Ohkubo, & Nakanishi, 1990). ET-1 is upregulated by astrocytes in a number of brain pathologies, including MS (D'haeseleer et al., 2013) and Alzheimer's disease (Palmer, Barker, Kehoe, & Love, 2012), as well as in rheumatoid arthritis (Haq, El-Ramahi, Al-Dalaan, & Al-Sedairy, 1999) and cancer (Wulfing et al., 2004). ET_B is upregulated in active MS lesions (Yuen et al., 2013), and ET-1 acts almost exclusively through ET_B, and not ET_A, on astrocytes to inhibit remyelination (Hammond et al., 2015). Therefore, it is reasonable to predict that alterations in the balance between the ET_A AR and its antagonist receptor, the ET_B, render the nervous system susceptible to anti-MBP immune responses. In humans, ET_A acts as an AR by inducing the expression of antiapoptotic genes in prostate cancer (Nelson et al., 2005). Its ligand, ET-1, acts as an AL and is produced by the prostate epithelia (Nelson et al., 2005). The ET-1/ET_A pathway is involved in the inhibition of apoptosis in melanocytes during UV irradiation (Swope & Abdel-Malek, 2016). In fact, a higher

responsiveness of melanoma patients to immunotherapy compared with patients with prostate cancer or ovarian cancer could be because the ET_A AR is upregulated in prostate and ovarian cancers but not in melanoma (Nelson, Bagnato, Battistini, & Nisen, 2003). The ET-1 AL is produced by the prostate epithelia (Nelson et al., 2005); in prostate cancer, not only a key component of ET-1 clearance, the ET_B receptor, is diminished (Nelson et al., 1996), but also the ET_A AR is upregulated (Nelson et al., 2003). These could make tumor-infiltrating T cells ineffective in patients with prostate cancer. Human DCs also produce ET-1 upon activation (Spirig et al., 2009), which in turn support survival of T cells during activation as well as tumor cells that express ET_A. ET-1 is also involved in the survival of activated T cells during autoimmune systemic sclerosis (Elisa et al., 2015). In rats, the ET-1/ET_A pathway is critical for thymocyte proliferation (Malendowicz, Brelinska, De Caro, Trejer, & Nussdorfer, 1998).

4.3 ARs and ALs: (ii) The PD-L1/PD-1 Checkpoint Pathway

The programmed cell death-1 (PD-1) receptor is expressed on activated T cells. Its ligands, PD-L1 and PD-L2, are commonly expressed on dendritic cells or macrophages. PD-L1 is a bidirectional membrane protein acting as a ligand to induce anergy in PD-1-positive T cells and acting as an AR to induce antiapoptotic genes in PD-L1-positive target cells (Azuma et al., 2008). Constitutive expression of PD-L1 in the immune-privileged sites such as cornea and retina protects them from GVHD following corneal allograft, despite infiltration of CD4+ T cells; however, blockade of PD-L1 accelerates allograft rejection (Hori et al., 2006). In a murine model, PD-L1 deficiency in pancreatic beta-cells triggers their destruction by CD8+ T cells (Rajasalu et al., 2010). An altered expression of PD-L1 correlates with not only autoimmune diseases but also cancer progression. For instance, PD-L1 loss was reported in children with systemic lupus erythematosus, and expression of PD-L1 is restored only during disease remission (Mozaffarian, Wiedeman, & Stevens, 2008). The expression of PD-L1 on activated T cells supports their survival such that PD-L1-deficient T cells express lower Bcl-xL, which is an antiapoptosis gene, than wild-type cells and are more sensitive to apoptosis in vivo (Pulko et al., 2011). Tumor cells exploit this pathway by the expression of PD-L1 in order to survive immune surveillance. Antitumor T cells can upregulate PD-L1 on tumor cells through the production of IFN-γ. For instance, upregulation of PD-L1 is only detected in tumor cells that are adjacent to IFN-γ-producing TILs in melanoma patients (Taube et al., 2012). Of note, tumor cells also

utilize IFN-γ-independent pathways for the expression of PD-L1 which involve *PTEN* (Parsa et al., 2007) or EGFR (Akbay et al., 2013). In phase I clinical trial, anti-PD-1 therapy showed cumulative response rates of 18%, 28%, and 27% among patients with non-small-cell lung cancer, melanoma, and renal cell carcinoma, respectively (Topalian et al., 2012). More recently, an objective response rate of 30%–40% in melanoma patients has been reported (Robert et al., 2015; Topalian et al., 2014). A high variety of response rates among different types of cancers to PD-1 immune-checkpoint inhibition therapy suggest the involvement of additional ARs that support tumor cell survival when the PD-L1 pathway is blocked. According to the adaptation model of immunity, antitumor efficacy of anti-PD-1/PD-L1 immunotherapy as a single agent is mainly due to the blockade of antiapoptotic gene expression downstream of PD-L1 on tumor cells. Therefore, the model predicts a higher efficacy of anti-PD-L1 therapy than anti-PD-1 therapy. In fact, some types of anti-PD-L1 antibodies can inhibit the interaction of not only PD-L1 and PD-1 but also PD-L1 and CD80 (Keir, Butte, Freeman, & Sharpe, 2008). On the other hand, blockade of PD-1 can rescue effector T cells from suppression, but the engagement of PD-L1 on tumor cells with CD80 on APCs can still induce survival signaling in tumor cells, facilitating resistance of tumor cells to antitumor effector T cells (Fig. 1). However, studies performed

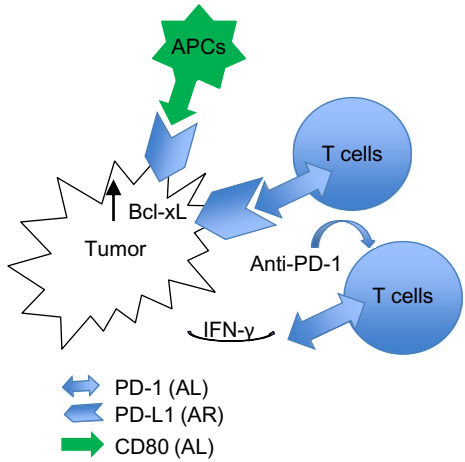

Fig. 1 PD-L1 acts as an AR on tumor cells. Anti-PD-1 could block PD-1/PD-L1 interaction and result in rescuing T cells from suppression. However, the engagement of CD80 on APCs with PD-L1 on tumor cells can upregulate the antiapoptotic gene Bcl-xL in tumor cells and support their survival in the presence of IFN-γ-producing antitumor T cells.

in the context of SNS model pay more attention to rescuing T cells from the suppression rather than blocking survival signaling in tumor cells following anti-PD-1/PD-L1 immunotherapy.

