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Potential therapeutic anti-tumor effect of a *Salmonella*-based vaccine

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One of the major obstacles to achieving complete eradication of tumors, even in the presence of circulating tumor-specific immunity, is the tumor-induced immunosuppressive environment, which includes myeloid-derived suppressor cells and regulatory T cells. Attenuated microorganisms have emerged as candidates for a novel anti-cancer approach in which they enhance anti-cancer immunity by boosting the innate immune system. Herein, we will discuss current innate-immunity activating strategies for anti-cancer therapy, with a focus on our recently reported approach involving the use of intratumoral injection of recombinant attenuated *Salmonella enterica* serovar Typhimurium vaccine; this approach elicits transformation of immunosuppressive myeloid-derived suppressor cells into TNF-α-secreting cells with characteristics of neutrophils, and reduces the generation of regulatory T cells, particularly in the presence of tumor-specific cytotoxic T lymphocytes.

Keywords: *Salmonella*, tumor, myeloid-derived suppressor cell, regulatory T cell, cytotoxic T lymphocytes

Abbreviations: RASV, recombinant attenuated *Salmonella enterica* serovar Typhimurium vaccine; MDSC, myeloid-derived suppressor cell; CTL, cytotoxic T lymphocyte; Treg, regulatory T cells; LN, lymph node

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Introduction

Ever since William B. Coley introduced "Coley's toxins" using the heat-killed bacteria *Streptococcus pyogenes* and *Serratia marcescens* for tumor therapy over a century ago,[1] many anti-tumor therapeutic approaches using attenuated microorganisms have been investigated. These include Bacillus Calmette-Guerin (BCG), *Listeria monocytogenes*, *Salmonella* spp, *Clostridium* spp and *Toxoplasma gondii*. These approaches are summarized in **Table 1**.

Our recently reported approach showed a potential therapeutic anti-tumor effect of intratumoral delivery of attenuated *Salmonella enterica* serovar Typhimurium.[15] Inflammatory responses were induced within the tumor microenvironment, consequently promoting conversion of immunosuppressive myeloid-derived suppressor cells (MDSCs) into TNF-α-secreting myeloid cells.[15] Similarly, others have recently reported that an attenuated but still invasive *Salmonella* spp preferentially invaded the tumor area, exerting both direct and indirect antitumor effects via recruitment of inflammatory cells and cross-presentation of the tumor antigen.[6] Interestingly, intratumoral administration of attenuated *Salmonella typhi* CVD915 elicited antitumor effects by recruitment of activated TNF-α-secreting neutrophils to the tumor site, and reducing regulatory T cells (Tregs) in tumor-draining lymph nodes (LNs).[7] In addition, a critical role of TNF-α in the anti-cancer effects of BCG-stimulated neutrophils in the immunotherapeutic treatment of bladder cancer has been suggested.[12]

Despite the immunostimulating effect of various immunotherapeutic approaches against cancer, the immunosuppressive environment produced by the tumor can restrict the antitumor potential of these approaches.[16] Thus, there is an urgent need to develop effective ways to subvert tumor-driven immune escape mechanisms, while potentiating tumoricidal effects. In this regard, *Salmonella*-based anti-tumor immunotherapies shed light on the development of effective ways to treat tumor patients, in that they can specifically target and colonize the tumor site, promote an inflammatory response by inducing infiltration of neutrophils, induce tumor-specific T-cell responses and

