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Simultaneous induction of NKG2D and NKG2D ligand for promoting immune surveillance by IL-12 plus doxorubicin treatment

Jiemiao Hu

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Simultaneous induction of NKG2D and NKG2D ligand for promoting immune
surveillance by IL-12 plus doxorubicin treatment

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**Simultaneous induction of NKG2D and NKG2D ligand for
promoting immune surveillance by IL-12 plus doxorubicin
treatment**

A

DISSERTATION

Presented to the Faculty of

The University of Texas

Health Science Center at Houston

And

The University of Texas

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Graduate School of Biomedical Sciences

In Partial Fulfillment

of the Requirements

for the Degree of

DOCTOR of PHILOSOPHY

By

Jiemiao Hu, MS

Houston, TX

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Dedication

I'm very blessed to have the most amazing parents Mr. Yizhi Hu and Mrs. Gefang Li who have always been supportive throughout my PhD study. They provided me the best education and taught me the correct personal values. It is really hard for them to let their only child go across the world, but they sacrificed a lot and encouraged me to pursue my dreams. They might not understand much English, but I'm sure they are very proud of me. Thank you, mom and dad.

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I would like to thank my mentor Dr. Shulin Li for giving me the opportunity to study and do research in his laboratory, for guiding me when I have troubles with the experiments, for sharing experience with me, and for supporting me on my way to be an independent scientist. I would never have accomplished any publications without his suggestions on my project every week. I would never have completed my dissertation without his encouragement all the way from Baton Rouge to Houston. I'd love to express my sincere gratitude to Dr. Li.

I would like to thank all of my past and current committee members. I'm very fortunate to have Drs. Joya Chandra and Dean A Lee in all of my three committees. They always give me advice on my committee meetings. They both wholeheartedly contributed to the success of my PhD study. Drs. Gary Gallick and Candelaria Gomez-Manzano served on two of my committees. Their encouragement supports me to conquer all the difficulties throughout my PhD study. Drs. Dennis PM Hughes and Randy J Legerski also gave me valuable advice on my project.

I would like to thank all the past and current members in Dr. Shulin Li's lab especially Summer Xia who has helped me with ordering, cloning and experiment design. Everybody in the Li lab collaborates and supports each other. I really enjoyed working with everyone and learnt a lot from each of you. I would like to thank everyone in the Pediatrics department. It is such a warm family to work in. I also would like to thank Ms. Donna Reynolds who helped to cut all the frozen slides for my staining experiments.

Thank you to all of my friends and family who are around me and love me unconditionally.

Abstract

NKG2D (natural killer group 2, member D) and its ligands interaction in tumor microenvironment directs tumor infiltrating immune cells to recognize tumor cells, stimulate cytotoxic effector immune cells, and therefore eradicate tumor cells. IL-12, a cytokine produced by antigen presenting cells, has remarkable antitumor effect by activating innate and adaptive immunity. Doxorubicin, a commonly used chemotherapeutic agent also boosts the host antitumor immune response to cause tumor cell death. Our previous publication suggests that IL-12 plus doxorubicin enhances NKG2D function-dependent inhibition of tumor progression and promotes CD8⁺T cells infiltrating into tumors. The purpose of this study is to determine the underlying mechanism.

Our study reveals a novel function of doxorubicin, which is to augment IL-12-induced NKG2D expression in CD8⁺T cells but not in NK or CD4⁺T cells. This observation was further validated by NK and CD8⁺T cell-depletion studies, in which only depletion of CD8⁺T cells abolished the expression of NKG2D in lymphocytes. The induced NKG2D expression in CD8⁺T cells is tightly associated with tumor-specific localization of CD8⁺T cells and improved antitumor efficacy.

The IL-12 plus doxorubicin treatment-induced antitumor efficacy is also due to NKG2D ligand Rae-1 induction in tumors. Rae-1 induction in tumors is a long term effect in multiple tumor models, but not in normal tissues. A novel CD8⁺T cell direct contact dependent mechanism accounts for Rae-1 induction *in vivo* and *in vitro*, and CD80 is the receptor through which CD8⁺T cells interplay with tumor cells to upregulate Rae-1 on tumor cells.

In summary, increased NKG2D expression in CD8⁺T cells in response to IL-12 plus doxorubicin was closely associated with tumor-specific localization of CD8⁺T cells and

greater antitumor efficacy of the combined regimen than either agent alone. NKG2D ligand Rae-1 induction is triggered by the interaction of CD80 on tumor cells with tumor infiltrating CD⁺8 T cells.

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Abbreviations

pCtrl	Control plasmid DNA
pIL-12 DNA	IL-12 plasmid DNA
NKG2D	Natural killer group 2
TIL	Tumor-infiltrating lymphocytes
Dox	doxorubicin
cy	cyclophosphamide
PBMCs	Peripheral blood mononuclear cells
PI3K	Phosphatidylinositol 3-kinases
TCR	T cell receptor
Rae-1	Retinoic acid induced early transcript
PAMPs	pathogen-associated molecular patterns
APCs	Antigen presenting cells
IL-12	Interleukin 12
MHC	Major histocompatibility complex
IFN	Interferon
ITAM motif	Immunoreceptor tyrosine-based activation motif

Chapter 1 Introduction

Background

Tumor immunosurveillance

In the tumor microenvironment, effector immune cells such as CD8⁺αβ T cells, natural killer (NK) cells, and macrophages bind circulating tumor cells, migrate to the microscopic tumor niche, and infiltrate tumors to lyse tumor cells and to suppress tumor progression and metastasis, which is referred as tumor immunosurveillance.

The immune system consists of innate and adaptive immune systems. When organisms are infected by bacteria or viruses, the innate immune system provides an acute but non-specific response. Most living creatures have an innate immune system. In vertebrates, the innate immune system can also activate the adaptive immune system, which generates responses that recognize specific antigens (1). Unlike the innate immune response, the response of the adaptive immune system lasts for a sustained period of time because it generates an immune memory for specific pathogens that enables the immune system to mount attacks on subsequent challenges (2).

Cells of the innate immune response include NK cells, γδ T cells, macrophages, dendritic cells, leukocytes, mast cells, phagocytes, neutrophils, basophils and eosinophils, etc. Innate immune cells recognize active groups of receptors called pathogen-associated molecular patterns (PAMPs) (3). After being taken up by the innate immune cells, with the assistance of cytokines and chemokines, antigens are processed and presented to T and B cells to activate the adaptive immune system. Unlike the innate immune system, cells in the adaptive immune system induce long-lasting antigen-specific immune responses against

non-self pathogens (4). The cell-mediated and antibody-mediated adaptive immune responses are carried out mainly by B and T lymphocytes. B cells are involved in the production of antigen-specific antibodies. T cells are categorized into $CD4^+$ T cells, $CD8^+$ T cells, and $\gamma\delta$ T cells based on cell-surface markers (Figure 1) (5). Naïve $CD4^+$ T cells become T_H0 cells after being activated by antigen-presenting cells (APC). T_H0 cells secrete interleukin 2 (IL-2), IL-4, and interferon gamma ($IFN\gamma$) (6). T_H0 cells respond to different cytokines and subsequently differentiate into T_H1 , T_H2 , T_H3 , T_H17 , and Treg cells. T_H1 cell proliferation is driven by IL-2, T-bet, and $IFN\gamma$ (7). T helper cells assist the $CD8^+$ T cytotoxic cell-mediated adaptive immune response and immune memory (8). The interaction between T cell receptor (TCR) on T cells and major histocompatibility complex class I (MHCI) on APCs and tumor cells, with the assistance of co-stimulatory receptors, can activate cytotoxic T cells (9). Active $CD8^+\alpha\beta$ T cells release $IFN\gamma$, perforin, and granzyme to kill target infected cells (10). Certain subpopulations of T and B cells can also convert to memory cells (11-13). T_H2 cell proliferation is stimulated by IL-4. T_H2 cells facilitate antibody-mediated adaptive immunity. Treg cells (T regulatory cells) are promoted by transforming growth factor beta ($TGF\beta$) and IL-10, which inhibits the immune response to prevent autoimmune diseases (14, 15). $TGF\beta$, together with IL-6, also facilitates T_H17 proliferation, which releases IL-17 to induce an inflammatory response and autoimmunity (16). The T_H3 cell population has recently been identified, but their function is still largely unknown.

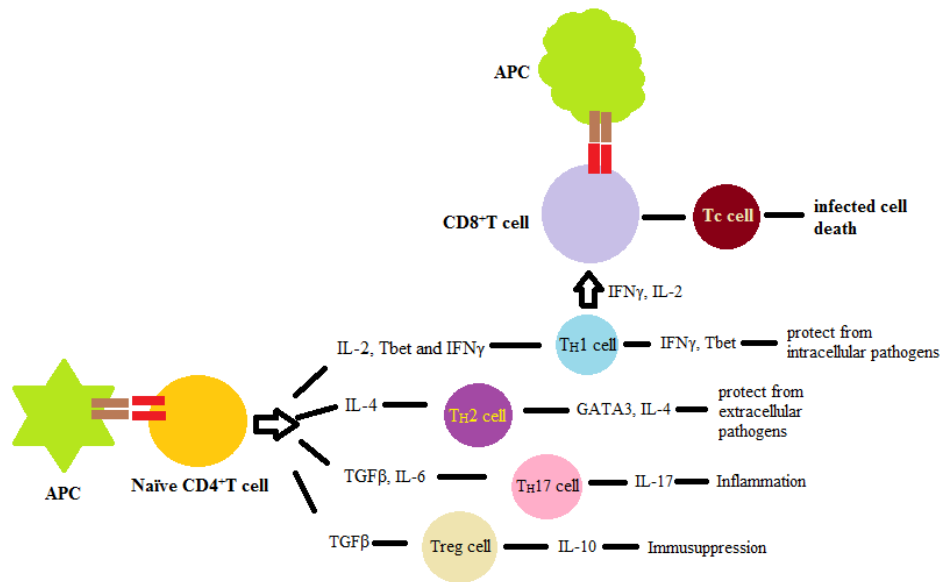


Figure 1 T cell classification. T cells are classified as CD4⁺T cells, CD8⁺T cells and $\gamma\delta$ T cells due to the cell surface markers. T cells can also be categorized as T_H1 cells, T_H2 cells, T_H3 cells, T_H17 cells, Treg cells and T cytotoxic cells based their function.

In this thesis, our interest will focus on tumor infiltrating T cells, because the number of tumor-infiltrating lymphocytes, especially CD8⁺ T cells, becomes a valuable prognostic marker of immunotherapy in many cancers (13, 17). In colorectal carcinoma (17), non-small cell lung cancer (18), ovarian cancer (19), , and uroepithelial carcinoma (20), an increased density of infiltrating CD8⁺ $\alpha\beta$ T cells as well as an enhanced level of IFN γ production in the tumor microenvironment suggest a positive prognostic value for tumor-free survival time. In ovarian carcinoma and breast cancer, T cell and APC levels become a marker of chemotherapy outcomes (21). In melanoma patients, the infiltrating NKG2D⁺ CD8⁺ $\alpha\beta$ T cells induced a dramatic anti-tumor response (22). Also, the presence of tumor-infiltrating lymphocytes in colorectal carcinoma (23), cutaneous melanoma (24), and breast cancer (25) has been shown to have a positive influence on the prognosis.

CD8⁺ $\alpha\beta$ T cells can recognize MHCI-expressing tumor cells, but many tumor cells are lost MHCI (26). Therefore, the interaction of NKG2D and NKG2D ligands mediates tumor

cell recognition and plays a critical role in tumor immunosurveillance to suppress the early stages of cell transformation (Figure 2) (27). The interaction between NKG2D and NKG2D ligands results in both an immune response and an immune escape (28). On one hand, NKG2D receptor expressed in immune cells, such as activated CD8⁺αβ T cells, NK cells, and γδ T cells, bind to various NKG2D ligands on tumor cells and trigger the phosphorylation of adapter protein DAP10/12, which recruits and activates the PI3K signaling pathway (28). The activated immune cells produce perforin and IFNγ to exert cytotoxicity against tumor cells (29). On the other hand, this NKG2D-NKG2D ligands interaction may cause NKG2D ligands to shed from tumor cells to block NKG2D function (30, 31). In cancer immunotherapy, strategies need to be investigated and developed to induce NKG2D receptor on immune cells in order to elevate the NKG2D-mediated immune response. Also, the tumor microenvironment may edit tumor cells to lose NKG2D-ligands (32). Therefore, it is also important to induce the expression of NKG2D-ligands in tumor cells when NKG2D⁺ effector cells are available to trigger tumor cell death.

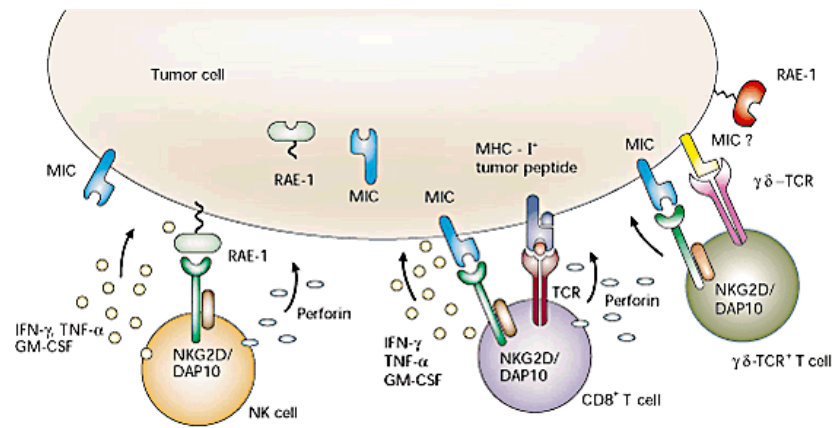


Figure 2 Over-expression of NKG2D ligands by transformed cells facilitates recognition by the immune system. In humans, NKG2D binds to MICA, MICB and ULBP. In mice, NKG2D binds to the RAE-1 family. NKG2D associates with the transmembrane-anchored adapter protein DAP10, which provides signal transduction by activating the PI3-kinase pathway. *NK cells, CD8⁺T cells and $\gamma\delta$ T cells kill tumors expressing NKG2D ligands and secrete cytokines.* *Nature Medicine* 7, 1178 - 1180 (2001)

NKG2D and NKG2D ligands

NK cell-activating receptors include NKp30, NKp44, NKp46, KIR2DS, CD94/NKG2C, NKG2D, CD16, and Ly49D/H. Most NK cell-activating receptors are transmembrane proteins with an intracellular domain lacking signaling activity. Therefore, NK cell-activating receptors always associate with adaptor proteins DAP-12 or DAP-10, which activate downstream cytotoxicity (Figure 3). The binding of activating receptors to their cognate ligands triggers cytotoxicity signaling, cytokine production, and cytolytic activity. As one of the NK cell-activating receptors, NKG2D belongs to the C-type lectin-like superfamily with a type II transmembrane-anchored dimer glycoprotein. NKG2D are expressed on all NK cells, NKT cells, $\gamma\delta^+$ T cells, some CD8⁺ $\alpha\beta^+$ T cells and on a small subset of CD4⁺ $\alpha\beta$ T cells (33, 34). The NKG2D-encoding gene is located at human chromosome 12 and mouse chromosome 6. NKG2D is found on all human CD8⁺ $\alpha\beta$ T cells

but only on active murine CD8⁺αβ T cells (35, 36). As part of tumor immunosurveillance, mouse NKG2D⁺ CD8⁺αβ T cells play a critical role in tumor cell recognition and the induction of TCR-independent anti-tumor cytotoxicity (37, 38). The NKG2D receptor on immune cells recognizes and binds to its non-MHCI ligands, leading to the phosphorylation of adaptor proteins DAP10 or DAP12 (33, 39). DAP10 contains a tyrosine-based (YINM) motif, which recruits Grb2 and activates PI3K signaling-mediated cytotoxicity (39). DAP12 has an ITAM motif (immunoreceptor tyrosine-based activation motif) that recruits and stimulates tyrosine kinases Syk/Zap70 signaling. There are two splice variants of NKG2D, a long variant (NKG2D-L) with an extension of 13 amino acids at the N terminus and a short variant (NKG2D-S). NKG2D-S associates with either DAP12 or DAP10, but NKG2D-L associates only with DAP10 (39). Both NKG2D-L and NKG2D-S are present on NK cells (39). NKG2D-L is primarily found in resting NK cells, and NKG2D-S is upregulated in active NK cells (27, 39). Therefore, NK cell signaling can be transduced through both DAP10 and DAP12. In contrast, resting T cells do not express NKG2D, and only the active CD8⁺αβ T cells display NKG2D (mainly NKG2D-S) (40). Because of the absence of DAP10 on T cells, the NKG2D signaling on CD8⁺αβ T cells can only be transmitted through DAP12 (40). In the tumor microenvironment, NKG2D activation inhibits tumor initiation. Myc transgenic mice with NKG2D knocked out had a much higher chance of developing spontaneous tumors than did mice with normal NKG2D function (41).