5. IMMUNOGENIC DORMANCY OF OCCULT TUMOR CELLS THROUGH ADAPTATION

An effective antitumor immune response, which is capable of inducing tumor regression, cannot guarantee elimination of tumor dormancy. In fact, immune responses induce the expression of an AR, PD-L1, on tumor cells through secretion of IFN-γ (Payne et al., 2016). IFN-γ is a dual-edged cytokine capable of inducing apoptosis and also facilitating tumor dormancy (Liu et al., 2017). Immunogenic tumor dormancy has been documented during unintentional transplantation of cancer into immunocompromised recipients from organ donors who were in clinical remission (Kauffman, McBride, & Delmonico, 2000) or with no clinical history of cancer (Myron Kauffman et al., 2002). Immunogenic tumor dormancy is defined by the expression of mutant antigens, increased MHC-I, cell membrane translocation of calreticulin, release of ATP, release of nonhistone chromatin-binding protein high-mobility group box 1, and secretion of immunostimulatory cytokines such as type I interferons (Michaud et al., 2011, 2014; Sistigu et al., 2014). A mechanism of immunogenic tumor dormancy was demonstrated in an animal model of methylcholanthrene-induced sarcoma (Koebel et al., 2007). Immunogenic dormancy is also evident in *Mycobacterium tuberculosis* infection keeping the infectious agent in dormant or latent state, thus protecting the host from active disease. Long latency before the appearance of AIDS is also evident in the presence of the immune response (Goonetilleke et al., 2009). HIV-infected CD4+ T cells express PD-L1 (Trabattoni et al., 2003), which could be kept dormant by HIV-specific PD-1^{low} CD8+ T cells during the latency period. Whereas PD-1^{high} effector T cells can be suppressed through PD-L1 engagement allowing tumor growth, the PD-1^{low} effector T cells could remain active and push PD-L1-positive tumor cells into the state of immunogenic dormancy by producing IFN-γ; dormant tumor cells will remain in check by the immune response until they escape from dormancy. Thus far, two types of tumor dormancy have been reported, which include $Ki67^{-}$ quiescent dormancy and $Ki67^{low}$ indolent dormancy (Payne et al., 2016). Similar to actively proliferating tumor cells, the indolent, but not quiescent, dormant cells can evolve through immunoediting and escape from the

immune response. Recently, an elegant study by Dr. Restifo's group demonstrated that tumor necrosis releases an intracellular ion, potassium, into the extracellular fluid at the tumor site and results in the suppression of effector T cells. They showed that ionic reprogramming of tumor-specific T cells can improve their effector functions and prolong survival of melanoma-bearing mice (Eil et al., 2016). In clinical settings, targeting neoantigens by immunotherapy resulted in the stabilization of metastatic cholangiocarcinoma for 13 months, and then, disease progression was observed in the lungs (Tran et al., 2014). In a separate study, adoptive T cell therapy using a polyclonal CD8+ TIL recognizing mutant KRAS G12D in a patient with metastatic colorectal cancer resulted in the regression of lung metastatic lesions. However, one lesion escaped through loss of heterozygosity of the copy of chromosome 6 that encoded HLA-C*08:02 (Tran et al., 2015; Tran et al., 2016). Complete regression of neu-overexpressing mammary carcinoma and subsequent relapse of antigen-negative tumor variant have been reported in a semiallogeneic model in which T cells and tumor cells were matched in major but not minor histocompatibility antigens (Kmieciak, Knutson, Dumur, & Manjili, 2007; Santisteban et al., 2009). Effectiveness of immunotherapy in some cancer patients but not others perhaps results from differences in the expression of ARs and/or ALs regulated by different oncogenes or epigenetic alterations. The adaptation model can also explain sterile chronic inflammation where the immune response to self-antigens is induced in the presence of signals I and II, but rather than destroying target organs, it initially inhibits cell growth because of the presence of ARs on target tissues, and eventually facilitates escape of natural malignant cells from dormancy (Manjili, 2017). Advances in our understanding of the AR/AL pathways are expected to lead to a breakthrough in immunotherapeutic treatment of cancer.

In summary, the adaptation model of immunity proposes that the status of ARs/ALs on tumor cells and T cells, respectively, determines the outcome of antitumor immune responses. There are four scenarios predicted by the adaptation model of immunity (Table 2). Tumor cells expressing ARs (ARs+) will receive survival signals from T cells by engaging with ALs on T cells (ALs+) and as a result become dormant as long as antitumor effector T cells are present. Other tumor-infiltrating cells such as myeloid cells could also express PD-1. Also, tumor cells expressing ALs will induce survival signals in effector T cells that express ARs (Scenario 1). Alterations in the expression of ARs/ALs on tumor cells could change the outcome, leading to the elimination of tumor cells that lack ARs (ARs−) by effector T cells (Scenarios 2). Tumor cells that do not express ALs fail to induce

Table 2 Outcomes of Antitumor Immune Responses

Scenarios	Tumor	Effector T Cells	Outcomes
1	AR +	AL +	Tumor dormancy
	AL +	AR +	T cell survival
2	AR–	AL +/–	Tumor elimination
	AL +	AR +	T cell survival
3	AR–	AL +/–	Tumor elimination
	AL–	AR +/–	T cells undergo AICD
4	AR +	AL +	Tumor escape and relapse
	AL–	AR–/+	T cells undergo AICD

survival signals in antitumor T cells, and these T cells will undergo AICD if they do not receive survival signals from stromal cells (Scenario 3). Finally, dormant tumor cells could escape from the immune response by down-regulating the expression of ALs on antitumor T cells (ALs −) and relapse (Scenario 4). Advances in our understanding and identification of ARs and ALs could lead to targeted therapies for epigenetic silencing of ARs on tumor cells, thereby rendering them vulnerable to immunotherapy.

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Review

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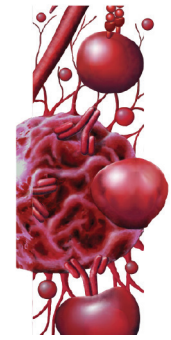

Immunotherapy of cancer: targeting cancer during active disease or during dormancy?