Table 1. Cancer immunotherapy based on killing of bacteria-infected tumor cells

Microorgamisms	Injection route	Model	Target cancer	Mechanism	Reference
Salmonella Typhimurium A1 (Leu and Arg auxotroph)	Intravenous or Intratumoral	Nu/nu mice	Human PC-3 prostate cancer cells	Apoptosis induction	2
			Primary orthotopic pancreatic tumor		3
			Orthotopic human breast tumor		4
Salmonella Typhimurium 14028 strain	Intraperitoneal	C57BL/6 mice	B16F1 melanoma cells	Downregulation of $CD44^{high}$ and $CD4^{+}CD25^{+}$Tregs	5
Salmonella Typhimurium SL3261AT InvA	Intratumoral	C57BL/6J mice	B16F10 and EG-7 cells	Cytotoxic T cells and intratumoral recruitment of $Gr1^{hi}$ granulocytes	6
Salmonella Typhi CVD915	Intratumoral and peritumoral	BALB/c mice	LM3 mammary adenocarcinoma	IFN-γ-secreting $CD4^{+}$ and $CD8^{+}$ T cells Reduction of Tregs	7
		C57BL/6 mice	EL4 T cell lymphoma	TNF-α-secreting neutrophils	8
Salmonella choleraesuis	Intraperitoneal	C3H/HeN and C3H/HeJ mice	Murine K1735 melanoma cells	TLR4-dependent T_H1 response	9
Propionibacterium acnes	Intratumoral	C57BL/6 mice	B16 melanoma cells	T_H1 immune responses and secretion of IL-12, IFN-γ, and TNF-α	10
Toxoplasma gondii (cps, uracil auxotroph)	Intratumoral	C57BL/6 mice	B16F10 melanoma cells	$CD8^{+}$ T cells and NK cells	11
Bacillus Calmette–Guérin	Intravesical	Human patients	Bladder cancer	TNF-α, TRAIL and neutrophils	12, 13
Listeria monocytogenes-LLO	Intraperitoneal	BALB/c mice	4T1 mammary carcinoma	$CD8^{+}$ T cells	14
Clostridium novyi non-toxic (NT) spore	Intravenous	C57BL/6N mice	Pancreatic tumor Panc02 cells	NK cells and innate immunity	46

importantly, reduce immunosuppressive cells including MDSCs and Tregs.

Ways to Subvert the Immunosuppressive Tumor Microenvironment

In the tumor microenvironment, there are various tumor-infiltrating immune cells, including immune effectors and immune suppressors.[17] Although some tumors are potentially immunogenic, immune suppressors present an obstacle to tumor rejection.[17] MDSCs are one of the critical immune suppressors.[16] The numbers of MDSCs increase in various inflammatory diseases, including cancer.[18] While the definition of MDSCs has been based on their immunosuppressive nature, MDSCs are a heterogeneous population and have diverse immunosuppressive mechanisms, including arginase 1, nitric oxide, reactive oxygen species and membrane-bounded TGF-β.[19-21] In a recent study by our group, two major subsets of MDSCs, Ly6-G^{high}Ly6-C^{inter} cells (granulocytic MDSCs) and Ly6-G^{inter}Ly6-C^{high} cells (monocytic MDSCs) were detected, but only the Ly6-G^{high}Ly6-C^{inter} subset increased by intratumoral injection of recombinant attenuated *Salmonella enterica* serovar Typhimurium vaccine (RASV)[15] (**Fig. 1**). These data suggest that each subset of MDSCs may constitute a separate population, induced under distinct circumstances.

Interestingly, there is plasticity in both the phenotype and suppressive function of MDSCs.[22] Tumor-derived factors induce the generation of an immunosuppressive subset of MDSCs.[23] On the other hand, several pharmacological approaches are competent in regulating MDSC-mediated immune suppression. Treatments with drugs that regulate myelopoiesis reduce the number of MDSCs, and some cytotoxic chemotherapeutic agents have the capacity to eliminate MDSCs selectively. To decrease the number of MDSCs at the tumor site, blockade of MDSC recruitment is one potential strategy. Lastly, various agents for neutralization of the immunosuppressive function of MDSCs have been reported (**Table 2**).

Some conditions that induce MDSC generation cause MDSCs to become immunostimulatory myeloid cells, including tumoricidal neutrophils.[12,15,42] Cuenca et al. have reported that in trauma and sepsis, MDSCs play the role of immune effector cells, increasing immune responses.[20] In a cancer model, immunogenic MDSCs mediating antitumor immunity were generated in epithelial ovarian cancer-bearing mice.[43] In our recently reported study,[15] we detected an accumulation of distinct TNF-α-producing Ly6-G^{high}Ly6-C^{inter} MDSCs in mice treated with intratumoral RASV (**Fig. 2**), and they exhibited a therapeutic antitumor effect. While activated neutrophils secreting TNF-α can act as direct effector cells in therapeutic anticancer therapy, many cytokines associated with chronic inflammatory status in the tumor microenvironment, including IL-6 and IL-1β, are associated with the accumulation of MDSCs.[33,44] Thus, further studies are required to identify the factors that may regulate MDSC conversion into TNF-α-producing neutrophils in the inflammatory tumor microenvironment after intratumoral injection of attenuated *Salmonella*.