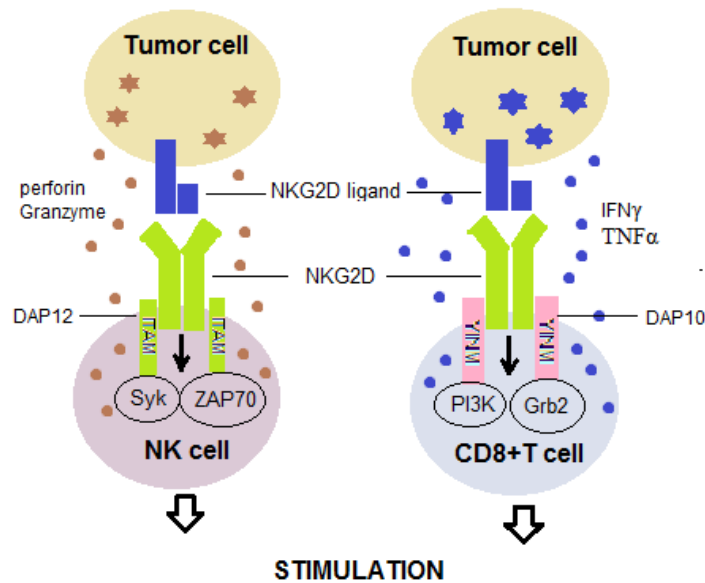


Figure 3 NKG2D signaling on NK and CD8⁺T cells. Binding to NKG2D ligands on tumor cells, NKG2D stimulates the downstream signaling by recruiting DAP12 which activates Syk and ZAP70 in NK cells, or by recruiting DAP10 which activates PI3K signaling. NKG2D signaling activation leads to cytokine-mediated cytotoxicity against tumor cells.

NKG2D binds to the cellular stress-inducible molecules NKG2D ligands. Human NKG2D ligands include major histocompatibility complex class I chain-related proteins A and B (MICA, MICB) (42) and the retinoic acid early transcript-1 (RAET1) family (43). Murine NKG2D ligands are classified into three families according to the protein homology: retinoic acid early inducible-1 (Rae-1, α - ϵ); histocompatibility antigen 60 (H60a-c); and murine ULBP-like transcript 1 (Mult1, only one member) (27, 44). Among all the murine NKG2D ligands, H60c and the Rae-1 family members have glycosylphosphatidylinositol (GPI) anchors, lacking cytoplasmic domains. The Rae-1 family contains distinct loci encoding polypeptides that share 92%-95% amino acid identity, anchored to the membrane via a GPI-linkage. The Rae-1 proteins are distantly related to MHCI and include only the α 1 and α 2 domains. All Rae-1 family members were shown to bind NKG2D to counteract immune invasion. Rae-1-positive cells were found to attract NKG2D⁺ CD8 T cells (45).

NKG2D ligands were reported with a very low level of expression in the normal tissues of adult mice (34); however, the expression can be upregulated in infected tissues or tumor cell lines. Normally, the NK inhibitory receptor ligands are expressed in the healthy cells to suppress NK cell cytotoxicity. However, these ligands are lost in the infected cells, and, instead, ligands for the NK cell-activating receptors, such as NKG2D ligands, are expressed in the infected cells to trigger immune response-mediated cytolytic activity. Additionally, Diefenbach *et al.* demonstrated that NKG2D ligands are highly expressed in the NK target cell line YAC-1, macrophage lines (RAW 309 Cr.1 and J774), a dendritic cell line (DC2.4), a colon carcinoma line (MC38), a B lymphoma line (A20), a prostate carcinoma line (TRAMP), T lymphomas lines (BW 5147, WEHI 7.1, and S49.1), and weakly expressed in the P815 mastocytoma cell line; and not expressed on 3T3 fibroblasts (27). In normal cells, NKG2D ligands are expressed in BALB/C mice CD4⁺CD8⁺ and CD4⁻CD8⁺ thymocytes but not on CD4⁺CD8⁻ thymocytes. NKG2D ligands were not found expressed in thymocytes from C57BL/6 mice. Other studies have shown that the Rae-1 family members are highly expressed in the brain tissue of embryos (46) but cannot be detected in healthy adult tissues.

NKG2D ligands are stimulated by heat shock, oxidative stress, DNA damage, oncogene exposure, viral infection, lipopolysaccharide (LPS), and chemicals. During early murine cytomegalovirus (MCMV) infection, Rae-1 α , β , and γ were found induced in BALB/C mice, whereas Rae-1 δ and ϵ were upregulated in C57BL/6 mice (47). H60a is detected in BALB/C but not in C57BL/6 mice, and H60b and c are expressed in both strains (48). In humans, MICA was found upregulated by bacteria (such as *E. coli*) infection (49). MICB can be induced by Influenza A or heat shock (50). MICs and RAET1 are expressed in various tumors, including leukemia, gliomas, melanomas, neuroblastomas, etc. (51, 52). Mouse and human NKG2D ligands can be increased by adenovirus infection (53, 54).

Chemicals reported to induce NKG2D ligands on tumor cells include proteasome inhibitors, HDAC inhibitors, valproic acid, and a variety of chemotherapeutic agents (30, 55-57). Several chemicals that have been shown to induce NKG2D ligands also affect post-translational events. For example, proteasome inhibitors prevent ubiquitination, HDAC inhibitors and valproic acid protect acetylation, and tunicamycin stabilizes glycosylation, suggesting that NKG2D ligands expression in tumor cells may be involved in multiple post-translational modifications. Chemotherapeutic agents induce NKG2D ligands through activating the DNA damage response pathway (58). In brief, the increase in NKG2D ligands is generally through stress-induced DNA damage signaling by activating ATM (Ataxiate, langiectasia, mutated)/ATR(ATM and Rad3-related) kinases that lead to the activation of checkpoint kinases chk1/chk2, which in turn switch on downstream factor p53 (59). Studies also show that NFκB activation is involved in NKG2D ligand upregulation (60). However, the DNA damage-signaling model can only explain NKG2D ligand induction in certain circumstances, and the precise mechanism of NKG2D ligand regulation still needs better definition.

It has been reported that mouse NKG2D ligands lead to tumor rejection in vivo: NKG2D-deficient mice had a much higher chance of developing cancer than did NKG2D normal mice (41). Under the stimulation of LPS, H60 or Rae-1 activates NKG2D receptor signaling in macrophages (27). Introducing NKG2D ligands Rae-1 or H60 to murine tumor cells effectively prevented tumor development in vivo (61). Immune cell depletion studies suggested that mouse NKG2D ligand-mediated tumor growth inhibition is dependent on NK or CD8⁺αβ T cells (62). Moreover, it was found that Rae-1 was induced by skin carcinogens but not on healthy skin, and skin-associated NKG2D⁺γδ⁺ T cells were found to exert cytotoxicity against the carcinoma cells (63).

In humans, NKG2D ligands were also shown to eliminate tumor cells. MICA was found to stimulate NKG2D-mediated cytotoxicity against target cells (64). MICA expression in tumor cells is positively correlated with NK cell cytotoxicity (65). While binding to ULBPs, NK cells are stimulated to produce multiple chemokines and cytokines such as tumor necrosis factor- α (TNF- α), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon- γ (IFN γ), macrophage inflammatory protein-1 β (MIP1- β), and IL-309. In addition, NKG2D ligand expression in RMA tumors extended the survival duration in mice (62). Colorectal cancer patients with high levels of NKG2D ligands were more likely to have a longer survival time than those with low levels of NKG2D ligands and the same grade of cancer (66). Data from several other groups also indicate that neuroblastoma and tracheal rhabdomyosarcoma patients with high levels of NKG2D ligands generally could survive longer than the patients with low levels of NKG2D.

Accumulating investigations suggest that the induction of NKG2D ligands boosts NKG2D-mediated tumor cell death (27, 43, 54, 67). We expect that developing a strategy to simultaneously boost induction of the NKG2D ligand in tumors and NKG2D expression in immune cells will greatly enhance the antitumor immune response and the treatment's antitumor efficacy.

co-stimulatory/co-inhibitory receptors play dual roles in antitumor immune response

Besides NKG2D-NKG2D ligands, other co-stimulatory/co-inhibitory receptors also engage in tumor immunosurveillance (Figure 4) (68-70). T cell activation usually requires the presence of co-stimulatory receptor interaction along with the TCR/MHC complex (71-73). Lymphocytes and APCs communicate through numerous stimulatory and inhibitory receptors/ligands on the cell surface. The interaction and signal transduction between receptors on immune cells and ligands on APCs is also known as an immune synapse (74).

On the surface of lymphocytes, stimulatory receptors TCR (which interacts with MHC), CD28 (which interacts with B7.1 and B7.2), CD40L (which interacts with CD40), inducible co-stimulator (ICOS, which interacts with ICOSL), CD27 (which interacts with CD70), OX40 (which interacts with OX40L), and 41BB (which interacts with 41BBL) transduce activated signals to immune cells to initiate immune responses (Figure 4) (75). Suppressive receptors such as programmed cell death protein 1 (PD-1, which interacts with PD-L1 and PD-L2) and cytotoxic T-lymphocyte antigen 4 (CTLA4, which interacts with B7.1 and B7.2) attenuate immune response (Figure 4) (75).

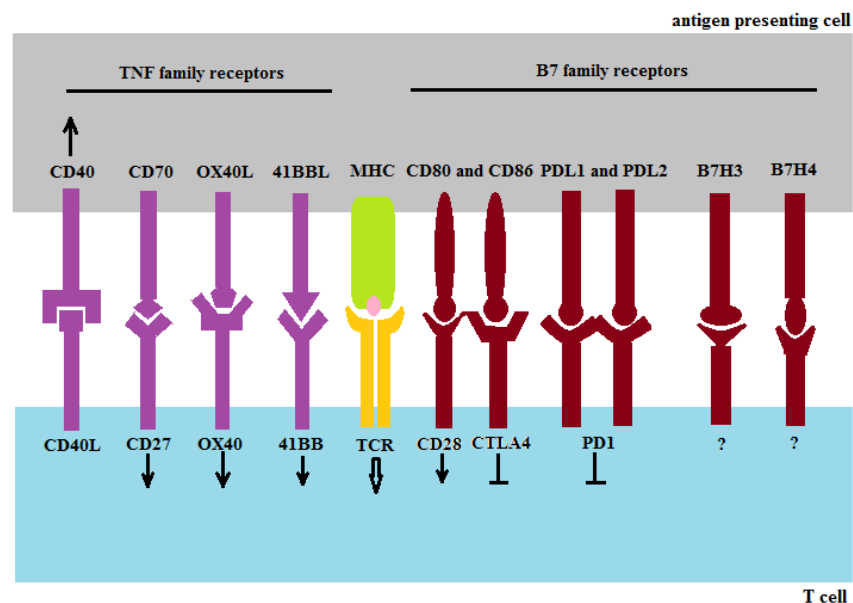


Figure 4 *Co-stimulatory/co-inhibitory receptors on antigen presenting cells.* B7 family receptors and TNF family receptors that display on antigen presenting cells interplay with corresponding receptors on T cells to transduce activatory or inhibitory signals.

Lymphocytes activation or regulation is through the “two signal” model. The first signal is the interaction between major histocompatibility complex (MHC) on APCs and T cell receptor on T cells. When APCs (including dendritic cells, macrophages, and B cells) digest and process pathogens, the fragments of pathogen peptides are delivered to the cell surface and presented to lymphocytes via MHC I or MHC II (76). The T cell receptor recognizes the

non-self peptides presented by MHC and in turn triggers the down-stream signaling for T cell augmentation and activation. However, this MHCI- and TCR-mediated primary signal is necessary but not sufficient for T cell activation. T cell activation also needs the co-stimulatory signal. Without the co-stimulatory signal, T cells cannot effectively respond but, instead, become anergic (77). CD28 was found to be a critical co-stimulatory receptor constitutively expressed in T cells. CD28 signaling can be stimulated by binding to the B7 family receptors CD80 (B7.1) and CD86 (B7.2), located on the surface of APCs and some tumor cells (77). The engagement of CD28 signaling in collaboration with TCR signaling facilitates the proliferation and activation of T cells in terms of cytokine production and T cell survival (78). CD80 (B7.1) forms a homodimer with a higher affinity to CD28 than to CD86 (B7.2) monomer (79).

Besides CD28, inhibitory receptor CTLA4 also recognizes CD80 (B7.1) and CD86 (B7.2) to suppress the immune responses (78). The CTLA4 receptor transmits a negative signal to recruit phosphatases and to upregulate indoleamine 2,3-dioxygenase (IDO) and tryptophan, which in turn inhibit TCR and MHC interaction-mediated immune cell proliferation (80). Unlike CD28, which is constitutively expressed in T cells, a very low level of CTLA4 is found on naïve T cells, but after T cell activation it can be immediately induced (81). Interestingly, the co-stimulatory receptor CD28 and the co-inhibitory receptor CTLA4 share the same ligands CD80 (B7.1) and CD86 (B7.2), although CD80 (B7.1) and CD86 (B7.2) show a much higher affinity for CTLA4 than for CD28 (81). However, since CD28, but not CTLA4, is constitutively expressed in immune cells, ectopic expression of CD80 (B7.1) and CD86 (B7.2) in tumor cells still induces immunogenic responses (82). In cancer cells, when CD80 (B7.1) was knocked down, the colon cancer cells failed to develop tumors in vivo in immunocompetent mice but not in immune deficient mice (83). This study also indicated that CD80 (B7.1) overexpression raises the immunogenicity of the tumor

cells via CD28 activation (83). This effect is very likely due to the fact that CD80 (B7.1) and CD86 (B7.2) have a higher affinity for the suppressive receptor CTLA4 than for the stimulatory receptor CD28. CD80 (B7.1) and CD86 (B7.2) at low expression levels are inclined to interact with CTLA4 to suppress immune response, but high levels of CD80 (B7.1) and CD86 (B7.2) trigger the positive signal to overwhelm the negative signal so as to induce the immune response. This tendency also explains why immature dendritic cells usually express low levels of CD80 (B7.1), which bind to the inhibitory receptor CTLA4 rather than to the stimulatory receptor CD28 (84, 85). CD80 and CD86 have been known to display and function in APCs, but their presence on tumor cells has not been thoroughly investigated. Although the roles of CD80 and CD86 on immune cell regulation have been thoroughly studied, how CD80 and CD86 affect tumor cellular signaling is still controversial.