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Immunotherapeutic targeting of advanced stage cancers has prolonged the survival of cancer patients, yet its curative efficacy is limited due to tumor immunoediting and escape. On the other hand, human vaccines have been able to eradicate smallpox and control several other infectious diseases. The success has resulted from the administration of vaccines in prophylactic settings, or during latency periods in order to protect an individual during future exposure to the disease rather than curing an established disease. Therefore, administration of immunotherapy at the right time is the key to success. However, instead of focusing on the prevention of cancer, current cancer immunotherapies are often being used in a therapeutic setting with the goal of eliminating tumor cells. The present review of evidence related to cancer immunotherapeutics suggests that immunotherapeutic targeting of tumor dormancy could be more promising than targeting of advanced stage disease to achieve a cure for cancer.

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Current strategies for improving the efficacy of cancer immunotherapy rely on: strengthening antitumor immune responses by modulating tumor cells to become highly immunogenic and/or reprogramming of T cells to increase their affinity and avidity for tumor antigens as well as their sustainability in the host in order to improve humoral and cell-mediated immune responses, overcoming immune suppressive pathways by targeting Tregs and myeloid-derived suppressor cells (MDSCs), and overcoming immune tolerance by the blockade of the immune checkpoint pathways. These strategies, alone or in combination, have shown promising results against established cancers in some, but not all, patients. Very recently, attempts were made to identify and target mutated neoantigens in order to develop personalized immunotherapy, and thus, make it effective for all cancer patients. Here, we provide a review of literature highlighting the challenges that these strategies are facing. This review demonstrates that immunotherapeutic strategies that improve efficacy of tumor-reactive T cells, modulate the tumor-immune cells crosstalk or target some tumor escape mechanisms can at best prolong survival of cancer patients and cannot guarantee cancer cure. Based on recent observations that quiescent dormant tumor cells are not able to undergo immunoediting [1], we suggest that the immunotherapeutic targeting of tumor dormancy with the goal of maintaining tumor dormancy and preventing cancer recurrence, would be an effective strategy in containment or cure of cancer.

Immunotherapeutic targeting of advanced cancer prolongs patient survival but comes short from achieving cancer cure

Studies which demonstrated that the cellular arm of the immune system might be responsible for tissue rejection [2] led investigators to postulate the use of immune cells for the treatment of tumors. The first clinical study in humans demonstrating immune responses generated by tumor infiltrating lymphocytes (TIL) against autologous

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tumors was published in 1987 [3]. TILs have been detected in the stroma of various cancers, and have been harnessed for adoptive cellular therapy (ACT). Conditioning the host environment by a nonmyeloablative (NMA) lymphodepleting regimen (cyclophosphamide and fludarabine) prior to ACT showed increased tumor responses [4]. In order to ascertain the degree of the effect of lymphodepletion, a pivotal follow-up study by Goff *et al.* randomized 51 patients to receive an NMA lymphodepleting regimen (cyclophosphamide and fludarabine) and 50 patients to receive an NMA regimen along with 1200 cGy of total body irradiation (TBI) prior to receiving autologous TIL. The results revealed that even though the objective response (OR) rate was higher in the NMA + 1200 cGy arm (62%) compared with the NMA arm alone (45%), both regimens had almost identical complete response (CR) rates of 24% [5]. In a prior study by the same group, the degree of lymphodepletion (chemotherapy alone) was noted to show increasing CRs of 12, 20 and 40%, respectively [5]. All these patients were previously heavily treated with other regimens for advanced melanoma (high-dose IL-2, anti-CTLA-4, anti-PD-1, a combination of anti-CTLA-4, anti-PD-1, IFN-α, dacarbazine, temizolamide, small molecule inhibitors and biochemotherapy). None of the prior treatment strategies were reflective of any correlation to observed tumor responses in either arm on subgroup analysis [5]. The duration of ongoing CR was 53.4 months as of the date of publication and one patient with CR recurred at 19 months. Even though these studies showed successful ACT and improvement in degree of tumor response with increasing lymphodepletion, this was not sustained in the partial responders and did not reach statistical significance. In a Phase II clinical trial, Chandran *et al.* [6] evaluated the effect of autologous $CD8^+$ T cells clones against MART-1 or gp100 in patients with refractory metastatic melanoma. Fifteen patients treated with these highly avid clones resulted in immune-mediated targeting of skin melanocytes in 11 patients (73%) with minor transient tumor response by Response Evaluation Criteria In Solid Tumors criteria [7] but no OR in spite of successful clonal repopulation and engraftment in the host [6]. Multiple studies in both murine and human models have shown that younger the T cells are the higher the likelihood of antitumor efficacy is [8–10]. In a pilot study, 33 patients were treated with lymphodepleting chemotherapy alone followed by $CD8^+$ enriched young TIL and 23 patients received lymphodepleting chemotherapy and 6Gy TBI followed by $CD8^+$ enriched young TIL (longer telomeres, higher expression of CD27/28). Nineteen of the 33 patients (58%) showed OR by Response Evaluation Criteria In Solid Tumors criteria, including three CR (9%) and 16 partial responders (48%). In the arm receiving additional TBI, 11 out of 23 patients showed an OR (48%) including two patients with CR (9%), with CR similar to previous cohort receiving lymphodepleting chemotherapy alone. It was noticed that in comparison to prior standard TIL therapies, this study cohort that received younger TIL following transfer showed higher level of absolute lymphocyte count on reconstitution suggesting as increased capacity for *in-vivo* expansion for younger TIL compared with selected TIL previously described [11,12]. Analysis among subsets of memory T cells in different studies has indicated that central memory T (T_{CM}) cells are more efficient in antiumor activity in comparison to effector memory T (T_{EM}) cells [13–15]. Among $CD8^+$ memory T cells, T memory stem cells (T_{SCM}) have been identified with even superior antitumor properties compared with other subsets of memory T cells [16].