Another type of immune suppressors, which control self-reactive T cells

to prevent autoimmunity and are a major obstacle for anti-tumor immunotherapy, is $CD4^+CD25^+$ Tregs.[19,45] The Treg population is reportedly expanded in some tumor patients, and they are recruited to tumor sites, where they exert a suppressive role against cytotoxic T lymphocytes (CTLs).[17] There was a close correlation between the expansion of Tregs and MDSCs,[19] and a recent report showed that the suppression of tumor growth by *Salmonella enterica* serovar Typhimurium was related to down regulation of $CD4^+CD25^+$ Tregs.[5] Likewise, *Salmonella typhi*-based immunotherapy reportedly mediated tumor-specific immune responses in tumor-draining LNs, with an associated reduction in the number of Tregs among the $CD4^+$ T cell population.[8] In our recently reported study, we also found that the percentage of $CD25^+FoxP3^+$ Tregs among the $CD4^+$ T cell population was significantly reduced in tumor-bearing mice intratumorally treated with RASV, compared with PBS-treated controls[15] (**Fig. 3**). However, it is not certain whether *Salmonella*-based immunotherapy directly inhibits the generation of Tregs in tumor-bearing mice, or whether reduction in MDSCs indirectly affects the expansion of Tregs.

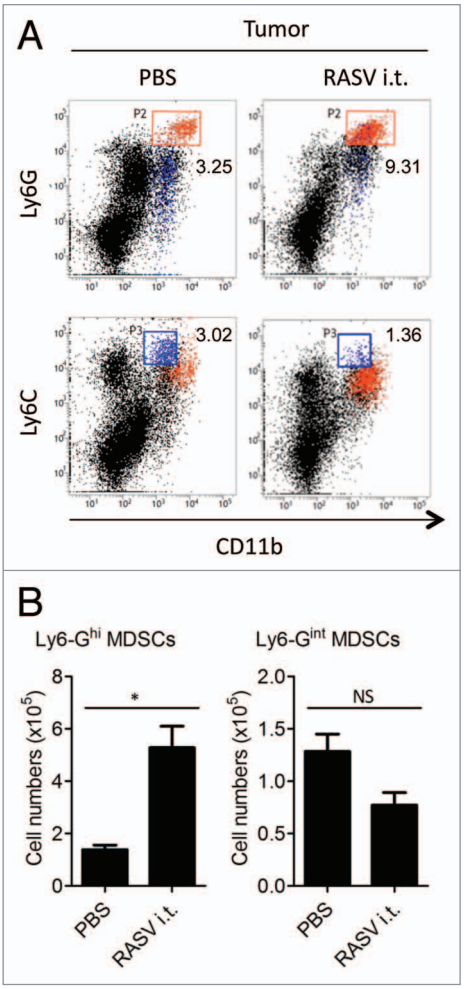

Figure 1. RASV increased Ly6-G^{high} MDSCs in the tumor. Two subsets of MDSCs were evident, Ly6-G^{high}Ly6-C^{inter} cells (upper panel) and Ly6-G^{inter}Ly6-C^{high} cells (lower panel). (**A**) FACS plot percentages and (**B**) absolute number of each MDSC subset in the tumor. *$p < 0.05$. Adapted with permission from Hong et al.[15]

Mechanism of Immune Reversion from Immunosuppressive into Anti-Cancer Immunity by Microorganisms

The mechanism underlying the induction of antitumor activity by treatment with attenuated microorganisms could be explained by several factors, including the regulation of Treg generation, conversion of MDSCs into immunostimulatory cells, and generation of IFN-γ-producing T_H1 and CTLs. In a RASV treatment model, we investigated the underlying mechanism by analyzing these factors.[15]

First, CTLs in untreated vs. intratumoral *Salmonella*-injected tumors were compared. When tumor-infiltrating $CD8^+$ T cells were restimulated with a tumor antigen-specific peptide, tumor antigen-specific IFN-γ secretion by CTLs was significantly lower in RASV-injected tumors compared with that in PBS-treated tumors. These results suggest that the anti-tumor effect of intratumoral RASV injection may not be mediated by circulating tumor antigen-specific CTLs (**Fig. 4**). However, the absolute number of tumor-infiltrating $CD8^+$ T cells per tumor weight significantly increased by intratumoral RASV administration. Thus, the effector function of tumor-infiltrating $CD8^+$ T cells can be significantly increased by RASV injection, and they exhibit important therapeutic anti-tumor effects, although it is uncertain whether they are reactive to other tumor associated antigens and *Salmonella*-infected tumors.