Antitumor cytokines and their function on tumor immunosurveillance

Cytokines, molecules involved in cellular communication, stimulate the activation of both innate and adaptive immune systems. NKG2D can be upregulated by cytokines IL-2, IL-12, IL-15, IL-18, IL-21, and IFN α , or downregulated by TGF β and exogenous IFN γ (86). Cytokines can also activate NKG2D-NKG2D ligand interaction-induced immune cell cytotoxicity (87). Cytokines were also found to trigger immune cell antitumor effects by inducing the activation markers CD25 and CD69 on NK cells (88) and to stimulate the secretion of IFN γ in the tumor microenvironment (89). The promising cytokines in cancer immunotherapy include IL-2, IL-12, IL-15, and IL-21 (89). With the ability to proliferate and activate T cells and NK cells, IL-2 has been used to manage melanoma and other cancers (90). The IL-2/IL-15 signaling pathway was shown to promote CD8⁺ T cell proliferation and antitumor activity in ovarian cancer (91). IL-15 alone could enhance NK cell and CD8⁺ T memory cell activities in the B16 tumor model (92). Although soluble IL-

IL-21 has little effect on NK cells, it has recently been found that membrane-bound IL-21 could stimulate NK cell expansion *ex vivo* (93). More importantly, efficient NK cell proliferation greatly supports the adoptive NK cell transfer in cancer immunotherapy (94).

In our lab, we applied IL-12 along with other therapeutic agents to treat various cancers *in vivo* and observed very exciting results in preclinical studies. IL-12 belongs to IL-12 family together with IL-23, IL-27 and IL-35 (95, 96). Heterodimeric cytokine IL-12 comprises two subunits, p35 and p40 (Figure 5). IL-12 binds to the heterodimeric receptors IL-12R- β 1 and IL-12R- β 2 which is associated with T-cell activation (97, 98).

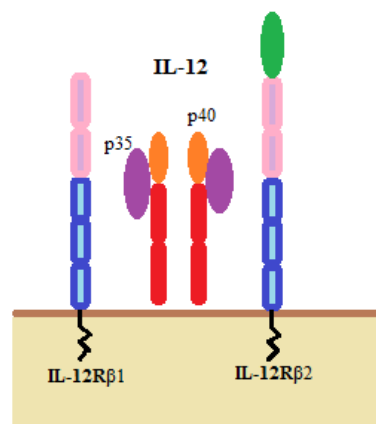


Figure 5 IL-12 and IL-12 receptor. IL-12 is composed of p35 and p40 subunits. IL-12 binds to IL-12 receptor to activate the signaling.

IL-12, produced mainly via APCs such as dendritic cells and macrophages, contributes to the innate and adaptive immune system communication (98). IL-12 production can be induced by bacteria, fungi, parasites, and CpG-containing oligonucleotides (99). Cytokines such as IFN γ and IL-4 form a positive feedback loop to enhance IL-12 production (100). However, IL-12 is inhibited by IL-10, which leads to immunosuppression. IL-12 is involved in the activation of both the adaptive and the innate immunity (100).

IL-12 stimulates T cell differentiation from naïve T cells into T_H1 or T_H2 cells to induce immune responses against pathogens (101). Like IL-2, IL-12 also stimulates TCR-CD3 as well as CD28 signaling to release IFN γ (102). IL-12 induces anti-angiogenic effect via IFN γ , which can augment the expression of anti-angiogenic effector chemokine IP-10 (inducible protein-10) or CXCL10 (103). As has been reported, IL-12, after binding to IL-12 receptor IL-12R- β 2, triggers tyrosine phosphorylation that recruits and activates TYK2 and JAK2 kinase activity, which in turn stimulates the JAK-STAT signaling pathway (Figure 6) (104, 105). IL-12 also leads to the activation of MKK6-p38 signaling, which contributes to the phosphorylation of transcriptional factor STAT4 (Figure 6) (106). Activated pSTAT4 translocates into the nucleus to induce another transcription factor Ets-related molecule (ERM), which is associated with the production of chemokine receptors CCR5, IL-18R, and IFN γ (107). STAT4-deficient mice have been reported to have a similar phenotype as IL-12-deficient mice (108).

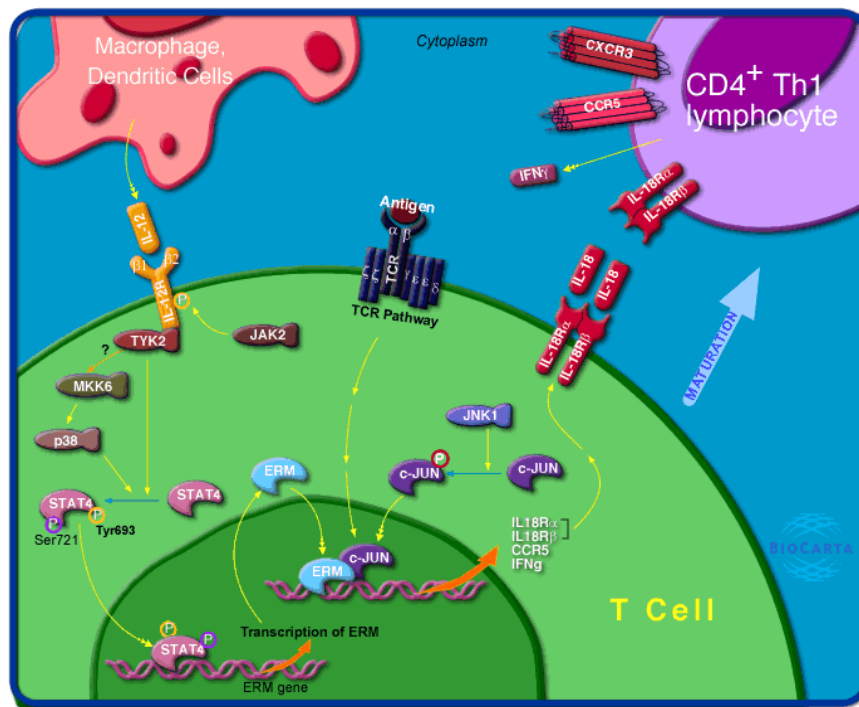


Figure 6 IL-12 signaling pathway. IL-12 signaling activates JAK-STAT signaling and MKK6-p38 signaling to induce STAT4-mediated chemokine production. http://www.biocarta.com/pathfiles/h_IL12Pathway.asp

Although IL-12 has little effect on NK cell proliferation, it can stimulate IFN γ as well as TNF α production in NK cells to enhance NK cell cytotoxicity. Several studies showed that IL-12 signaling increased the production of effector molecules IFN γ , perforin, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in NK92, NKL, and PB-NK, which substantially boosted the cytotoxicity of NK cells toward hematological and solid tumor cell lines (29, 65). When IL-12 binds to IL-12 receptors on NK cells (in the same way that it binds on T cells), it activates the downstream kinases JAK2 and TYK2 (109), triggering the phosphorylation of transcription factor STAT4. Dimerized STAT4 translocates into the cell nucleus to promote downstream gene transcription. NK cells can be stimulated by IL-12 signaling through the activation of p38 and ERK1/2 pathways (110, 111).

IL-12 has remarkable anti-tumor effects by activating both innate and adaptive immune cells including cytotoxic T cells (109), NK cells, and NKT cells (Figure 7). The production of IFN γ and other cytokines induced by IL-12 signaling not only has cytotoxicity against tumor cells but also, more importantly (112), promotes the expansion and maturation of T_H1 cytotoxic T cells, and tumor-targeting IgG antibodies (101). Recently, IL-12 was shown to stimulate immune responses especially CD8⁺T cells by inhibiting myeloid-derived suppressor cells (MDSCs), which, as suppressive APCs, create an immunosuppressive microenvironment in tumors (112). IL-12 also prevents tumor progression via the activation of NK cell cytolytic activity against tumor cells. Together with soluble NKG2D ligands, IL-12 seems to have great potential to enhance NK cell-mediated cytotoxicity to tumor cells in vivo in a perforin-dependent manner (29).

Some studies have suggested that IL-12 treatment might cause tumor rejection in an NKG2D-dependent manner. IL-12 treatment leads to NKG2D-dependent inhibition of MCA-induced sarcoma (113). Another study showed that the IL-12-induced immune cell cytotoxicity against tumor cells was dependent on NKG2D-NKG2D ligand interaction (29). Since IL-12 upregulates both NKG2D and NKG2D ligand ULBP (114), IL-12 could prevent tumor growing through an NKG2D-NKG2D ligand-mediated mechanism.

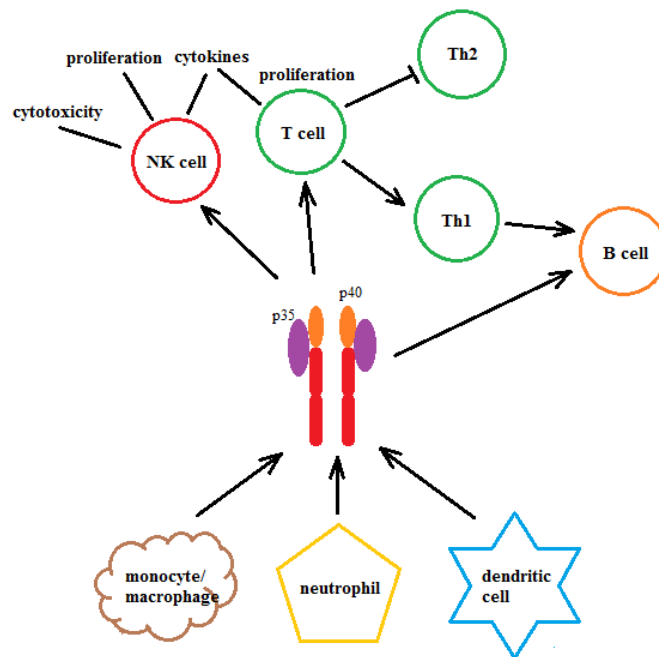


Figure 7 *IL-12 activates innate and adaptive immune systems.* IL-12 released by antigen presenting cells stimulates the proliferation and cytokine production of NK, T and B cells.

Antitumor drug doxorubicin induced antitumor immune response

Doxorubicin, an anthracycline antibiotic drug in cancer chemotherapy, has primarily been used to treat lymphomas, breast cancer, and sarcomas (115). Doxorubicin is able to intercalate into DNA, to inhibit DNA topoisomerase II, and to break DNA double strands (116). Previous studies suggested that doxorubicin not only can cause cytotoxicity and cytostasis responses to tumor cells but also can suppress the occurrence of metastasis (117). Besides, doxorubicin was demonstrated to suppress the TGF β -Smad3-signaling pathway in tumor cells by downregulating Smad2, Smad3, and Smad4 but upregulating the inhibitory protein Smad7, which blocks the transcription factor Smad3 from translocating into the nucleus (118). TGF β expression is usually increased in human cancers to promote tumor-cell proliferation and could be a potential novel mechanism of doxorubicin's ability to inhibit tumor growth.

Recent studies have also found that chemotherapeutic agents inhibit immunosuppressive effects in the tumor microenvironment. For instance, neoadjuvant taxane and doxorubicin treatment for breast cancer (119), cyclophosphamide treatment for early stage breast cancer (120), and trastuzumab treatment for HER2-positive breast cancer were shown to reduce the immunosuppressive Foxp3/Treg cell ratio in the tumor microenvironment (120).

Besides its effects on tumor cells and suppressive immune cells, doxorubicin can also stimulate the immune system to activate macrophages, to induce CTL activity, to enhance NK cell responses, and to increase IL-1 and IL-2 expression (121). Accumulating evidence suggests that chemotherapeutic agents may facilitate the infiltration of immune effector cells into tumors and may sensitize tumor cells to immune cell attack (122). Doxorubicin, in combination with IL-12, showed improved antitumor effects on murine melanoma, bladder carcinoma, and breast carcinoma (123, 124). The likely mechanism could involve NKG2D induction because chemotherapeutic agents could induce DNA damage-mediated NKG2D ligands expression increase on human fibroblasts (58). Likewise, MICA was upregulated in RPMI-8226 and SKO-007(J3) cell lines after doxorubicin treatment (125).

Chemotherapeutic agents, especially DNA damage agents, could activate DNA damage signaling, which in turn induces activation of NKG2D ligands and death receptors on tumor cells and sensitizes tumor cells to immune responses (125). Chemotherapeutic agents cisplatin and doxorubicin in cervical cancer treatment may induce mannose-6-phosphate receptors (MPR) in tumor cells, which could sensitize tumor cells to effector T cells' cytotoxicity (122).

In fact, the immune response has the potential to become a predictor of therapeutic outcome of chemotherapy in cancer patients (126). Supporting this potential are reports that immunocompetent tumor-bearing mice were much more susceptible to chemotherapy than

were immunodeficient mice (e.g., Rag2 knockout mice and IFN γ knockout mice) (127, 128). Some chemotherapeutic agents exhibit a more promising antitumor effect in patients who have tumor-infiltrating lymphocytes after treatment than in patients who lack tumor-infiltrating lymphocytes (129, 130). Cancer patients with severe lymphopenia usually have little response to chemotherapy (131). Despite these advances, the mechanism by which chemotherapy promotes immune responses, such as driving immune effector cells into the tumor microenvironment, is largely unknown.

In our earlier study, we discovered that compared with single treatment alone, the co-administration of IL-12 and doxorubicin induced more promising NKG2D function-dependent therapeutic effect (124). The co-administration effectively stimulates immune cell antitumor cytolytic activity *in vitro* (unpublished data), and also suppresses primary and metastatic tumor progression *in vivo* (124). However, NKG2D blocking antibody (clone C7) impairs the co-administration mediated *in vitro* and *in vivo* antitumor effect (unpublished data). The combination treatment also substantially induces IFN γ accumulation in tumors, which is possible due to a dramatic increase of immune cell infiltration into tumors, but no IFN γ level increase in blood and normal organs, and no IL-12 level increase in any organs is observed (124). Also, the co-administration of IL-12 and doxorubicin dramatically facilitates immune cells especially CD8 $^{+}$ $\alpha\beta$ T cells infiltrating into tumors (124). In addition, we demonstrated that the co-administration of IL-12 and doxorubicin had the anti-angiogenic effects to tumors through the upregulation of anti-angiogenic genes IP-10 and Mig. These antitumor effects could be reversed after depleting specific immune cells. We also showed that the co-administration of IL-12 and doxorubicin significantly suppressed tumor growth and vessel density in tumors in an IFN γ -stat1 signaling pathway dependent manner (124). However, it was still unclear about the underlying mechanism by which doxorubicin promotes IL-12-induced cytotoxicity against tumor growth and metastasis, and

how the co-administration facilitates CD8⁺T cells to localize into tumor microenvironment to enhance the tumor immunosurveillance.

Aims of the study

We have observed that doxorubicin improves IL-12-induced inhibition of tumor growth and even promotes CD8⁺T cell localization into tumors (124), but the underlying mechanism was poorly understood. Results from others suggested that IL-12 could induce NKG2D receptor at modest levels, therefore stimulating NKG2D-dependent tumor immunosurveillance (65). We also found that the antitumor effect induced by IL-12 plus doxorubicin is NKG2D function dependent. Several recent studies showed that doxorubicin boosts antitumor immune response in cancer treatment (126). We hypothesized that the co-administration-induced antitumor effect is due to the facilitation of NKG2D signaling dependent immune surveillance. Since both NKG2D receptor on immune cells and NKG2D ligands on tumor cells contribute to the NKG2D dependent tumor immunosurveillance, to illustrate the reason why the co-administration enhances the antitumor immunity, we investigated NKG2D receptor and ligand expression and function after the co-administration.

Aim one was to better understand whether doxorubicin can promote IL-12-mediated NKG2D induction. To address this question, we examined NKG2D expression level after each treatment, and also identified the crucial types of immune cells in which NKG2D receptor could be promoted by the co-administration.

Aim two was to illustrate the association between NKG2D expression level and tumor infiltrating immune cells. We examined NKG2D level in tumors after each treatment and

further determined the types of the tumor infiltrating immune cells as well as the NKG2D expression levels in such immune cells in the tumor microenvironment.