Modulating the crosstalk between T cells & tumor cells improves the efficacy of cancer immunotherapy but could also induce tumor immunoediting & escape

According to the self-nonself theory of immunity, tumors are often incapable of inducing an effective antitumor immune response because of the expression of self-antigens. Therefore, enhancing immunogenicity of tumor cells and increasing the affinity of T cells for the antigen are expected to modulate the crosstalk between tumor cells and T cells, thereby improving the efficacy of cancer immunotherapy. To test this hypothesis, Yu and colleagues used double-transgenic mice engineered to express both human T-cell receptor chains against gp100 antigenic peptides in T cells and human MHC-I domains in somatic cells. They demonstrated that a mutant gp100 peptide serving as a foreign-like antigen, induced a stronger immune response leading to tumor inhibition compared with a native peptide. However, a complete regression of the tumor was not achieved [17]. In clinical settings, targeting mutant peptides or neopeptides by means of adoptive T-cell therapy resulted in the stabilization of metastatic cholangiocarcinoma for 13 months, and then, disease progression was observed in the lungs [18]. In a separate study, adoptive T-cell therapy using a polyclonal $CD8^+$ TIL recognizing mutant KRAS G12D in a patient with metastatic colorectal cancer resulted in the objective regression of all seven lung metastatic lesions. However, one lesion escaped through loss of heterozygosity of the copy of chromosome 6 that encoded HLA-C*08:02 [19,20]. We also observed complete regression of neu overexpressing mammary carcinoma in wild-type FVB mice in a T-cell-dependent manner recognizing the rat neu protein as a foreign protein. However, a fraction of animals experienced tumor recurrence due to neu antigen loss [21,22]. Similar observations were made in a preclinical

model of breast cancer, and in patients with multiple myeloma when tumor cells were epigenetically modulated by the administration of hypomethylating drugs in order to express cancer testis antigens (CTA) [1,23]. ACT by means of genetically engineered T cell receptor recognizing a cancer testes antigen NY-ESO in patients with either melanoma or synovial cell sarcoma, showed an OR of nine out of 17 patients (52%). Five patients with metastatic melanoma showed OR including two CR (on going at 22, 20 months as of the date of publication), and four out of six patients (66%) with synovial sarcoma showed OR though partial with one lasting 18 months [24]. In the FVBN202 transgenic mouse model of breast carcinoma, adoptive T-cell therapy combined with decitabine prolonged survival of animals bearing lung metastasis, but animals eventually succumbed to metastatic tumors due to tumor immunoediting characterized by the downregulation and loss of tumor antigens as well as upregulation of PD-L1 [1]. In patients with multiple myeloma, use of azacytidine resulted in the expression of CTA in tumor cells and the induction of CTA-reactive immune responses, leading to tumor regression following autologous stem cell transplantation [23]. However, some patients experienced tumor relapse associated with loss of CTA in their tumor cells (Payne *et al.*, Unpublished Data). To this end, modulation of the antigenic profile of tumors improved the efficacy of immunotherapy but was not able to overcome tumor immunoediting and escape from immunotherapy. Similar results were obtained using engineered T cells. Chimeric antigen receptor (CAR) T-cell therapy targeting CD19 resulted in complete remissions in some patients with relapsed/refractory acute lymphocytic leukemia (ALL) [25,26]. This therapy also induced CD19 loss, which is a limiting factor for its therapeutic efficacy. In two patients with refractory $CD19^+$ ALL, CAR T-cell therapy led to a complete remission, which was sustained in one patient during a follow-up period of 9 months, and led to relapse of CD19 negative ALL after 1 month [27]. To overcome tumor escape, T cells were collected from patients whose tumors lost CD19, and modified to target CD22. Again, tumor relapse was evident as a result of CD22 downregulaion or total loss [28]. It appears that IFN-γ produced by T cells is responsible for inducing tumor immunoediting [29,30]. Such tumor immunoediting has not been observed in adults with chronic lymphocytic leukemia [31]. This could be due to the state of dormancy in residual tumor cells since CAR therapy was used after the establishment of stable disease by using bendamustine with rituximab chemotherapies in adults with chronic lymphocytic leukemia. The study did not examine whether stable disease was in the state of cellular dormancy. We have recently reported that quiescent, but not indolent, dormant tumor cells are resistant to immunoediting [1].

Targeting tumor escape mechanisms: MDSCs, Tregs and immune checkpoints

Active solid tumors often induce and recruit MDSCs and/or Tregs, thereby inhibiting the efficacy of antitumor immune responses. A meta-analysis of eight studies that included 442 patients with solid tumors showed that MDSCs were associated with poor overall survival [32]. In patients with advanced non-small-cell lung cancer (NSCLC), multivariate analysis revealed an independent association of MDSCs with decreased progression free-survival and overall survival [33]. A meta-analysis of 18 published studies that included 8562 patients with breast cancer showed an association between Tregs infiltration and poorer prognosis [34]. Similar results were reported from patients with prostate cancer [35]. Analysis of the peripheral blood of 41 patients with prostate cancer and 36 healthy controls showed an increased frequency of MDSCs and Tregs in patients with prostate cancer associated with poor prognosis [35]. In addition, FOXP3 immunohistochemistry analysis of tissue microarray from 2002 prostate cancer patients revealed a higher number of intratumoral $FOXP3^+$ Tregs associated with a more advanced tumor stage [36]. Although, control of MDSCs and Tregs restored antitumor immune responses, it did not produce a curative outcome in cancer patients. In order to target MDSCs and Tregs as well as to increase the efficacy of adoptively transferred TIL, conditioning regimens were used prior to ACT. Murine models and follow-up human studies demonstrated that use of lymphodepletion prior to cell transfer increased the effectiveness of ACT significantly [11]. Lymphodepleting regimens could increase the persistence of transferred T cells [4], deplete endogenous lymphocytes and myeloid cells containing Tregs [37], increase levels of homeostatic cytokines (IL-7 and IL15) as well as remove their sink as seen in both murine and human studies [38]; and finally, they enhance the efficacy of ACT by activating antigen presenting cells via stimulation of toll-like receptors resulting from translocation of commensal microflora across mucosal barriers [39]. Addition of the immune checkpoint inhibitors, however, produced OR in some patients. Use of the phosphodiesterase-5 inhibitor tadalafil has also been associated with depletion in MDSCs [40]. In patients with head and neck squamous cell carcinoma, tadalafil treatment significantly reduced both MDSCs and Tregs, and increased tumor-specific immune responses, though no OR was reported [41]. Therapeutic targeting of immune checkpoints pathways has found to be effective in producing objective clinical responses. The use of neoadjuvant anti-CTLA4, ipilimumab, in patients with regionally advanced melanoma resulted in elevated T-cell responses

against NY-ESO-1, MART-1 and gp100 antigens associated with decreased tumor infiltrating Tregs and MDSCs, and improved progression-free survival for 1 year [42]. Anti-PD1 and anti-PD-L1 immunotherapies have been highly effective for patients with NSCLC, bladder cancer, head and neck cancer and Merckel cell carcinoma. These immune checkpoint inhibitors are the only US FDA approved drugs for bladder cancer in the past 20 years [43].