With regard to the involvement of NK cells and T_H1 cells in anti-tumor activity after RASV treatment, the percentages and absolute numbers of $NK1.1^+CD3^-$ NK cells in the draining LNs were significantly increased but they did not secrete IFN-γ at all, whereas IFN-γ-secreting $CD4^+$ T cells (T_H1) marginally increased.[15] Thus, NK cells and T_H1 cells could participate in the anti-tumor activity of RASV treatment, but may only play a minor role.

The absolute number of tumor-infiltrating Tregs reduced slightly in the spleen, draining LNs, and tumors.[15] In the draining LNs and spleen, the numbers of $CD4^+$ T cells increased consistently with enlarged LNs and the spleen after RASV treatment. Therefore, the absolute number of Tregs in these tissues was similar (the spleen) or rather increased (draining LNs) after RASV injection, although the percentages of Tregs reduced. Collectively, RASV treatment could reduce the percentages of Tregs among $CD4^+$ T cells by increasing effector $CD4^+$ T cells.

Table 2. Approaches to overcome the immune suppression mediated by MDSCs

Major goal	Approach	Result	Reference
Regulation of MDSC generation	Anti-c-kit mAb	Blockade of stem cell factor (SCF)-c-kit signaling and reduction of MDSC number	24
	Tyrosine kinase inhibitor (sunitinib)	Blockade of vascular endothelial growth factor receptors (VEGFR), c-Kit, STAT3, etc., and reduction of MDSC number	25, 26
Further differentiation of MDSC	All-trans-retinoic acid	MDSC differentiation into mature myeloid cells	27
	Vitamin D3	$CD34^+$ cell maturation	28, 29
Depletion of MDSC	Gemcitabine	Elimination of MDSCs	30, 31
	5-fluorouracil		32
	Anti-IL-6 receptor mAb		33
Prevention of MDSC recruitment to tumor	COX-2 inhibitor (celecoxib)	Downregulation of CCL2 production and decrease in MDSC recruitment	34
	Inhibitor of CSF1R signaling (GW2580)	Decrease in monocytic MDSC recruitment	35
Inhibition of MDSC immunosuppressive function	PDE-5 inhibitor (sildenafil)	Inhibition of iNOS and/or ARG-1 activities	36
	COX-2 inhibitor (celecoxib)		37
	Nitroaspirin		38
	CpG ODNs	Reduction of suppressive function of $Ly6G^{high}$ MDSC	39
	Triterpenoid	Inhibition of MDSC immune suppressive effect	40
	Rapamycin	Downregulation of ARG1, iNOS and Nox2 in MDSC	41
	α-galactosylceramide	Conversion of MDSC into nonsuppressor cells and increase in immunogenecity of MDSC	47, 48

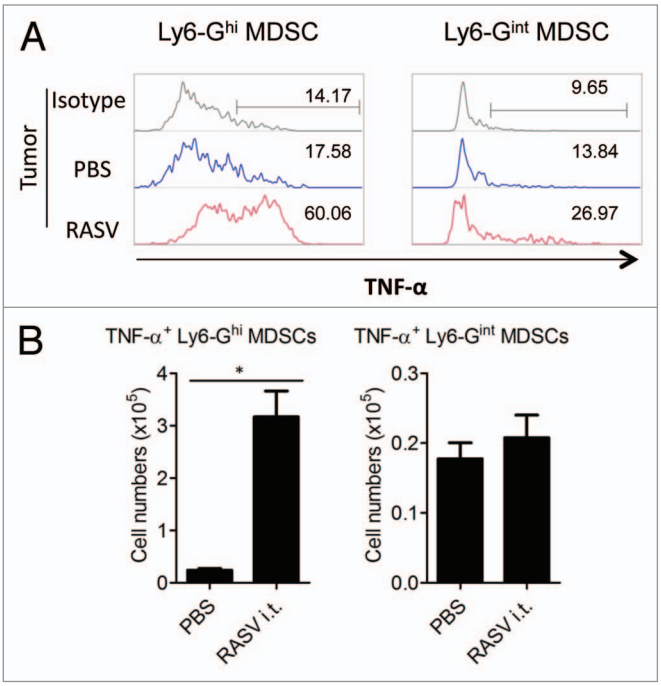

Figure 2. Intratumoral injection of RASV induced Ly6-G^{high} granulocytic MDSCs highly secreting TNF-α in the tumor. Tumor-infiltrating cells were stimulated with 200 ng/ml LPS for 2 h, and then, TNF-α secretion by Ly6-G^{high}Ly6-C^{inter} and Ly6-G^{inter}Ly6-C^{high} MDSCs was analyzed by intracellular staining. (**A**) Percentages of TNF-α^+ MDSCs in the tumor. (**B**) The absolute number of TNF-α^+ MDSC subsets in the tumor. *$p < 0.05$. Adapted from Hong et al.[15]