Aim three was to determine whether IL-12 and doxorubicin can induce Rae-1 expression in tumor cells. To identify the optimal strategy to induce Rae-1 in tumors, we tried various combinations of cytokines and chemotherapeutic agents. We also tested the duration of Rae-1 induction after each treatment. To ensure Rae-1 induction is tumor specific, we detected multiple normal tissues after each treatment.

Aim four was to illustrate the mechanism of Rae-1 induction by the co-administration of IL-12 and doxorubicin. To demonstrate Rae-1 induction is through the immune cell dependent mechanism, we established the *in vitro* model of Rae-1 induction. To identify the crucial types of immune cells that involve in Rae-1 induction, we conducted different immune cell subpopulation depletion and enrichment and then examined Rae-1 induction. To illustrate the required receptor through which immune cells interplay with tumor cells to induce Rae-1, we firstly detected the expression levels of each receptor on tumor cells, and then overexpressed or reduced CD80 expression on different cell lines to validate that CD80 plays the most important role in splenocytes-mediated Rae-1 induction.

Chapter 2

Materials and Methods

Rae-1 monoclonal antibody

The murine colon cancer cell lines CT26 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cell lines were grown in Dulbecco's modified Eagle medium supplemented with glutamine and 10% FCS, and 10 U/mL penicillin and streptomycin. Murine Rae-1 β gene (Open Biosystems) was subcloned into pBMNGreen fluorescent protein (GFP) plasmid (Phoenix Retrovirus Expression System). Retrovirus was produced by transfecting mRae-1 β /pBMN-GFP constructs into Phoenix eco packaging cells. Infect CT26 cells with retroviral containing supernatant derived from the transduced HEK293 cells. The transduction was confirmed by detecting GFP-expressing cells under the fluorescence microscope. Cell colonies with GFP expression from a single cell were picked and expanded. Further confirm Rae-1 expression using flow cytometry. Using this approach, both Rae-1 β /GFP and GFP-positive CT26 cells were obtained.

Cells were washed twice in PBS, counted and suspended in 100 μ l of sterile PBS, and then transferred in 0.5cc tuberculin syringe. 6-7 week old balb/c mice were injected with 3~5 $\times 10^6$ cells/foot in 50ul of volume to each foot. Immunize 6 times and at three days intervals. Isolate B cells from lymph node for fusion on day 18. One week before fusion, begin expansion of SP2/0-Ag14 myeloma cells in RPMI medium with 10% FBS in 10cm petri dish. On the day fusion is to be performed, SP2/0 cells must reach 1 $\times 10^8$ cells in density. Perform cell fusion. Once candidate hybridomas are identified, they are expanded and fed.

Coat EIA/RIA Costar plate with 20 $\times 10^6$ cells/plate overnight and allow it to dry then store it in -20 freezer until use. Wash with PBST (0.05% tween 20) 3 times. Block with

PBSTB (2%BSA) for 1 hour at RT. Add 100 μ l of culture supernatant, incubate 1 hour at room temperature. Wash with PBST 3 times. Add 100 μ l of goat anti-mouse IgG FC HRP (Jackson ImmunoResearch: 115-035-071) Incubate at RT for 1 hour. Wash 5 times with PBST, then add substrate. Absorbance is read at 450/620nm.

Gene construct and doxorubicin

The IL-12 DNA construct was purchased from Valantis, Inc. (Vilnius, Lithuania) as described in a previous publication (132). Plasmid DNA was prepared by using the endotoxin-free Mega preparation kit from Qiagen, Inc. (Valencia, CA) by following the manufacturer's instructions. Doxorubicin (Bedford Laboratories, Bedford, OH) was purchased from the pharmacy at the Louisiana State University or UT MD Anderson Cancer Center.

Tumor models and DNA delivery via intramuscular electroporation

BALB/C mice or C57BL/6 mice of age six to eight weeks, weighing 18-20 g were used for this study. The mice handling procedures were approved by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center.

Cell lines 4T1 (murine invasive breast carcinoma), CT26 (murine colon cancer), LLC (murine Lewis lung carcinoma), B16F10 (murine melanoma), K7M3 (murine osteosarcoma), LM8 (murine osteosarcoma lung metastasis), HCT116 (human colon cancer), CCH.OS.D (human osteosarcoma) were cultured in DMEM containing 10% FBS (Life Technologies, Grand Island, NY). 2×10^5 4T1, CT26, LLC, B16F10 and K7M3 tumor cells were subcutaneously inoculated into BALB/C mice 30 μ L PBS. Tumor volume was measured with calipers every 3 days, and calculated using the following formula: $V =$

$(\pi/8)a \times b^2$, where V = tumor volume in cubic centimeters, a = maximum tumor diameter, and b = diameter at 90° to a (133).

As in our previous publication (124), IL-12 encoding DNA and control DNA were injected via intramuscular injection into mouse tibia muscles by using electroporation (134). This procedure yields over 100 pg IL-12 protein in a week (135). The optimal electroporation parameters for intramuscular injection were set at 350 V/cm and 20 ms pulse for 2 pulses (134).

Mice received one of four standard treatments: control plasmid DNA alone (control DNA), doxorubicin plus control plasmid DNA (control DNA plus doxorubicin), IL-12 plasmid DNA alone (IL-12), or doxorubicin plus IL-12 plasmid DNA (doxorubicin plus IL-12). The treatments were administered twice, on days 6 and 16 after tumor cell inoculation. Mice were euthanized 4 days after the second treatment and their tissues subjected to the analyses described in subsequent sections. For each round of treatment, each mouse received 5 μ g DNA for each muscle, a total of 10 μ g DNA. Each doxorubicin treatment was 5mg/kg. doxorubicin was administered at the same time as the plasmid DNA.

CD8⁺T/NK cell depletion in vivo

CD8⁺T cell-depletion antibody (clone 2.43) and NK cell-depletion antibody (anti-Asialo GM1) were applied to deplete CD8⁺T cells and NK cells, respectively. Tumor-bearing BALB/C mice were inoculated intraperitoneally with one of the antibodies (50 μ g of antibody in 50 μ l PBS) on day 7 after tumor cell inoculation along with the first treatment. Injection of the antibody was repeated twice a week.

Flow cytometry analysis of NKG2D

Spleens from the treated mice were homogenized gently in a 40- μ m nylon strainer, and red blood cells were subjected to lysis with Puregene red blood cell lysis solution (Gentra Systems, Minneapolis, MN). Spleen cells (10,000 cells/sample) were treated with various antibodies to identify immune cell types: FITC-conjugated anti-mouse NKG2D or isotype control antibody (BioLegend, San Diego, CA); PE-Cy7-conjugated anti-mouse CD4 and anti-mouse CD8 antibody or their isotype control antibodies (Pharmingen, San Diego, CA); and FITC-conjugated anti-mouse NKp46 antibody or its isotype control antibody (eBioscience, San Diego, CA). NKp46 was recently identified as a NK cell marker (136). Anti-NKG2D C7 was generously provided by Dr. Wayne Yokoyama (Washington University School of Medicine). The stained cells were analyzed on an Attune acoustic focusing cytometer (Applied Biosystems, Inc., Carlsbad, CA). Data were analyzed by Attune (Applied Biosystems, Inc.) or Flowjo software (BD Biosciences, San Jose, CA). Lymphocytes were gated from total splenocytes for further analysis.

RNA isolation and Northern blot analysis of gene expression

RNA was isolated from tumors with TRIzol reagent (Invitrogen, Carlsbad, CA) as described previously (137). Northern blot analysis was performed as a previous publication (138). 4 μ g of total RNA was subjected to denaturing agarose gel electrophoresis, and ribosomal RNA was examined as the loading control of all samples. The Northern blot was scanned using a Molecular Imager (Bio-Rad, Hercules, CA). The signal intensity was normalized to the level of the total ribosomal RNA.

Immunocytochemical analysis.

Frozen tumor sections were fixed with cold acetone, acetone plus chloroform (1:1), and acetone. Tissue sections were blocked with blocking buffer (5% normal horse serum and 1% normal goat serum in PBS) and incubated with NKG2D-biotin antibody (1:50) (R&D Systems) overnight at 4°C. The next day, tissues were blocked and incubated with streptavidin conjugated goat anti-mouse Alexa flour 594 (Invitrogen) for 1 h at room temperature. Tissue sections were then blocked with normal rat serum (Jackson Immuno) for 1 h and F(ab')₂ fragment goat anti Rat (Jackson Immuno) for 1-2 h. After the blocking steps, tissues were incubated with second primary antibody (rat anti-mouse CD8, rat anti-mouse CD4 or rat anti-mouse NKp46) overnight at 4°C and second secondary antibody goat anti-mouse Alexa flour 488 (Invitrogen) for 1 h at room temperature. Rat IgG was used as the negative control. Nuclear was counterstained with Hoechst. Tumor sections were mounted in antifade fluorescence mounting media. Slides were visualized with fluorescence microscope with appropriate filters.

Western blot assay

Tissue samples from tumor-bearing mice were flash frozen in liquid nitrogen. Frozen tissues were smashed and placed in 0.4mL ice-cold lysis buffer with 5-8 beating beads. Tissue samples were dissociated by bead beater for 1 minute and centrifuged for 20 minutes at highest speed in 4°C. Supernatant was placed in a new tube and total protein concentration was measured by using BCA protein assay kit (Thermo scientific). 60 µg of the total protein were separated by 10% SDS-polyacrylamide gel electrophoresis, then transferred to nitrocellulose membranes by using iblot gel transfer device (Invitrogen). Membranes were developed by using WESTERN LIGHTNING Plus-ECL Oxidizing Reagent Plus. Rae-1 antibody was acquired and purified from hybridoma as described in Appendix. Antibody for beta-actin was purchased from santacruz. Antibody for B7.1 was purchased from R&D.

Immune cells enrichment

Spleens taken from mice euthanized by CO₂ were homogenized gently in a 40- μ m nylon strainer, and red blood cells were lysed with Puregene RBC lysis solution (Gentra Systems). NK, CD4 T and CD8⁺T cells were enriched by using Easysep mouse NK, CD4 T or CD8⁺T cell enrichment kits (Stem cell technology). Add cocktail of biotinylated antibodies to single cell suspension at 50 μ L/mL of cells and incubate at room temperature for 15 minutes. Add Biotin Selection Cocktail at 200 μ L/mL cells and incubate at room temperature for 15 minutes. Add the magnetic particles at 200 μ L/mL and incubate at room temperature for 10 minutes. Add recommended medium to the cell suspension to a total volume of 2.5 mL. Place the tube into the magnet for 5 minutes. Pick up the Magnet, and pour off the desired fraction into a new tube. The magnetically labeled unwanted cells will remain bound inside the original tube, held by the magnetic. Leave the magnet and the tube in inverted position for 2-3 seconds, then return to upright position. Place the new tube containing the desired enriched cells inside the magnet to perform a second round of magnetic separation.

DNA transfection

LM8 cells (1×10^6) are harvested and washed in PBS. Resuspend the cell pellet carefully in 100 μ l room temperature Nucleofector™ Solution V (Lonza Group Ltd) per sample. Combine 100 μ l of cell suspension with 3 μ g DNA pVC1157 vector or CD80 pVC1157. Transfer cell/DNA suspension into certified cuvette. Select Nucleofector™ Program T-020 (Nucleofector™ 2b Device). Take the cuvette out of the holder once the program is finished. Add ~500 μ l of the pre-equilibrated culture media to the cuvette and gently transfer the sample immediately into the culture dish.

siRNA transfection

CT26 cells (1×10^6) are harvested and washed in PBS. Resuspend the cell pellet carefully in 100 μ l room temperature Nucleofector™ Solution V (Lonza Group Ltd) per sample. Combine 100 μ l of cell suspension with 100 pmol sham siRNA or CD80 siRNA (sense: GAAUUACCUUGGCAUCAUA[dT][dT]; antisense: UAAUUGAUGCCAGGUAAUUC[dT][dT]). Transfer cell/DNA suspension into certified cuvette. Select Nucleofector™ Program T-016 (Nucleofector™ 2b Device). Take the cuvette out of the holder once the program is finished. Add ~500 μ l of the pre-equilibrated culture media to the cuvette and gently transfer the sample immediately into the culture dish.

MTT assay

Cells were plated in 96 well plates (1×10^3 /well, 5 replications for each time point) for 24, 48, 72, 96 and 120 hours, respectively. 20 μ L of 5 mg/mL MTT solution was added to the culture medium in each well and incubated for 5 h, followed by 200 μ L of stopping reagent dimethyl sulfoxide (DMSO). The light absorbance was read at 540 nm using a spectrometer.

Statistical analysis

The directly measured outcomes were analyzed by using the 2-sided Student *t*-test to compare 2 treatments or one-way ANOVA to compare more than 2 treatments. Graphpad software was used to determine the statistical significance. *P* values < 0.05 shows statistically significant.

Chapter 3 IL-12 plus doxorubicin augments NKG2D positive CD8⁺T cells from tumor-bearing mice.

Rationale and hypothesis

NKG2D receptor, as a critical mediator in tumor immunosurveillance, is mainly expressed on NK cells and CD8⁺T cells (33). Since most tumor cells are missing MHC I which presents antigen fragments to activate T cells, inducing NKG2D receptor so as to promote NKG2D-NKG2D ligand interaction could be a novel strategy to enhance tumor immunosurveillance against tumor progression. Our previous publication suggests that IL-12 plus doxorubicin treatment induces much more promising NKG2D-dependent antitumor therapeutic effect than IL-12 or doxorubicin treatment alone (124). We also demonstrated the co-administration of IL-12 and doxorubicin dramatically promotes CD8⁺T cells infiltration into tumors (124). CD8⁺T cells seemed to be the most crucial subtype of effector immune cells with cytolytic activity against tumor cells after the co-administration. However, it remained unclear about the mechanism by which the co-administration could better suppress tumor progression, and CD8⁺T cells were able to massively localize into tumors. Results from another group suggest that IL-12 induces NKG2D receptor on lymphocytes at a modest level (65). Also, IL-12 and doxorubicin are known to induce antitumor immune response. Therefore, the mechanism of the co-administration could be immune response dependent via the induction of NKG2D positive lymphocytes.

Here, we hypothesize IL-12 plus doxorubicin treatment leads to the increase of NKG2D positive lymphocytes, which mainly occurs on specific subpopulations of immune cells.

Results

IL-12 plus doxorubicin co-administration increases NKG2D positive lymphocytes

To test our hypothesis that IL-12 plus doxorubicin could increase NKG2D positive lymphocyte population, we prepared splenocytes from tumor-bearing BALB/C mice that were treated with control DNA, control DNA plus doxorubicin, IL-12 alone, or doxorubicin plus IL-12, and the levels of NKG2D expression in splenic lymphocytes were determined (Figure 8). All the DNA administrations were followed by electroporation for gene delivery to systemically generate IL-12 protein. The IL-12 protein level can retain to 10 days, and we gave the two treatments 10 days apart. These four standard treatments were used in the entire study as described in the Materials and Methods. Splenocytes were stained with isotype control antibody or NKG2D receptor antibody. We selected the lymphocytes population and identified NKG2D receptor level via flow cytometry. Lymphocytes from control groups (control DNA and control DNA plus doxorubicin) had very small percentages of lymphocytes (1-2%) expressing NKG2D and at low levels (Figure 8). IL-12 alone induced less than a 2-fold increase in the percentage of lymphocytes expressing NKG2D, whereas doxorubicin plus IL-12 induced a 7-fold increase over control DNA and a 4-fold increase over IL-12 alone (Figure 8). This finding suggests, notably, that doxorubicin greatly augments IL-12-mediated NKG2D expression on lymphocytes.