Immunotherapeutic targeting of tumor dormancy

Four decades ago, Gray & Watkins published a comprehensive review article related to cancer immunotherapy in which they attributed spontaneous regression of neuroblastoma, hypernephroma, choriocarcinoma and melanoma as well as the existence of tumor dormancy to the host-immune system [44]. The notion that tumor dormancy is controlled by the immune system was further supported in six cases of NSCLC exhibiting strong delayed hypersensitivity reactions to the soluble tumor antigens following immunotherapy. These patients ended up with tumor recurrence after an immunosuppressive event or drug treatment [45]. It was also reported that immunization by means of irradiated tumor cells can establish and maintain tumor dormancy in a murine model of B-cell leukemia/lymphoma [46]. Antibody response [47,48] and IFN-γ producing $CD8^+$ T cells [49] were found to be responsible for maintaining the murine B-cell lymphoma in a dormant state. In breast cancer patients, presence of tumor dormancy in the bone marrow was associated with an increase in $CD8^+$ T memory cells that were reactive against HLA-A2/HER-2/neu(p369–377) tumor antigen [50]. Two FDA-approved monoclonal antibodies, trastuzumab and pertuzumab, targeting HER2/neu can also prolong tumor dormancy as evidenced by delaying tumor recurrence and increasing progression free survival and overall survival of patients with invasive breast cancer [51]. Similar observations were made in patients with prostate cancer. Approximately, 70% of patients with prostate cancer have disseminated dormant cells in the bone marrow [52]. Recently, TGF-β was reported to be involved in maintaining prostate cancer dormancy in the bone marrow [53]. It remains to be determined whether TGF-β producing Tregs may contribute to prostate cancer dormancy.

Recent reviews of literature on tumor dormancy and immune response suggest tumor dormancy as the best target for immunotherapeutic prevention of tumor recurrence and advanced disease prophylaxis [54–56]. This is because dormant tumor cells that have been established by chemotherapy or radiation therapy remain susceptible to immunotherapy [1]. A prospective, randomized, multicenter Phase II clinical trial evaluated the efficacy of GP2+GM-CSF vaccine in HLA-A2+, $HER2^+$, node-positive and high-risk node-negative breast cancer patients. The vaccine was administered when patients were found to be disease-free, though might have harbored dormant tumor cells, in other words micrometastatic disease. This vaccination during tumor dormancy resulted in 5-year disease-free survival in 100% of $HER2^+$ patients compared with 89% disease-free in control patients [57]. However, the caveat is that dormant tumor cells could undergo immunoediting and eventually escape and relapse. In particular, indolent dormant cells are susceptible to immunoediting and escape. High grade tumor clones that are susceptible to chemotherapy or radiation therapy could become dormant but low grade tumor clones that do not respond well to these treatments could establish micrometastatic minimal residual disease. While dormant cells contain $Ki67^{low}$ indolent and $Ki67^-$ quiescent tumor cells [1], minimal residual disease is composed of indolent tumor cells, and more susceptible to immunoediting compared with dormant cells. In general, proliferating tumor cells either in the form of active disease or in the form of minimal residual disease or indolent dormancy are prone to immunoediting depending on the selective therapeutic pressure. Cancer therapeutics that could induce G0 cell cycle arrest could establish a quiescent type of tumor dormancy that is incapable of change and escape from therapy. It was reported that IFN-γ producing $CD8^+$ T cells were responsible for establishing and maintaining tumor dormancy, as well as inducing tumor immunoediting and subsequent tumor recurrence [1,21,29]. A very recent report identified IFN-γ as a key cytokine responsible for tumor immunoediting [30]. To this end, we reported that Ki67- quiescent, but not $Ki67^{low}$ indolent, dormant cells were resistant to immunoediting [1]. Therefore, the challenge in immunotherapeutic targeting of tumor dormancy is to dominate a quiescent type of tumor dormancy by means of conditioning regimens prior to immunotherapy in order to overcome tumor immunoediting and escape from immunotherapy. Alternatively, combination of targeted therapies with immunotherapy could inhibit certain immunoediting pathways in indolent dormant tumor cells. For instance, MYC inhibitors could prevent the expression of PD-L1 and CD47, because these immunoediting pathways are regulated by MYC [58]. Also, immunotherapeutic targeting of escape mechanisms such as PD-L1 or CTLA-4 expression could be overcome by immune checkpoint inhibitors [59]. The challenge is that tumor cells utilize numerous escape mechanisms; thus, some tumor clones could still escape from targeted therapies or immune checkpoint inhibitors.

Future perspective

Recently, there have been significant advances in the field of cancer immunotherapy. However, these advances have been limited to increasing patients' survival for a limited period of time when immunotherapeutics are administered in a therapeutic setting against advanced stage disease. For instance, T-cell-based therapies could produce CRs, yet they could not overcome tumor escape and recurrence in some patients. Similar observations were made in other immunotherapeutic approaches when targeting advanced stage diseases. For instance, Sipuleucel-T (Provenge) has extended survival of patients with metastatic prostate cancer by median 4.1 months [60]. The significance of immune checkpoint inhibitors is an increased survival tail in some patients with certain types of cancer, which has not been achieved by standard-of-care chemotherapies. Cumulative response rates for the anti-PD-1 antibody among patients with NSCLC, melanoma and renal cell cancer were 18, 28 and 27%, respectively. Responses were durable such that 20 of 31 responses lasted 1 year or more in patients with 1 year or more of follow-up [61]. To increase the size of survival tails, other checkpoint pathways should be identified and targeted; yet, immune checkpoint inhibitors cannot work for certain types of cancer that are weakly immunogenic to induce antitumor immune responses. To this end, immunogenic chemotherapies or radiation therapies should be considered to render all types of cancer responsive to immune checkpoint inhibitors. Alternatively, administration of immunotherapy including immune checkpoint inhibitors during tumor dormancy as a relapse prophylaxis regimen could be more effective, as prophylactic vaccines have been successful against many infectious diseases, as well as against HPV-associated cervical cancer [62]. In addition, application of stem cell transplantation and donor-derived lymphocyte infusion is successful only against minimal residual disease rather than against active and advanced stage disease. Therefore, it is reasonable to expect that the administration of immunotherapy during minimal residual disease or tumor dormancy could deliver a curative outcome.