With regard to MDSC modulation, of the two major subsets of MDSCs, Ly6-G^{inter}Ly6-C^{high} (monocytic) MDSCs and Ly6-G^{high}Ly6-C^{inter} (granulocytic) MDSCs, intratumoral RASV injection significantly increased only the latter in the spleen and tumor as compared with PBS-injected tumor-bearing mice[15] (**Fig. 1**). In particular, Ly6-G^{high}Ly6-C^{inter} MDSCs are significantly increased in the tumor by RASV injection, and they become a major population. Thus, we postulated that the increased MDSC populations in RASV-injected mice may not be immunosuppressive, but instead may help stimulate antitumor immune activity.

Upon assessing the characteristics of MDSCs, we found that tumor-infiltrating Ly6-G^{high} populations secreted more TNF-α than that secreted by Ly6-G^{inter} populations, and over 60% of tumor-infiltrating Ly6-G^{high} populations expressed TNF-α after lipopolysaccharide (LPS) restimulation[15] (**Fig. 2**). These data suggested that intratumoral injection of RASV can induce TNF-α-secreting Gr-1^{high}Ly6-G^{high}Ly6-C^{inter} populations, which have neutrophil-like characteristics. Intratumoral injection of RASV increased sub-populations of $CD11b^+$Gr-1^+ cells,

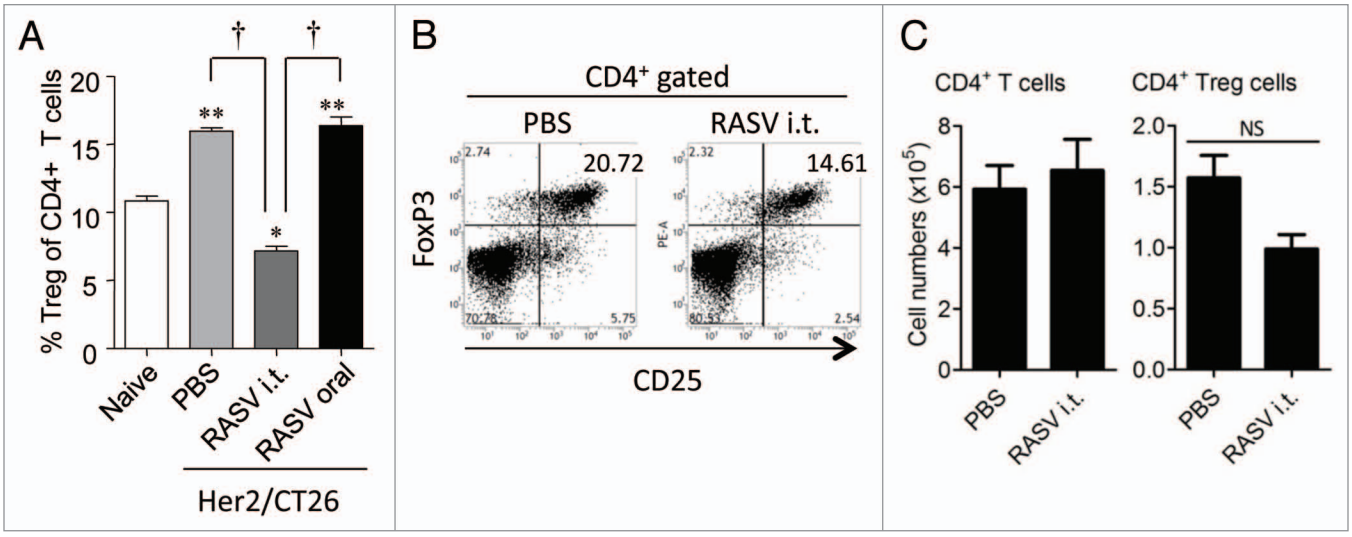

Figure 3. CD4⁺CD25⁺FoxP3⁺ regulatory T-cell levels decreased in tumor-bearing mice after i.t. injection of RASV. (**A**) The percentages of CD4⁺CD25⁺FoxP3⁺ Tregs among the CD4⁺ T cell population in splenocytes (n = 6 mice per group). **p < 0.01, ***p < 0.001 compared with naïve control mice. †p < 0.01, Her2/CT26-PBS vs. Her2/CT26-RASV i.t. and Her2/CT26-RASV i.t. vs. Her2/CT26-RASV oral. (**B**) The percentages of FoxP3⁺ Tregs among the CD25⁺ cells are shown after gating the tumor-infiltrating CD4⁺ T cells. (**C**) The absolute number of tumor-infiltrating CD4⁺ T cells and CD4⁺ CD25⁺ FoxP3⁺ Tregs in the tumors. NS, not significant. Adapted from Hong et al.[15]