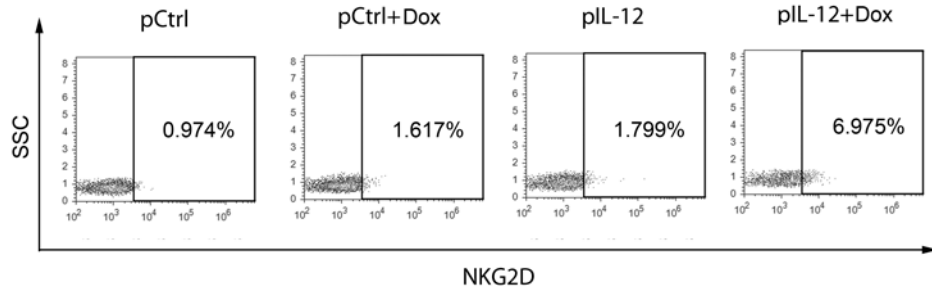


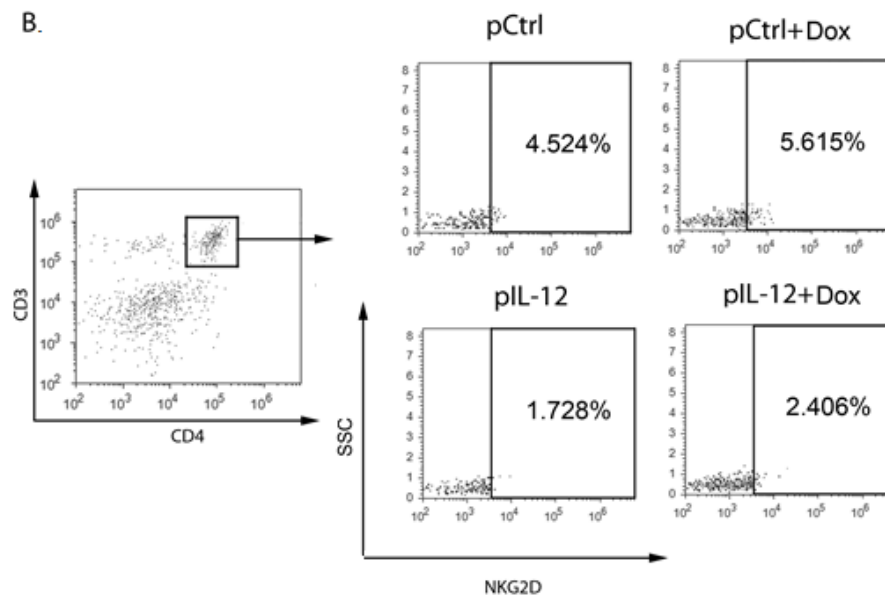
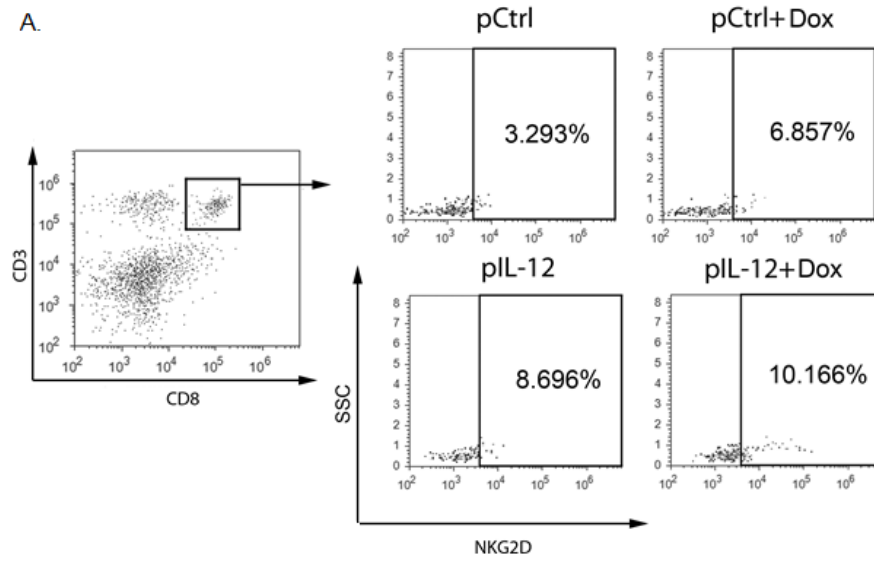
Figure 8 *Induction of NKG2D positive lymphocytes by IL-12 plus doxorubicin in vivo.* pCtrl, pIL-12, and doxorubicin represent control plasmid DNA, IL-12-encoding plasmid DNA, and doxorubicin, respectively. Tumor-bearing BALB/C mice were coadministered doxorubicin and plasmid DNA via intraperitoneal injection and intramuscular electroporation, respectively. A total of 2 administrations were performed with 10 days apart. The doses of DNA and doxorubicin were 10 μ g and 5 mg/kg per mouse, respectively. Spleens were collected 4 days after the second administration. Splenocytes were isolated and stained with FITC-NKG2D. Flow cytometry assay was performed to gate lymphocytes and then analyze NKG2D-positive lymphocytes. Data shown are representative of 3 independent experiments.

The co-administration of IL-12 plus doxorubicin mainly augments NKG2D positive CD8⁺T cells

Our previous result suggested that NKG2D induction occurred on only certain type(s) of lymphocytes instead of the total lymphocytes (Figure 8). Our next aim was to identify on which types of lymphocytes NKG2D could be induced by the co-administration. To determine the types of immune cells, splenocytes from the treated mice (including mice from all the control groups) were stained with CD3/CD8/NKG2D (Figure 9A), CD3/CD4/NKG2D (Figure 9B), or NKp46/NKG2D (Figure 9C) antibodies to examine NKG2D on CD8⁺T cells (Figure 9A), CD4⁺T (Figure 9B) cells or NK cells (Figure 9C), respectively, and the NKG2D-positive cells were identified via flow cytometry. We first gated CD3CD8-positive, CD3CD4-positive, and NKp46-positive lymphocytes, respectively, and then examined the percentage of NKG2D-positive cells in the gated cells by selecting NKG2D-positive CD8⁺T, CD4⁺T, and NK cells, respectively (Figure 9). Previously published results showed that NKG2D is constitutively expressed on NK and activated CD8⁺T cells (27, 139, 140). In our results, only NKG2D positive CD8⁺T cell population was increased, primarily in those treated with doxorubicin plus IL-12 (Figure 9A). IL-12 seemed to inhibit NKG2D expression in both NK and CD4⁺T cells (Figures 9B, 9C). This result was consistent in at least three repeated experiments. The induction of NKG2D in CD8⁺T cells lasted for at least 15 days after the second treatment (unpublished data).

To validate the possibility that doxorubicin plus IL-12 increases NKG2D positive CD8⁺T cells but not on other types of immune cells, we determined the NKG2D expression in NK or CD8⁺T cell-depleted mice by using depleting antibodies (Figures 10,11). The working hypothesis was that only depleting CD8⁺T cells would eliminate NKG2D positive cells in splenocytes if CD8⁺T cells were the true primary cell type in which NKG2D was induced by doxorubicin plus IL-12. To test this hypothesis, we depleted NK cells (Figure

10) or CD8⁺T cells (Figure 11) from tumor-bearing BALB/C mice by using depleting antibodies, and then administered the same four treatments already described. The splenocytes harvested from these mice were subjected to the same flow cytometry analysis as described for wild-type mice (Figure 9). As shown in Figure 10A and Figure 11A, NK cell-depletion antibody and CD8⁺T cell-depletion antibody effectively removed NK and CD8⁺T cells, respectively. In the NK cell-depleted mice, NKG2D positive lymphocytes and CD8⁺T cells were increased by IL-12 plus doxorubicin treatment(Figure 10B, 10C), but not on CD4⁺T cells (Figure 10D). This result was the same as the result from mice in which NK cells were present (Figure 9). This result clearly shows that NKG2D induction by IL-12 plus doxorubicin is independent of NK cells, and NK cell depletion does not affect NKG2D induction on CD8⁺T cells. In the CD8⁺T cell-depleted mice, no increase of NKG2D positive lymphocytes(Figure 11B), CD4⁺T cells (Figure 11C) or NK cells (Figure 11D)was observed after IL-12 plus doxorubicin treatment. This result validates the conclusion that the increase of NKG2D positive lymphocytes by IL-12 plus doxorubicin occurs primarily on CD8⁺T cells.



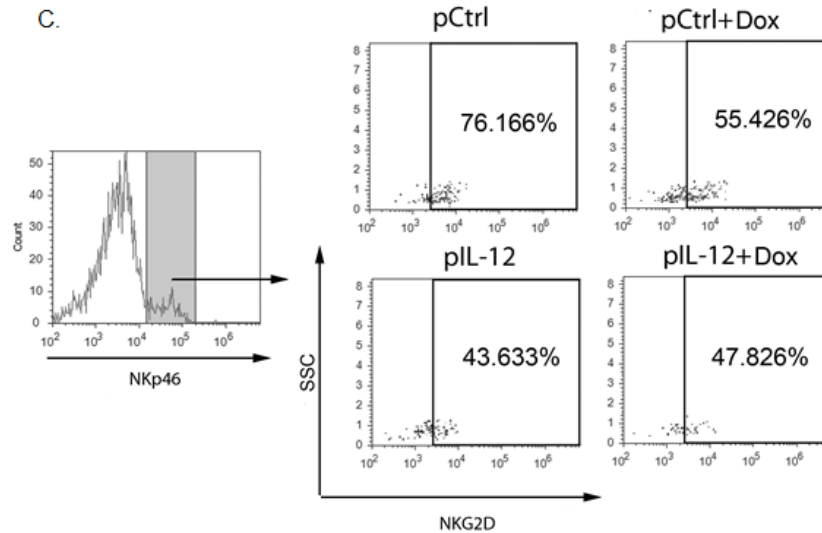
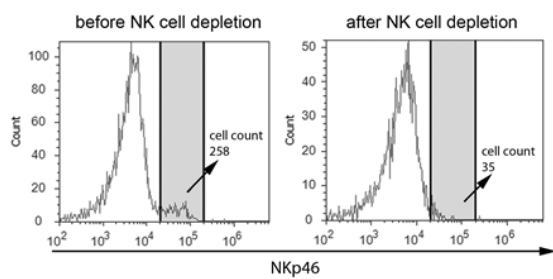
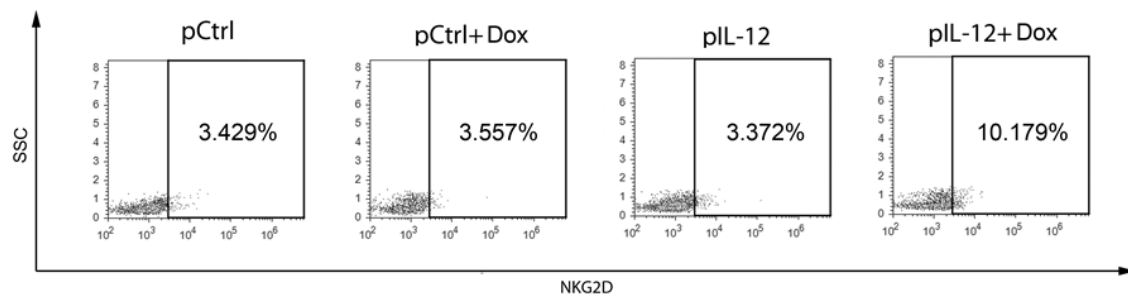


Figure 9 *CD8⁺T cell specific NKG2D induction by the co-administration of IL-12 and doxorubicin.* Tumor-bearing BALB/C mice were administered control DNA, control DNA plus doxorubicin, IL-12 DNA, IL-12 DNA plus doxorubicin as described in Figure 1. In each group, n = 5. Tumor-bearing mice were euthanized on day 4 after the second treatment and splenocytes were collected. In each group, splenocytes of five mice were mixed. **(A)** NKG2D expression in CD8⁺T cells. Splenocytes were collected and stained with anti-mouse NKG2D-FITC, anti-mouse CD8-PE-Cy7 and anti-mouse CD3-PE antibodies or cognate isotype control antibodies. Flow cytometry was performed to select CD3 and CD8 positive lymphocytes, and then further gated to determine the NKG2D level on these CD3CD8⁺T positive cells from the different treatments. **(B)** NKG2D expression in CD4⁺T cells. Splenocytes were collected and stained with anti-mouse NKG2D-FITC, anti-mouse CD4-PE-Cy7 and anti-mouse CD3-PE antibodies or cognate isotype control antibodies. Flow cytometry was performed to select CD3 and CD4 positive lymphocytes, and then gated to determine the NKG2D level on CD3CD4 positive cells. **(C)** NKG2D expression in NK cells. Splenocytes were collected and stained with anti-mouse NKG2D-FITC, anti-mouse NKp46 followed by efluor 710 anti-rat secondary antibody or corresponding isotype control antibodies. Flow cytometry was performed to select NKp46 positive lymphocytes, and then gated to determine the NKG2D positive cells on the gated NK cells.

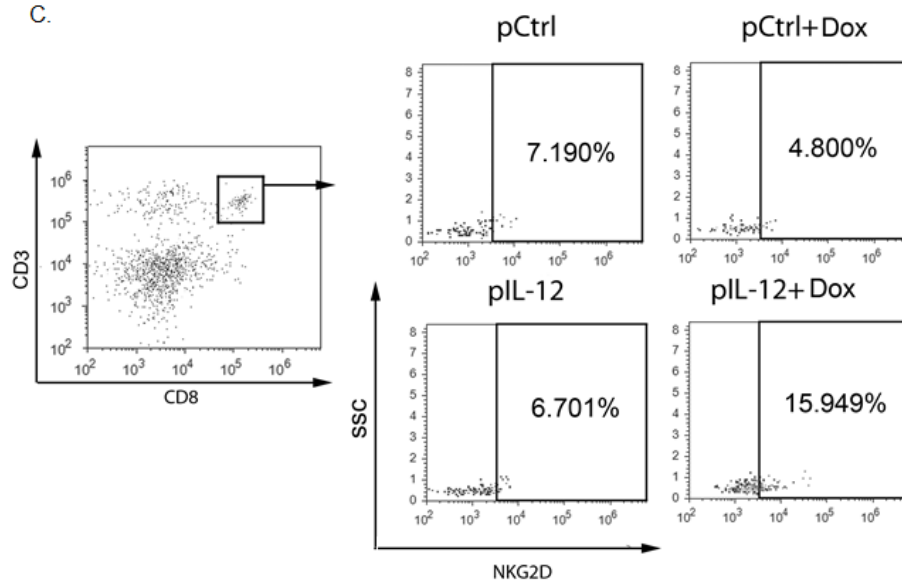
A



B



C.