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Commentary

Conditioning neoadjuvant therapies for improved immunotherapy of cancer

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ABSTRACT

Recent advances in the treatment of melanoma and non-small cell lung cancer (NSCLC) by combining conventional therapies with anti-PD1/PD-L1 immunotherapies, have renewed interests in immunotherapy of cancer. The emerging concept of conventional cancer therapies combined with immunotherapy differs from the classical concept in that it is not simply taking advantage of their additive anti-tumor effects, but it is to use certain therapeutic regimens to condition the tumor microenvironment for optimal response to immunotherapy. To this end, low dose immunogenic chemotherapies, epigenetic modulators and inhibitors of cell cycle progression are potential candidates for rendering tumors highly responsive to immunotherapy. Next generation immunotherapeutics are therefore predicted to be highly effective against cancer, when they are used following appropriate immune modulatory compounds or targeted delivery of tumor cell cycle inhibitors using nanotechnology.

Contents

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1. Introduction

Combinatorial cancer therapies such as chemo-immunotherapy, radio-immunotherapy, or targeted therapies combined with immunotherapy have been rationally designed to impinge on different pathways of tumor growth in order to achieve additive or synergistic anti-tumor effects. For instance, patients with HER2/neu overexpressing breast cancer receive chemotherapy and anti-HER2/neu antibody therapy using Trastuzumab and Pertuzumab. Chemotherapeutics such as doxorubicin increase free radicals that cause DNA damage, as well as intercalate into DNA and disrupt the DNA repairing function of topoisomerase-II [1]. Trastuzumab induces antibody dependent cellular cytotoxicity (ADCC), increases endocytotic destruction of the receptor, and inhibits shedding of the extracellular domain of HER2/neu [2] while Pertuzumab inhibits homo- and hetero-dimerization of HER2/neu, thereby blocking signalling pathways of tumor cell proliferation [3]. The caveat for such traditional chemo-immunotherapies is that standard dose chemotherapies are highly toxic to the host immune system and thus less effective for being simultaneously combined with immunotherapy (Table 1). Recent advances in our understanding of the mechanisms of action of low dose versus high dose chemotherapies are changing the concept of and approaches to chemo-immunotherapeutic design. Many studies demonstrated that certain chemotherapeutics at low doses induce immunogenic tumor cell death (ICD) and confer immune stimulatory effects. Therefore, the rationale for low dose chemotherapies is to condition tumor cells to become highly responsive to immunotherapies. A similar concept applies to the combined use of other cancer therapies, particularly those that induce cell cycle arrest, as conditioning regimens for an effective immunotherapy of cancer. The new chemo-immunotherapeutic approaches are predicted to make immunotherapies highly effective against cancer (Table 1).

2. Low dose metronomic (LDM) chemotherapy for an effective immunotherapy of cancer

Standard chemotherapy dosing regimens have traditionally used the maximum tolerated dose (MTD) of a drug administered with acceptable side effects as determined through clinical trials. In addition to targeting the malignant cells, the nonspecific cytotoxic drugs damage healthy cells with a high proliferation rate such as gastrointestinal mucosal and immune cells. Consequently, an extended time period is required between treatments in order to allow for tissue recovery. LDM chemotherapy is an alternative dosing regimen that is characterized by administering a cytotoxic drug at a low dose scheduled at a regular interval in order to minimize the drug-free time periods. Metronomic dosing schedules aim to achieve adequate disease control with less toxicity than MTD chemotherapy. The rationale for LDM is to not only inhibit tumor growth but also induce ICD and anti-tumor immune responses [4–7] to make patients highly responsive to immunotherapies. A LDM chemotherapy can control tumor progression in patients with early stage as well as those with advanced-stage cancers [8].

2.1. Non-immunogenic mechanisms of LDM chemotherapy

Proliferating malignant cells' oxygen requirements are met by forming inappropriate vascularization to the tumor. Tumor hypoxia results in the production and release of angiogenic cytokines, which leads to resistance to both antiangiogenic and chemotherapeutic regimens [9,10]. One of the earliest studies using low dose chemotherapy at regular intervals referred to the dosing regimen as antiangiogenic scheduling [11]. The study found that low dose cyclophosphamide given at regular schedule was able to kill cells that were resistant to a standard dose chemotherapy. The results have been reproducible [12,13], though the efficacy of low dose chemotherapy as a first line treatment for untreated cancers is yet to be determined. The tumor regression was attributed to sustained endothelial cell apoptosis that occurred due to the higher frequency dosing, which did not occur during the drug-free periods used in MTD chemotherapy. In fact, circulating endothelial cells are released from the bone marrow as an adaptive response to marrow suppression induced by MTD chemotherapy, allowing for damaged tumor cells to regenerate. In this aspect LDM chemotherapy has a unique mechanism in suppressing vasculogenesis by suppressing the source of vascular growth factors [14]. Promotion and maintenance of angiogenesis involves a balance of proangiogenic and antiangiogenic molecules acting within the tumor microenvironment. One of the earliest growth factors released from the tumor site in response to hypoxia is the transcriptional regulator, HIF-1alpha. Doxorubicin at a LDM regimen has been reported to block this transcription factor, the inhibition of which has been shown to overcome resistance to antiangiogenic therapies and promote tumor regression [15,16]. LDM chemotherapy has been shown to decrease expression of proangiogenic molecules VEGF and VEGF receptor 2 [17] and increase the expression of the antiangiogenic thrombospondin 1 [18]. Taken together these data indicate that LDM chemotherapies suppress the tumor microenvironment's response to hypoxia by suppressing angiogenesis.

2.2. Immunogenic mechanisms of LDM chemotherapy

Certain chemotherapies at the MTD have been associated with immune stimulation through the induction of ICD. The term ICD was first introduced over a decade ago by Dr. Kroemer's group to indicate a functionally peculiar type of cell death induced by certain chemotherapeutics that can elicit an immune response against damage associated molecular patterns (DAMPs) in the absence of any adjuvant [19]. Inducers of ICD include doxorubicin, cyclophosphamide, epirubicin, idarubicin, mitoxantrone, bleomycin, bortezomib, 5-fluorouracil, paclitaxel and oxaliplatin [20,21]. On the other hand, some other chemotherapeutics such as cisplatin fail to induce ICD [22]. Animals challenged with doxorubicin-sensitized tumor cells were able to mount anti-tumor immune responses that protected them from re-challenge with tumor cells of the same type [19]. Recent studies demonstrated that the lack of ICD is correlated with poor prognosis for breast cancer patients

Table 1
Current concepts on combinatorial cancer immunotherapies.