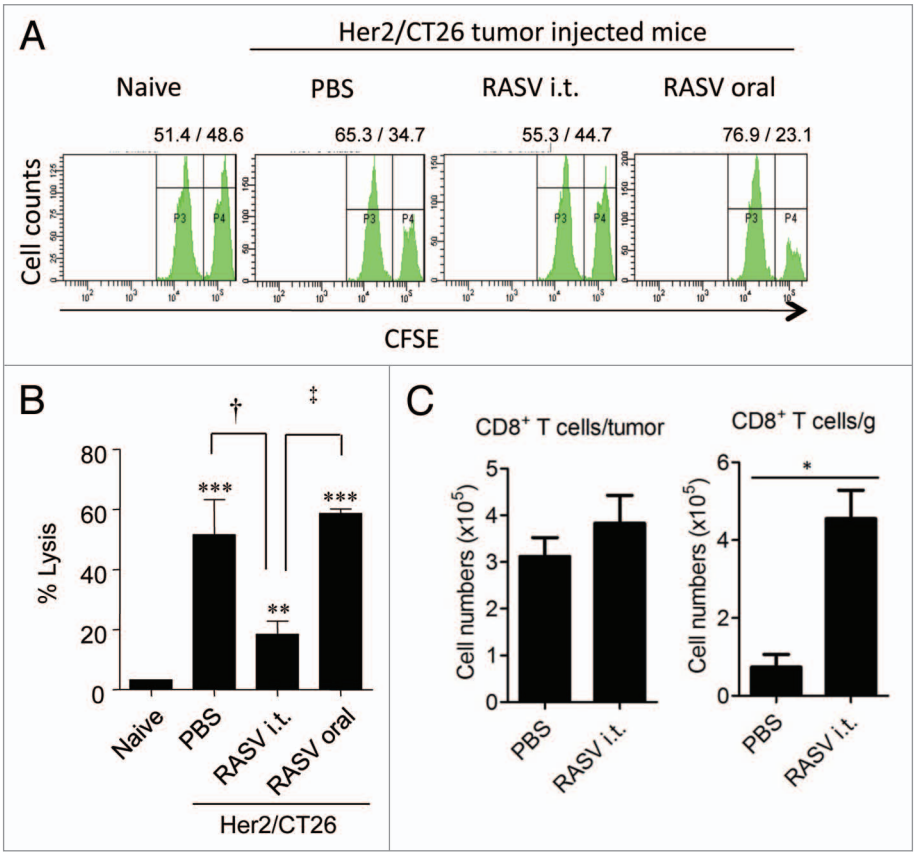

Figure 4. Tumor antigen-specific CTL activity and tumor-infiltrating CD8⁺ T cells. (**A**) Spleens from RASV-treated mice were obtained, and specific lysis of hP63 (TYLPTNASL) peptide-loaded target cells was estimated by in vivo CTL levels. (**B**) Results are expressed as the mean cytotoxicity ± SEM from in vivo CTL assays. (**C**) The absolute number of tumor-infiltrating CD8⁺ T cells per tumor (left) and per tumor weight (right). *p < 0.05. Adapted with permission from Hong et al.[15]

which are distinct from classical suppressive MDSCs because they secrete TNF-α, and consequently resulted in tumor regression.

Conclusions and Future Prospects

Attenuated *Salmonella* can be used as a therapeutic anti-tumor vaccine, mediating conversion of immunosuppressive MDSCs into TNF-α-secreting neutrophil-like myeloid cells. Intratumoral administration of attenuated *Salmonella* induced $CD8^+$ T cell-dependent tumor regression. Thus, intratumoral injection of attenuated *Salmonella* vaccine can be a successful therapeutic anti-tumor regimen, inducing anti-tumor effectors including CTL and TNF-α-secreting neutrophils, as well as overcoming aspects of the immunosuppressive tumor environment including MDSCs and Tregs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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