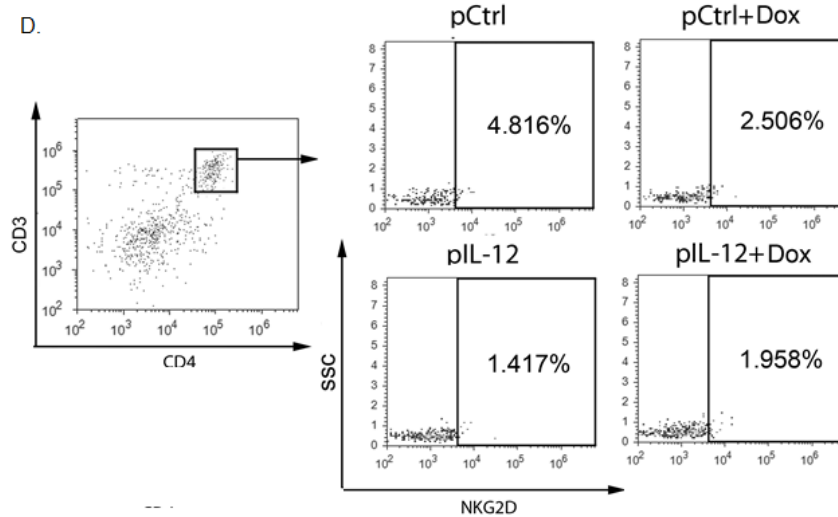
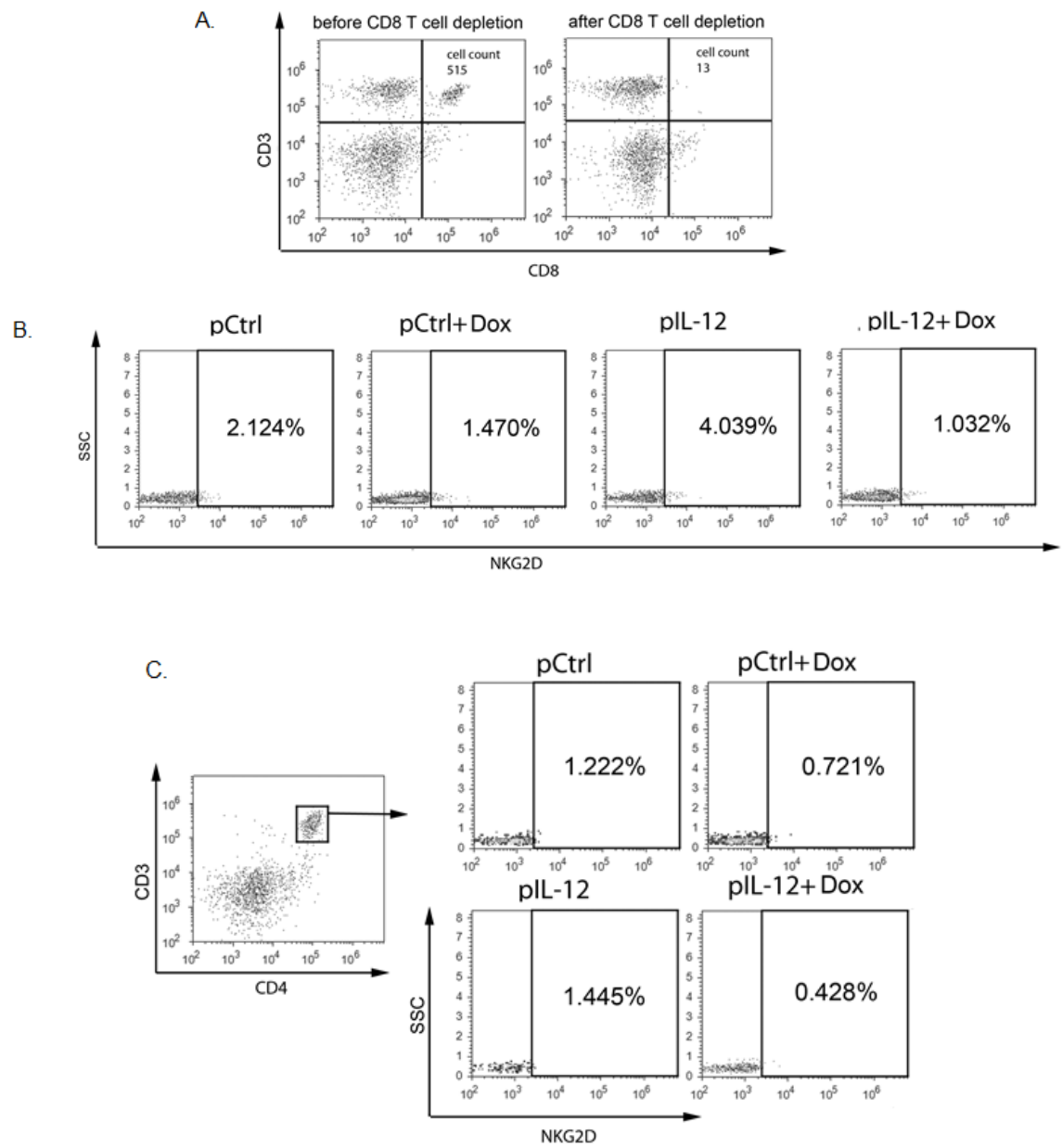


Figure 10 Analysis of NKG2D expression from NK cell-depleted tumor-bearing mice. Control DNA, control DNA plus doxorubicin, IL-12 DNA, or IL-12 DNA plus doxorubicin was administered to tumor-bearing BALB/C mice as described in Figure 2, $n = 3$. (A) NK cells depletion on NKG2D expression on CD8⁺T cells. Each tumor-bearing BALB/C mouse received 50 μ g of NK cell depletion antibody (anti-Asialo GM1) in a volume of 50 μ L PBS via intraperitoneal injection. The NK depletion was initiated at the same time as the first therapeutic treatment was applied to the mice; the antibody was administered twice per week. Splenocytes were collected and stained with NKp46 to confirm NK cell depletion. (B) Analysis of NKG2D induction on total lymphocytes by different treatments after NK cell depletion. Splenocytes were collected and stained with anti-mouse NKG2D-FITC or isotype control antibodies to determine the level of NKG2D induction using flow cytometry. (C) NKG2D expression in CD8⁺T cells in NK cell-depleted mice. Splenocytes were collected and stained with anti-mouse NKG2D-FITC, anti-mouse CD8-PE-Cy7 and anti-mouse CD3-PE antibodies or corresponding isotype control antibodies. Flow cytometry was performed to select CD3 and CD8 positive lymphocytes, and then gated to determine the NKG2D level on these CD3CD8 positive cells. (D) NKG2D expression on CD4 T cells. Splenocytes were collected and stained with anti-mouse NKG2D-FITC, anti-mouse CD4-PE-Cy7 and anti-mouse CD3-PE antibodies or corresponding isotype control antibodies. Flow cytometry was performed to select CD3 and CD4 positive lymphocytes, and then gated to determine the NKG2D level on these CD3CD4 T positive cells.



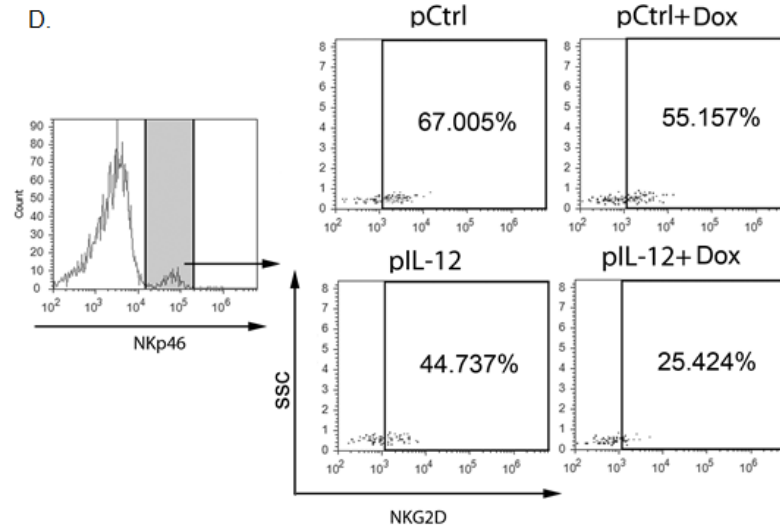


Figure 11 Analysis of NKG2D expression from CD8⁺T cell-depleted tumor-bearing mice. Control DNA, control DNA plus doxorubicin, IL-12 DNA, or IL-12 DNA plus doxorubicin was administered to tumor-bearing BALB/C mice as described in Figure 2. Different from Figure 2, these mice were also subjected to CD8⁺T cell depletion. *n* = 3. (A) Effect of CD8⁺T cells depletion on NKG2D detection. Tumor-bearing BALB/C mice were treated with 50 μ g of CD8⁺T cell depletion antibody (clone 2.43) in a volume of 50 μ L PBS via intraperitoneal injection. The depletion was initiated at the same time as the first therapeutic treatment; the antibody was administered twice per week. Splenocytes were collected and stained with CD3-PE and CD8-PE-Cy7 to confirm CD8⁺T cell depletion. (B) Analysis of NKG2D induction on lymphocytes by different treatments after CD8⁺T cell depletion. Splenocytes were collected and stained with anti-mouse NKG2D-FITC or isotype control antibodies to check for NKG2D level using flow cytometry. (C) NKG2D expression in CD4⁺T cells. Splenocytes were collected and stained with anti-mouse NKG2D-FITC, anti-mouse CD4-PE-Cy7 and anti-mouse CD3-PE antibodies or corresponding isotype control antibodies. Flow cytometry was performed to select CD3 and CD4 positive lymphocytes, and then gated to determine the NKG2D level on these CD3CD4 positive cells after the depletion of CD8⁺T cells. (D) NKG2D expression in NK cells. Splenocytes were collected and stained with anti-mouse NKp46 antibody or corresponding isotype control antibody. Flow cytometry was performed to select NKp46 positive lymphocytes, and then gated to determine the NKG2D level on NKp46 positive cells after the CD8⁺T cell depletion.

Summary

To investigate the possible mechanism by which the co-administration of IL-12 and doxorubicin effectively prevents tumor progression, we tested NKG2D receptor level on lymphocytes after the second treatment of control DNA, control DNA plus doxorubicin, IL-12 DNA or IL-12 DNA plus doxorubicin. Our results suggested IL-12 could induce NKG2D receptor at a modest level on lymphocytes, and IL-12 plus doxorubicin treatment could dramatically increase NKG2D positive lymphocytes (Figure 8).

The next question we studied was on which subtypes of immune cells NKG2D receptor was induced after the co-administration. We stained lymphocytes with different immune cell markers: CD3CD8, CD3CD4 or Nkp46 to examine CD8⁺T cells, CD4⁺T cells, and NK cells, respectively (Figure 9). We identified the NKG2D receptor level on different subtypes of immune cells after each treatment. We discovered that the co-administration of IL-12 and doxorubicin induces NKG2D receptor positive CD8⁺T cells by 3 fold than control DNA treatment (Figure 9A), and the induction could maintain up to 15 days after the second treatment. We could not detect any NKG2D induction on CD4⁺T cells or NK cells (Figure 9B, 9C). To confirm this observation, we performed the treatments on tumor-bearing NK cell-depleted or CD8⁺T cell-depleted mice (Figure 10, 11). We found the depleting NK cells could not affect the co-administration-induced NKG2D (Figure 10). However, we failed to detect any NKG2D induction in mice lacking mature CD8⁺T cells (Figure 11).

Here, we concluded doxorubicin could augment IL-12-induced NKG2D receptor on CD8⁺T cells in tumor-bearing mice. CD8⁺T cells are needed for NKG2D induction by co-administration.

Chapter 4 IL-12 plus doxorubicin treatment facilitates NKG2D positive immune cells to localize in tumors

Rationale and hypothesis

The number of tumor infiltrating lymphocytes in tumor microenvironment becomes a valuable prognostic marker of immunotherapy in many cancers (141-144). After immunotherapy, lymphocytes localize into tumor microenvironment and recognize specific tumor cell markers on the cell surface. The interaction between tumor cells and immune cells stimulates immune cells' antitumor cytotoxicity, and in turn eliminate tumor cells.

In our previous publication, we noticed that after the co-administration of IL-12 plus doxorubicin, there was an obvious increase of CD8⁺T cells in tumor sections, suggesting that the co-administration facilitates CD8⁺T cells infiltration into tumors. We also detected a significant increase of IFN γ in tumors, but not in blood, which means the infiltrating CD8⁺T cells exert cytotoxicity against tumor cells. In Chapter 3, we discovered a dramatic increase of NKG2D positive CD8⁺T cells after the co-administration of IL-12 and doxorubicin. We next needed to determine whether the IL-12 plus doxorubicin treatment also promotes NKG2D positive immune cell infiltration into tumors and what subpopulations of immune cells are the tumor infiltrating NKG2D positive cells.

Therefore, we hypothesized that increased expression of NKG2D may contribute to the increased infiltration of immune cells in tumors receiving IL-12 plus doxorubicin.

Results

IL-12 plus doxorubicin treatment facilitated NKG2D⁺ CD8⁺T cells infiltrating into tumors

To test this hypothesis, we needed to demonstrate that increased infiltration of immune cells into tumors is dependent on the presence of NKG2D on the immune cells.

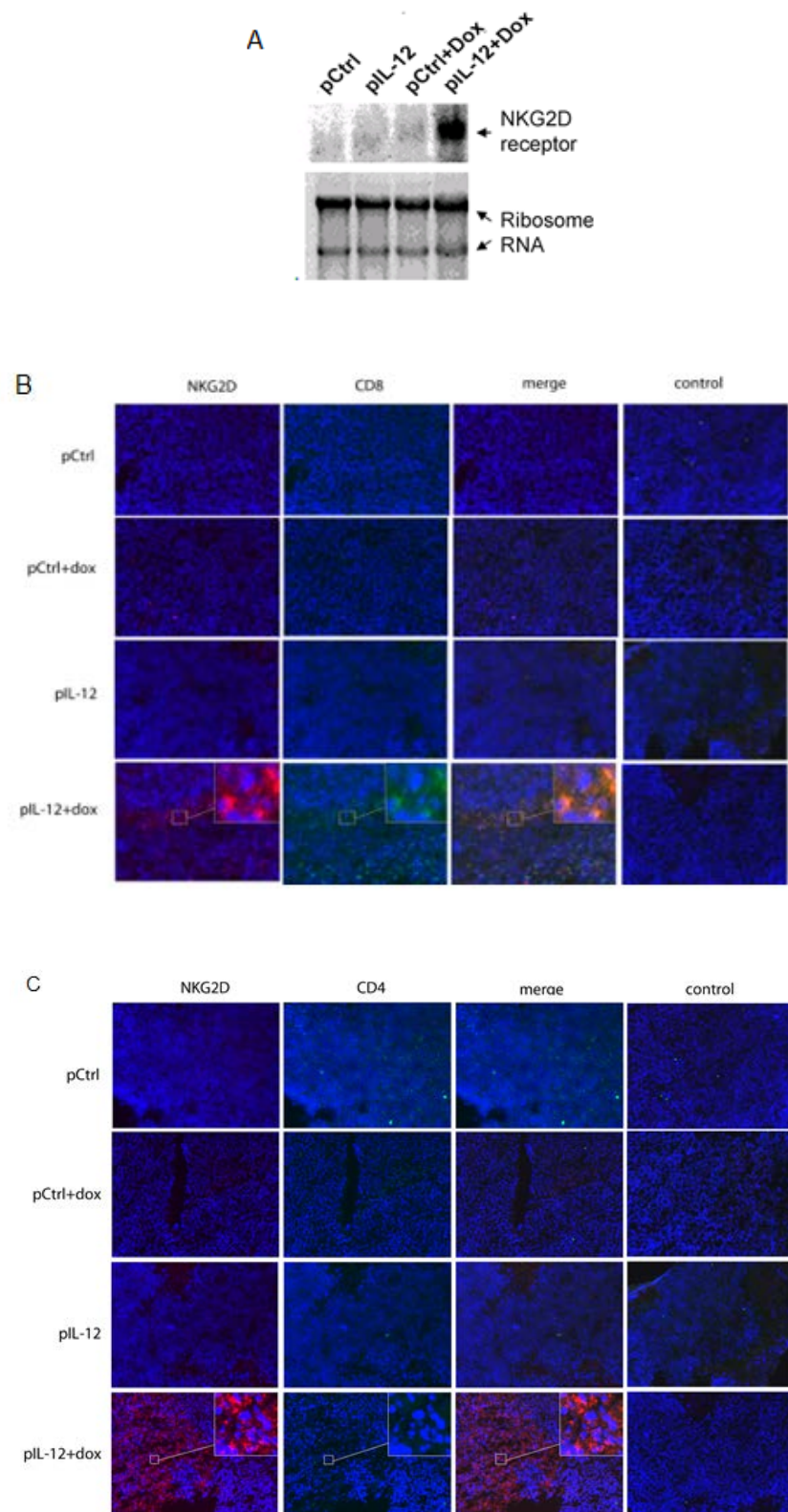
Accordingly, our first experiment was designed to determine if the numbers of NKG2D-positive immune cells were increased in tumors. Since only IL-12 plus doxorubicin induced a high level of NKG2D expression on CD8⁺T cells (Figures 9-11), we expected that NKG2D would be detected only in tumors receiving IL-12 plus doxorubicin, but not in tumors receiving any other treatments (doxorubicin, IL-12, or control DNA).