Concept	Objective	Approach	Weakness	Strength
Traditional	To impinge on different pathways of tumor growth in order to achieve additive or synergistic anti-tumor effects	Adjuvant therapies at maximum tolerated doses	Toxicity Immune suppression	Tackle multiple drug resistant mechanisms
New	To condition the tumor microenvironment and make tumor cells highly responsive to immunotherapy	Low dose neoadjuvant conventional therapies and standard dose adjuvant immunotherapy	Tumor immunoediting and escape	Immune stimulatory Safe

[23], and ongoing clinical studies have identified some standard-of-care chemotherapeutics that induce ICD [20,24].

Molecular components of DAMPs that induce ICD following chemotherapy have been identified as: i) cell surface expression of endoplasmic reticulum (ER) chaperones such as calreticulin (CRT), ii) release of ATP, iii) release of non-histone chromatin-binding protein high mobility group box 1 (HMGB1), and iv) secretion of immunostimulatory cytokines such as type I interferons [25–27]. ICD is induced even prior to cell death such as during autophagy or senescence [26]. Such chemotherapy-induced ICD recruited dendritic cells (DCs) to the tumor site and activated them to take up dead-cell associated antigens. The activated DCs undergo maturation and present tumor antigens to T cells, resulting in the induction of tumor-specific immune responses [26]. CRT, HMGB1, and ATP interact with CD91, TLR-4, and purinergic P2RX7 receptors on DCs, respectively. These interactions, in turn, result in antigen uptake, antigen presentation and production of IL-1β by DCs [22,28,29]. Any defects in the DAMP-sensing machinery, such as type I interferon receptor alpha and beta, CD91, TLR4 or P2RX7 could alter the immune response to chemotherapy-induced ICD. Other chaperones such as HSP70 and HSP90, as well as uric acid are also considered as markers of ICD that interact with CD91 or TLRs [30]. Unfortunately, standard chemotherapies are also known to compromise immune surveillance by killing proliferating effector T cells, and contribute to treatment resistance [31]. On the other hand, LDM chemotherapies can induce ICD and also confer additional immune stimulatory effects without the significant killing of anti-tumor T cells. This immune stimulatory function of LDM chemotherapies is important because tumor cells are able to unleash an immunosuppressive network of cells composed of M2-polarized macrophages, regulatory T (Treg) cells and myeloid-derived suppressor cells (MDSCs) [32], which leads to tumor cell evasion by dampening anti-tumor immune responses. Breast cancer patients who were treated with low dose cyclophosphamide showed decreased Tregs and increased effector T cells as well as NK-cell-dependent anti-tumor immunity [4,5]. Cyclophosphamide also enhances Th-17 and Th1 immune responses, and expands NK-cell and DCs in multiple mouse tumor models [33,34]. Other immune stimulatory chemotherapies that deplete circulating or tumor-infiltrating Tregs and/or circulating MDSCs include 5-fluorouracil [35,36], gemcitabine [37,38], oxaliplatin [39], paclitaxel [40], and docetaxel [41], and decitabine [42]. In addition, oxaliplatin promotes anti-tumor function of macrophages and neutrophils [43]; paclitaxel induces maturation of DCs [44] and tumor infiltration of NK cells in breast cancer patients [45]. In fact, the anti-tumor efficacy of doxorubicin has been suggested to depend on the host immune system [46] such that depletion of T cells compromises anti-tumor efficacy of doxorubicin [47]. LDM chemotherapy also has been shown to be a suitable preparative regimen for vaccination approach in order to boost anti-tumor immune responses against dormant cells [7]. Similarly, whereas fractionated radiation therapy (RT) is immunogenic and generates abscopal responses in mice, single high-dose RT fails to do so [48]. This failure is because of the upregulation of three prime repair exonuclease 1 (TREX1) which in turn inhibits type I interferon secretion, an ICD signal, by irradiated tumor cells [49].

3. Epigenetic targeting of tumor cells for immune modulation against cancer

Spontaneous cancers arise in immunocompetent individuals with active immunoediting mechanisms that make tumor cells weakly immunogenic [50]. Therefore, improving the immunogenicity of cancer is essential to improving cancer immunotherapy. Epigenetic modulators such as azacytidine (Aza) and decitabine (Dec) function as cytosine analogs, which lead to their incorporation into newly synthesized DNA strands during S phase of the cell cycle; these agents have been shown to enhance immunogenicity of tumor cells by inducing the expression of a panel of highly immunogenic cancer testis antigens (CTAs), and result in improved immunotherapy of cancer [51,52]. Both Aza and Dec also induce the expression of tumor suppressor gene p53 [53] and the death receptor Fas [54] on tumor cells. These functions are attributed to the capacity of these agents to mechanistically operate as potent DNA methyltransferase (DNMT) inhibitors through the formation of a covalent complex with a cysteine residue at the active site of DNMT1. This results in CpG island demethylation during cellular proliferation, which, in turn, results in hypomethylation within the promoter of tumor suppressor genes as well as highly immunogenic CTAs [55,56], leading to their enhanced transcription. Ultimately, the use of such epigenetic modulating agents renders tumor cells susceptible to CTA-reactive immune responses while potentially reducing the proliferative capacity of tumor cells by restoring p53 expression. In fact, aberrant CTA expression has been shown to elicit CTA-specific cytotoxic T cell responses in melanoma; treatment of CTA-expressing metastatic melanoma with autologous CTA-specific T cells has elicited long-term complete remission [57,58]. Dec in particular is an attractive therapeutic because it requires activation by deoxycytidine kinase (DCK), an enzyme preferentially expressed in tumor cells and myeloid cells. Therefore, it is expected to specifically kill tumor cells and MDSCs while leaving T and B cells unharmed. In addition, DCK has been found to be overexpressed in poor outcome breast cancer [59], suggesting that epigenetic therapy to induce CTA expression may prove to be an efficacious approach in breast cancer patients with poor prognosis. A low dose regimen of Dec was shown to render mouse mammary carcinoma highly susceptible to immunotherapy [52]. In colorectal cancer, Aza at a low dose increased type I interferon production within the tumor through the re-activation of endogenous retroviruses, and as a result enhanced anti-tumor immune responses [60]. In patients with solid tumors, low dose Dec increased TcR diversity, which is important for T cells to respond to antigenic diversity of tumor cells [61]. A low dose regimen of Dec was also reported to induce the expression of CD80 co-stimulatory molecule on tumor cells associated with enhanced anti-tumor immune responses [62]. Aza therapy in patients with relapsed or refractory Hodgkin lymphoma resulted in a complete response to immune checkpoint inhibitors [63]. We have also reported that the use of Aza combined with the immune modulatory lenalidomide induced the expression of CTAs within tumor cells, and generated CTA-specific immune responses in patients with multiple myeloma [64]. Similar results were observed in a mouse model of experimental metastatic breast cancer [65]. Therefore, Dec is an attractive candidate as a neoadjuvant immune modulator when combined with immunotherapy.