To detect the infiltration of NKG2D-positive immune cells into tumors, we analyzed the levels of NKG2D mRNA in the treated tumors using Northern blotting (Figure 12A). Since NKG2D are not expressed on tumor cells, any detected level of NKG2D expression could be attributed to tumor-infiltrating immune cells. We collected tumors from mice after different treatments to isolate RNA. As expected, a high level of NKG2D expression was detected only in the tumors treated with IL-12 plus doxorubicin (Figure 12A), but there was very low level of NKG2D receptor expression in the tumors that received other three treatments.

To validate the Northern blotting result, we determined the expression and co-localization of NKG2D and CD8 in tumor sections via immunocytochemistry analysis (Figure 12B). Tumor samples were collected from mice that received the 4 standard treatments. Tumor samples were embedded in O.C.T freezing medium and flash frozen in liquid nitrogen. We stained tumor sections from mice receiving each treatment with NKG2D and CD8, NKG2D and CD4, NKG2D and NKp46 or isotype control. This analysis corroborated the Northern blotting results: a high number of NKG2D/CD8-positive immune cells were detected and co-localized in tumors receiving IL-12 plus doxorubicin but not in

tumors receiving other treatments (Figure 12B). The NKG2D signal was not co-localized with CD4 (Figure 12C) or NKp46 (Figure 12D). In fact, neither CD4 nor NKp46 was detectable in any tumors. This result is consistent with the flow cytometry result (Figure 9).

To confirm that the NKG2D/CD8 double-positive cells detected in tumors that were treated with doxorubicin plus IL-12 (Figure 12B) are CD8⁺T cells, the same immune cell depletion approach as described in Figures 10 and 11 was used. The rationale was the same—that depletion of CD8⁺T cells would eliminate detectable NKG2D/CD8–positive cells in tumor tissues, while depletion of NK cells would not affect these signals. As expected, we detected a high number of NKG2D/CD8 double-positive cells in tumors from mice receiving IL-12 plus doxorubicin in the absence of NK cells (Figure 13A) but none were detected in CD8⁺T cell-depleted mice (Figure 13B). These results clearly confirmed that NK cell depletion does not influence the infiltration of NKG2D-positive CD8⁺T cells.



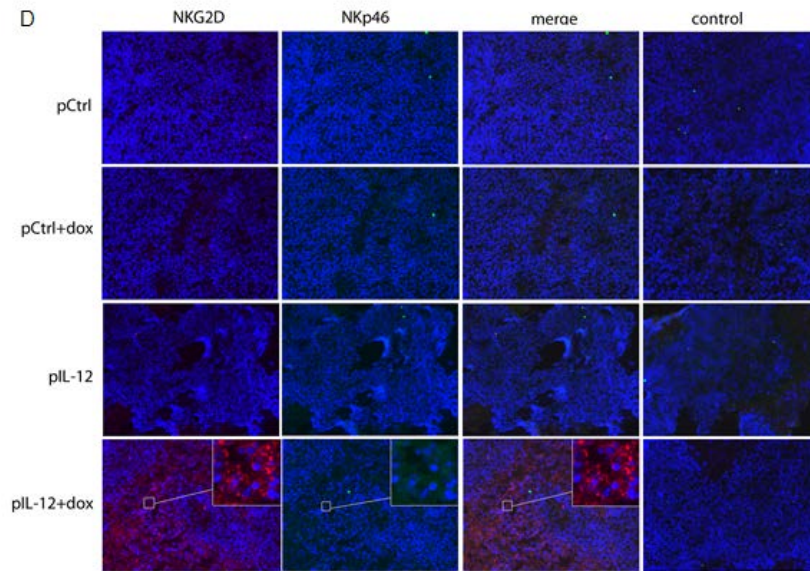


Figure 12 *NKG2D-dependent infiltration of CD8⁺T cells in tumors.* (A) Analysis of NKG2D cell infiltration using Northern blot analysis. Tumors were collected from mice receiving the indicated treatments, as described in Figure 2. Northern blot analysis was performed to detect the NKG2D expression in tumors. Ribosomal RNA was used to confirm equal loading among samples. (B) Detection of NKG2D/CD8 double positive cells. Frozen tumor sections from wild-type tumor-bearing BALB/C mice that received the treatments described in Figure 2 were stained with anti-mouse NKG2D-biotin and anti-mouse CD8 or isotype antibody followed by streptavidin conjugated Alexa fluor 594 or Alexa fluor 488 secondary antibodies. (C) Detection of NKG2D/CD4 double positive cells. Frozen tumor sections from wild-type tumor-bearing BALB/C mice that received the treatments described in Figure 2 were stained with anti-mouse NKG2D-biotin and anti-mouse CD4 or isotype antibody followed by streptavidin conjugated Alexa fluor 594 or Alexa fluor 488 secondary antibodies. (D) Detection of NKG2D/NKp46 double positive cells. Frozen tumor sections from wild-type tumor-bearing BALB/C mice that received the treatments described in Figure 2 were stained with anti-mouse NKG2D-biotin and anti-mouse NKp46 or isotype antibody followed by streptavidin conjugated Alexa fluor 594 or Alexa fluor 488 secondary antibodies.

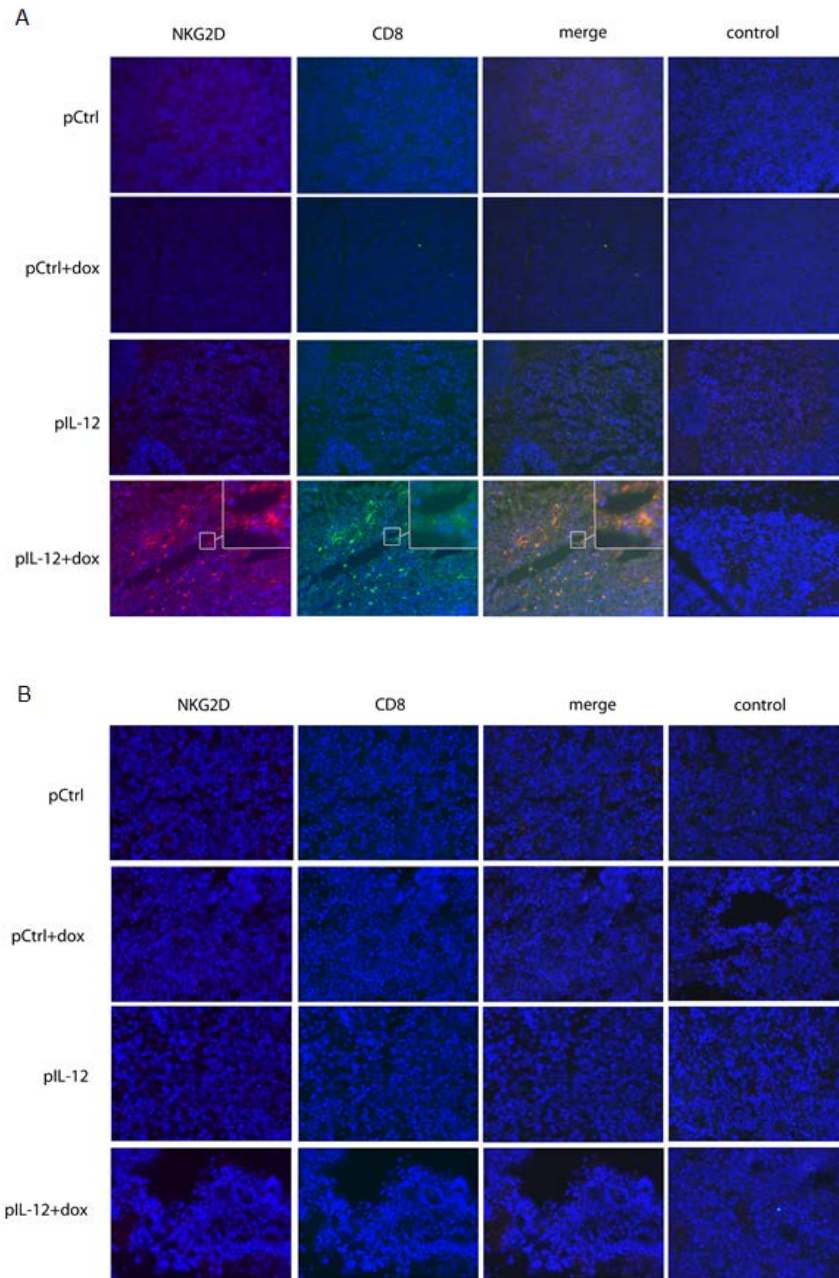


Figure 13 *NKG2D positive lymphocytes infiltration in NK cell- or CD8⁺T cell-depleted tumor-bearing mice.* (A, B) Detection of NKG2D and CD8 double positive cells in tumors bearing on NK- or CD8-depleted mice. These mice received the same treatments as detailed in Figure 2. Frozen tumor sections from NK cell-depleted (A) or CD8⁺T cell-depleted (B) tumor-bearing BALB/C mice were stained with anti-mouse NKG2D-biotin and anti-mouse CD8, or anti-mouse NKG2D-biotin and anti-mouse CD4, or anti-mouse NKG2D-biotin and anti-mouse Nkp46 antibodies or isotype antibody followed by streptavidin conjugated Alexa fluor 594 or Alexa fluor 488 secondary antibodies.

Summary

In this chapter, we demonstrated that IL-12 plus doxorubicin could enhance NKG2D⁺ CD8⁺T cells infiltration into tumors. We performed Northern blotting assay and immunocytochemistry double staining to support our hypothesis.

In the northern blotting assay, we observed more than 10 fold increase of NKG2D receptor mRNA in tumors receiving the co-administration than the tumors receiving IL-12 single treatment (Figure 12A). This result suggested the co-administration could substantially boost NKG2D positive lymphocytes infiltrating to tumors.

In the immunocytochemistry staining, we observed increasing number of NKG2D⁺CD8⁺T cell in frozen tumor sections from mice receiving the co-administration (Figure 12B), but we did not observe any CD4⁺T cells or NK cells in tumor sections (Figure 12C, 12D), suggesting that the tumor infiltrating NKG2D positive immune cells were CD8⁺T cells. In the tumor sections from NK cell depleted mice, the same as in the wild-type mice, we detected NKG2D⁺CD8⁺T cells in the tumor sections receiving the co-administration, meaning that the tumor infiltrating immune cells promoted by the co-administration were not NK cells (Figure 13A). However, in the tumor sections from CD8⁺T cell depleted mice, no infiltrating NKG2D positive immune cells were observed. Our result clearly suggested that the co-administration could promote NKG2D⁺CD8⁺T cells infiltrating into tumors (Figure 13B). CD8⁺T cells, but not NK cells were needed for the infiltration.

In this chapter, we conducted different experiments to demonstrate that the co-administration of IL-12 and doxorubicin could facilitate NKG2D⁺CD8⁺T cells infiltration into tumors. The increased infiltrating CD8⁺T cells could be activated by the NKG2D-NKG2D ligand interplay and therefore eliminate tumor cells. The fact that the co-administration could dramatically increase NKG2D positive lymphocytes infiltrating into tumors also explains why IL-12 plus doxorubicin induces NKG2D-dependent antitumor effect.

Chapter 5---The co-administration of IL-12 and doxorubicin upregulates Rae-1 on tumor cells *in vivo*

Rationale and Hypothesis

We have illustrated that IL-12 plus doxorubicin induced NKG2D-function dependent antitumor cytotoxicity by increasing the NKG2D receptor expression level on CD8⁺T cells and promoting NKG2D⁺CD8⁺T cells infiltration into tumors (Figure 8-13). The unanswered question is how the NKG2D⁺CD8⁺T cells efficiently localize in tumor microenvironment. One plausible hypothesis is that NKG2D ligand is induced in tumors by IL-12 and doxorubicin treatment. The rationale for generating such a hypothesis is that overexpression of NKG2D ligand Rae-1 could attract NKG2D receptor positive CD8⁺T cells infiltrating as effector immune cells (45). In this chapter, we will provide results to support our hypothesis that the co-administration of IL-12 plus doxorubicin induces NKG2D ligand expression on tumor cells. If this hypothesis is confirmed, then the NKG2D ligand positive tumor cells may interact with infiltrating NKG2D⁺CD8⁺T cells, inducing the NKG2D-dependent tumor cell death progression. It also explains why the co-administration could induce more promising antitumor therapeutic effect compared to either single agent treatment alone.

Others have reported that a variety of chemotherapeutic agents could induce NKG2D ligands Rae-1 on tumor cells *in vitro* (58). In their studies, a relatively high dose of chemotherapeutic agents were applied to tumor cells for forty eight hours, which caused stress-induced NKG2D ligand Rae-1 expression on tumor cells (58). However, no study has shown that chemotherapeutic agents induce NKG2D ligands *in vivo*. Unlike *in vitro* treatment which is specific on tumor cells, *in vivo* treatment may be very different as the reaction occurs in physiological conditions.

Results

Chemotherapy induces Rae-1 on tumor cells in vitro but not in vivo

Results from others indicated that various chemotherapeutic agents induced NKG2D ligands on tumor cells *in vitro* (58). To validate whether chemotherapy can induce NKG2D ligand Rae-1 on murine colon cancer cells CT26 and murine osteosarcoma cells K7M3, we treated CT26 cells and K7M3 cells with various chemotherapeutic agents including sham, doxorubicin (100 μ M), cisplatin (10 μ g/ml), cyclophosphamide (10 μ g/ml), chloroquine (2 μ g/ml), bleomycin (50 μ g/ml), methotrexate (5 μ g/ml) and ifosfamide (2 μ g/ml) (Figure 14A, 14B). Forty eight hours after the treatment, we collected the tumor cells and stained with the Rae-1 antibody followed by Alexa fluor 405 secondary antibody to identify Rae-1 level via flow cytometry (Figure 14A, 14B). Our results agreed with what others have discovered in previous publication, Rae-1 was induced at different levels by the chemotherapeutic agents (Figure 14A, 14B). It confirmed other investigators' observation that chemotherapeutic agents are able to cause tumor cell stress-induced NKG2D ligand Rae-1 expression on tumor cells *in vitro*.

However, there is no publication showing whether systemic administration of chemotherapeutic agents can induce Rae-1 *in vivo*. To address this question, we treated K7M3 tumor-bearing BALB/C mice with saline or the same chemotherapeutic agents we used *in vitro* via intraperitoneal injection (Figure 14C). The chemotherapy treatment was performed twice 10 days apart. On day 4 after the first and second treatment, respectively, we collected the tumors and extracted the total proteins. We performed western blotting to determine Rae-1 expression levels after each chemotherapeutic agent

treatment. Unlike the result from *in vitro* study, systemic administration of chemotherapeutic agents at low dose did not seem to increase Rae-1 expression in tumors (Figure 14C). Saline was used as the negative control. Different from direct *in vitro* treatment in tissue culture dishes, none of these chemotherapeutic agents induced Rae-1 in tumors. Our result suggested that although the chemotherapeutic agents induce Rae-1 on tumor cells *in vitro*, they failed to trigger stress-induced Rae-1 expression in tumors *in vivo*.