4. Control of cell cycle progression prior to immunotherapy

The dysregulation of the cell cycle is a classic hallmark of cancer growth and metastasis. Cyclin-dependent kinases (CDKs) are a family of multifunctional enzymes that can modify various protein substrates involved in cell cycle progression. All eukaryotic cells have multiple cyclins, which act during a specific stage of the cell cycle. Common cyclins include G0/G1-phase cyclins, G1/S-phase cyclins, S-phase cyclins, and M-phase cyclins. CDK4 and CDK6 are important for progression during the G_1 cell cycle phase [66], CDK2 is important for transition from G1 to S-phase [67], and CDK1 is important during G2 and M progression [68]. The inhibition of tumor cell cycle progression through CDK inhibitors has emerged as an attractive option for targeted cancer therapy. Three

specific CDK4/6 inhibitors palbociclib, abemaciclib and ribociclib have been successfully tested in patients with hormone receptor-positive HER2-negative breast cancer [69]. Palbociclib inhibits cell growth and DNA replication in a number of retinoblastoma (Rb) proficient human cancer cells, including breast cancer because over 70% of breast cancers are Rb proficient [70]. Palbociclib is a well-tolerated cancer therapeutic [71] that induces G0/G1 arrest in HER2/neu− and HER2/neu+ breast tumor cells [69,72] as well as in neu positive murine mammary tumor cells [73]. Palbociclib can be administered at a concentration of 150 mg/kg through oral gavage and given daily for 3–4 weeks either alone or after the completion of chemotherapy in order to further reduce tumor cell burden. Palbociclib and chemotherapies may not be used simultaneously, because G0/G1 arrest by Palbociclib could render tumor cells resistant to cytotoxic function of chemotherapy [74]. Palbociclib does not induce apoptosis in bone marrow hematopoietic cells, and its function as a cell cycle arresting agent is reversible upon its removal [75]. Unlike pan-CDK inhibitors, palbociclib is not toxic to T cells [76]; thus, it can be used with immunotherapeutics. Roscovitine is a selective CDK5 inhibitor that is able to induce the apoptosis of drug-resistant breast cancer cells [77]. A sequential use of chemotherapy and roscovitine can induce G2/M arrest and apoptosis in highly invasive triple negative breast cancers [78]. Rescovitine is not toxic for tumor-reactive T cells [79] and could sensitize breast cancer cells to immunotherapy by TRAIL-induced apoptosis [80]. Very recently, it was reported that IFN-γ-induced immunoediting via PDL-1 expression is a CDK5-dependent event; thus, roscovitine can suppress IFN-γ-induced expression of PD-L1 [81].

The rationale for use of CDK inhibitor is to push tumor cells towards dormancy so that the immune system can control tumor growth by inducing tumor cell death. It has been reported that dormant tumor cells, while become refractory to chemotherapy, remain susceptible to immunotherapy [65]. Therefore, the next generation immunotherapeutics are expected to be highly effective against cancer, when combined with immune modulatory compounds.

5. Targeted delivery of tumor cell inhibitors: nanotechnology

Although LDM chemotherapies were found to be immunogenic, they could still affect the normal cells because of their administration over an extended period of time. Therefore, tumor immune modulatory chemotherapies that induce ICD and increase the expression of MHC or other immune modulatory receptors such as Fas or CD80 on tumor cells would be more effective through targeted delivery than systemic administration of the drug. To this end, targeted delivery of cell cycle inhibitors prior to immunotherapy would be desirable. Folate (vitamin B9) receptor (FR) is an attractive target because of high level of expression on tumors of epithelial origin compared to normal tissue, including ovarian, breast, brain, lung and colorectal cancers [82]. Folate-conjugated nanoparticles that carry desirable drugs are internalized upon binding to FR and the acidic microenvironment inside tumor cells dissociates FR from the drug-carrying nanoparticles resulting in drug-induced apoptosis or inhibition of tumor cell proliferation. Cytotoxic drugs have been successfully delivered to tumor cells via targeting the FR and using nanoparticles as a potent drug carrier. In a breast tumor model, paclitaxel-loaded folate modified lipid-shell and polymer-core nanoparticles (FLPNPs) showed similar antitumor efficacy but lower toxicity compared to paclitaxel. The paclitaxel-loaded FLPNPs confer a higher tumor inhibitory effect than the nontargeted paclitaxel-loaded LPNPs [83]. Cases of successful oral delivery of Dec to abdominal tumors by means of nanostructured lipid carrier have been reported [84]. It was demonstrated that nanoconjugated delivery of Dec to human glioblastoma cells could overcome chemo-resistance by rendering tumor cells susceptible to alkylating chemotherapy [85]. In humans, carbon nanoparticles have been successfully used to protect parathyroid glands in patients with thyroid cancer. Such a nanotechnology approach reduced incidence of hypoparathyroidism and hypocalcemia after surgical removal of thyroid tumor [86]. Safety and efficacy of SGT-53 liposomal nanoparticle delivery of p53 gene into refractory solid tumors in combination with chemotherapy have also been established [87]. Very recently, therapeutic nanoparticles plus trastuzumab with and without cyclophosphamide were successfully tested through enhanced permeability and retention in patients with HER2 positive metastatic breast cancer [88]. These trials suggest the feasibility of tumor-targeted nanoparticle drug delivery using cell cycle inhibitors as a conditioning regimen for immunotherapy of cancer. However, the feasibility and efficacy of such targeted delivery of tumor immune modulators in combination with immunotherapy remain to be investigated.

Conflict of interest

The authors declare no conflict of interest.

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