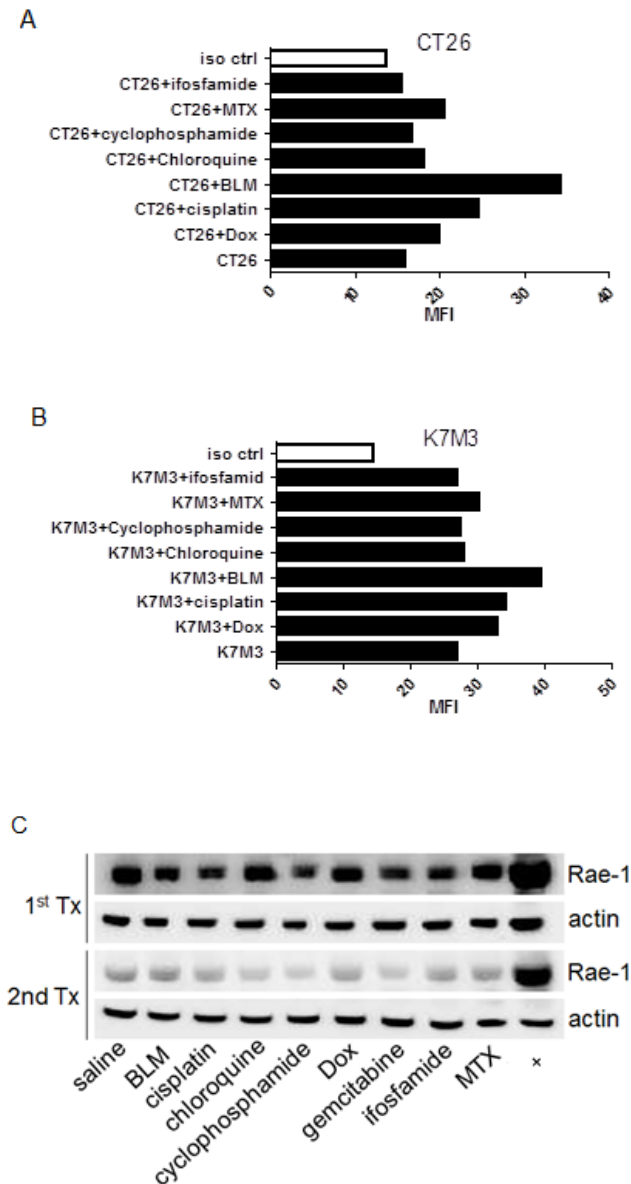


Figure 14 *Rae-1* induction in tumors by chemotherapeutic agents *in vitro* and *in vivo*. CT26 cells (A) or K7M3 cells (B) were treated with chemotherapeutic agents ifosfamide (2 μ g/ml), metrotrexate (5 μ g/ml), cyclophosphamide (10 μ g/ml), bleomycin (50 μ g/ml), cisplatin (10 μ g/ml), doxorubicin(100 μ M) or saline for 48 hours. Rae-1 level on tumor cell surface was determined by using flow cytometry. (C) CT26 tumor-bearing BALB/C mice were treated with chemotherapeutic agents bleomycin (5U/kg), cisplatin (20mg/kg), chloroquine (10mg/kg), cyclophosphamide (60mg/kg), doxorubicin (5mg/kg), gemcitabine (20mg/kg), ifosfamide (80mg/kg), metrotrexate (25mg/kg), doxorubicin (5 μ g/mg) or saline control via i.p. injection. The treatment was conducted on day 7 and day 17 after the inoculation. Tumors were collected and processed on day 9 and day 19. Rae-1 level was detected by western blotting assay. Actin was used as the loading control.

The combination of IL-12 and a few specific chemotherapeutic agents can increase Rae-1 in tumors

Since chemotherapeutic agents alone failed to increase Rae-1 *in vivo*, we tested combinations of chemotherapeutic agents and cytokines to induce Rae-1 expression in tumors. We treated K7M3 tumor-bearing mice with IL-12 DNA or control DNA plus one of the chemotherapeutic agents including doxorubicin, cyclophosphamide (cy) and ifosfamide (Figure 15A). The combination of IL-12 DNA plus doxorubicin induces the highest level of Rae-1 in tumors than any other combinations (Figure 15A). To validate this observation on another tumor model, we treated LLC tumor-bearing mice with IL-12 or IFN γ plus various chemotherapeutic agents including doxorubicin, cisplatin, cyclophosphamide, bleomycin and chloroquine (Figure 15B). In agreement with the observation in K7M3 tumor model, IL-12 and doxorubicin induced the highest level of Rae-1 expression in LLC tumors (Figure 15B). To determine the cytokine specificity for Rae-1 induction, we treated tumor-bearing mice with doxorubicin plus various other cytokines such as IL-4, IL-6, IFN α , IFN β and IFN γ (Figure 15C). Surprisingly, only IL-12 plus doxorubicin could induce Rae-1 in tumors (Figure 15C). After testing multiple combinations of cytokines and chemotherapeutic agents, we concluded only the co-administration could most dramatically induce Rae-1 in tumors.

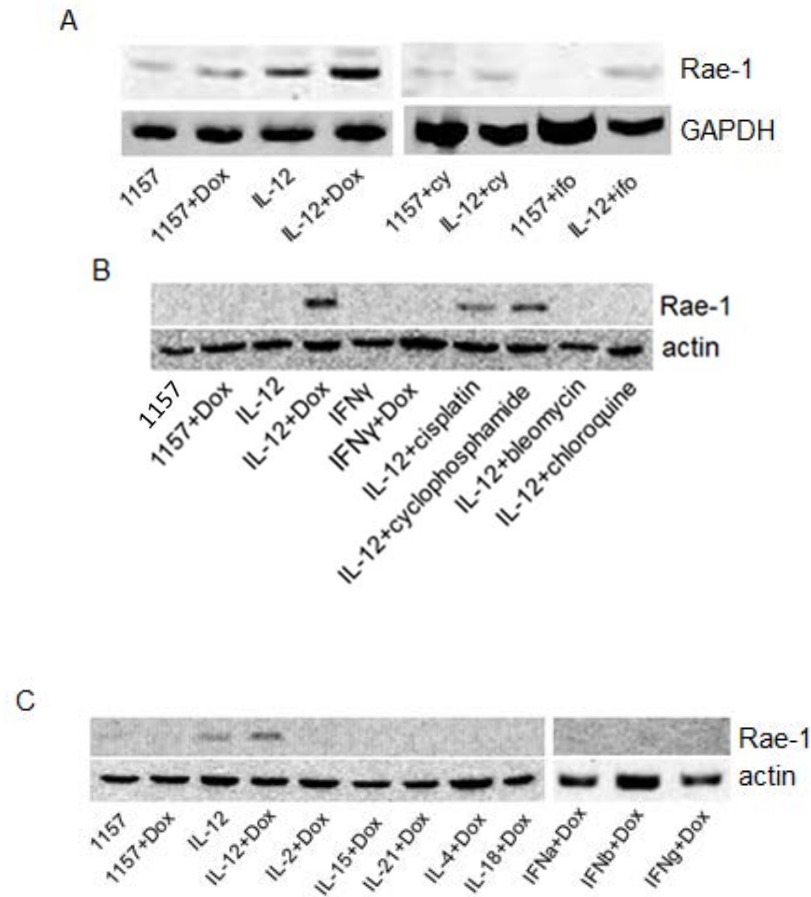


Figure 15 *Rae-1* expression levels in tumors after different treatments. 10 μ g of DNA was administered to each mouse. The same chemotherapeutic agent was administered the same dose to various mouse strains. (A) K7M3 tumor-bearing mice received the treatment of control DNA, control DNA plus doxorubicin (5mg/kg), IL-12 DNA, IL-12 DNA plus doxorubicin, control DNA plus cyclophosphamide (60mg/kg), IL-12 DNA plus cyclophosphamide, control DNA plus ifosfamide (80mg/kg), or IL-12 DNA plus ifosfamide. (B) LLC tumor-bearing mice received the treatments of control DNA, control DNA plus doxorubicin, IL-12 DNA, IL-12 DNA plus doxorubicin, IFN γ DNA, IFN γ DNA plus doxorubicin, IL-12 DNA plus cisplatin, IL-12 DNA plus cyclophosphamide, IL-12 DNA plus bleomycin or IL-12 DNA plus chloroquine. (C) LLC tumor-bearing mice received control DNA, control DNA plus doxorubicin, IL-12 DNA, IL-12 DNA plus doxorubicin, IL-2 DNA plus doxorubicin, IL-15 DNA plus doxorubicin, IL-21 DNA plus doxorubicin, IL-18 DNA plus doxorubicin, IFN α DNA plus doxorubicin, IFN β DNA plus doxorubicin or IFN γ DNA plus doxorubicin. In the above three experiments, the treatments were performed on day 7 and day 17 after the tumor cell inoculation. Tumors were collected and processed on day 19 to detect Rae-1 via western blotting assay. GAPDH (A) or actin (B, C) were also tested as the loading control.

IL-12 plus doxorubicin induces tumor specific and long duration of Rae-1 expression

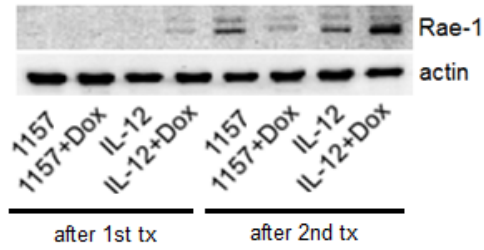
The next question we wanted to address was whether Rae-1 induction by IL-12 plus doxorubicin treatment occurs in most tumor models. If it is a general effect, we should be able to observe Rae-1 induction in various types of tumor models with the same treatment of IL-12 plus doxorubicin. We treated BALB/C or C57BL/6 mice bearing different tumor models with our 4 standard treatments: control DNA, control DNA plus doxorubicin, IL-12 DNA or IL-12 DNA plus doxorubicin (Figure 16). The tumor models we tested include 4T-1 (breast carcinoma) (Figure 16A), LLC (lewis lung carcinoma) (Figure 16B), B16F10 (melanoma) (Figure 16C), CT26 (colon cancer) (Figure 16D) and K7M3 (osteosarcoma) (Figure 18E). Four days after the first or second treatment, tumors were processed to dissociate the cells for flow cytometry assay or to extract the total proteins for western blotting assay. Frozen tumor sections were stained with Rae-1 antibody followed by Alexa fluor 405 secondary antibody in immunohistochemistry staining assay. Our results from western blotting assay, flow cytometry assay and immunohistochemistry staining assay all suggested that in all the tested tumor models, the co-administration of IL-12 DNA plus doxorubicin could substantially induce Rae-1 expression in tumors (Figure 16). These results strongly support the hypothesis that the co-administration-mediated Rae-1 induction in tumors is a general effect applied for a variety of tumor models.

One concern was that if IL-12 plus doxorubicin treatment could also induce Rae-1 expression in normal tissues, it may cause severe immune toxicity. To test Rae-1 expression level in normal tissues after each treatment, we collected normal tissues including hearts, livers, spleens, lungs and kidneys from LLC (Figure 17A) and K7M3 (Figure 17B) tumor model bearing mice after the second time treatment. In both tumor models and all the normal tissues tested, we did not detect any Rae-1 induction in any normal tissue after either treatment (Figure 17). This result suggested that the Rae-1 induction by the co-

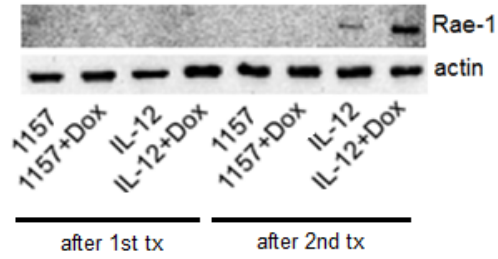
administration only occurs in tumors but not normal tissues. The specificity of Rae-1 induction in tumors greatly reduces safety concern of this treatment.

Since we observed a long term induction of NKG2D after the IL-12 plus doxorubicin co-administration, we were interested in knowing whether the co-administration induces a long duration of Rae-1 expression. The long term induction of both NKG2D and NKG2D ligand could further facilitate immune cell infiltrating into tumors to trigger a long term antitumor response. To illustrate whether the Rae-1 induction by the co-administration is a transient or a long term effect, tumors were collected from LLC tumor model bearing mice 1 day and 4 days after the first treatment, as well as 1 day, 4 days and 8 days after the second treatment (Figure 18). After the first treatment on day 1 or day 4, we did not observe any Rae-1 induction after any treatment. One day after the second treatment, after the co-administration, weak Rae-1 induction was detected. Four days after the second treatment, IL-12 and IL-12 plus doxorubicin could both increase Rae-1 in tumors, but the co-administration increased Rae-1 at a higher level. Interestingly, eight days after the second treatment, high levels of Rae-1 were still maintained in the tumors that received the co-administration, but tumors received other treatments didn't have detectable level of Rae-1 (Figure 18). Our results suggested that the co-administration of IL-12 and doxorubicin had a long term effect of Rae-1 induction in tumors. Rae-1 induction by the co-administration starts right after the second treatment, but is maintained at high levels until after 8 days. It also explains why the co-administration had continuously inhibitory effect on tumor growth, while IL-12 DNA treatment only inhibited tumor growth during and shortly after the treatment.

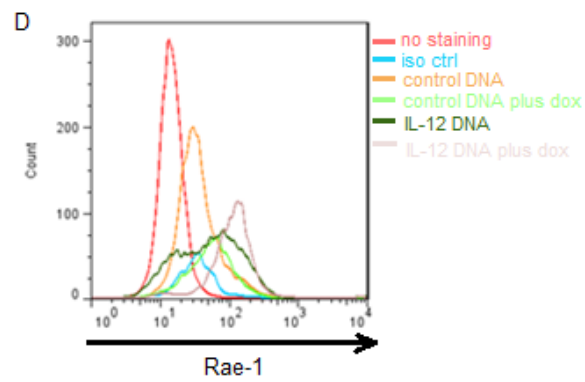
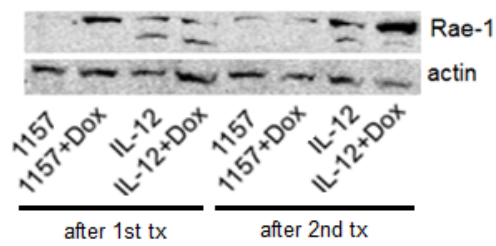
A 4T-1



B LLC



C B16F10



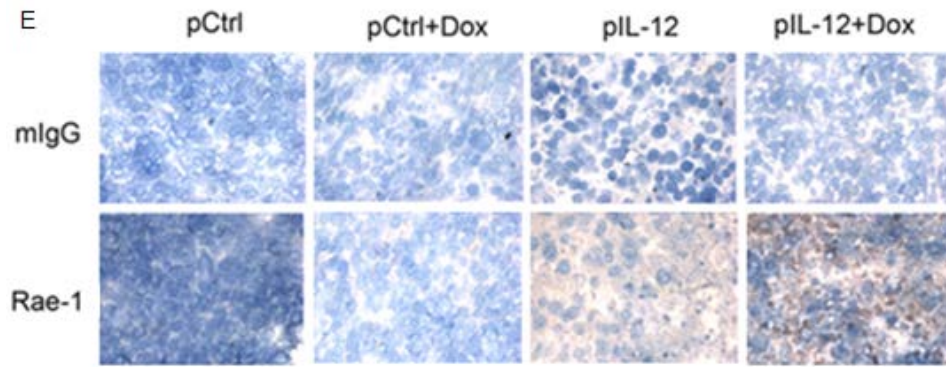


Figure 16 *Rae-1* expression levels in multiple tumor models after the co-administration of *IL-12* plus doxorubicin. 4T-1 (A), LLC (B), B16F10 (C), CT26 (D) and K7M3 (E) tumor-bearing mice were given 4 standard treatments as described in Material and Methods. Western blotting (A-C), flow cytometry (D) and immunohistochemistry (E) were performed to detect Rae-1 level in tumors after different treatments. The result represents three independent experiments.

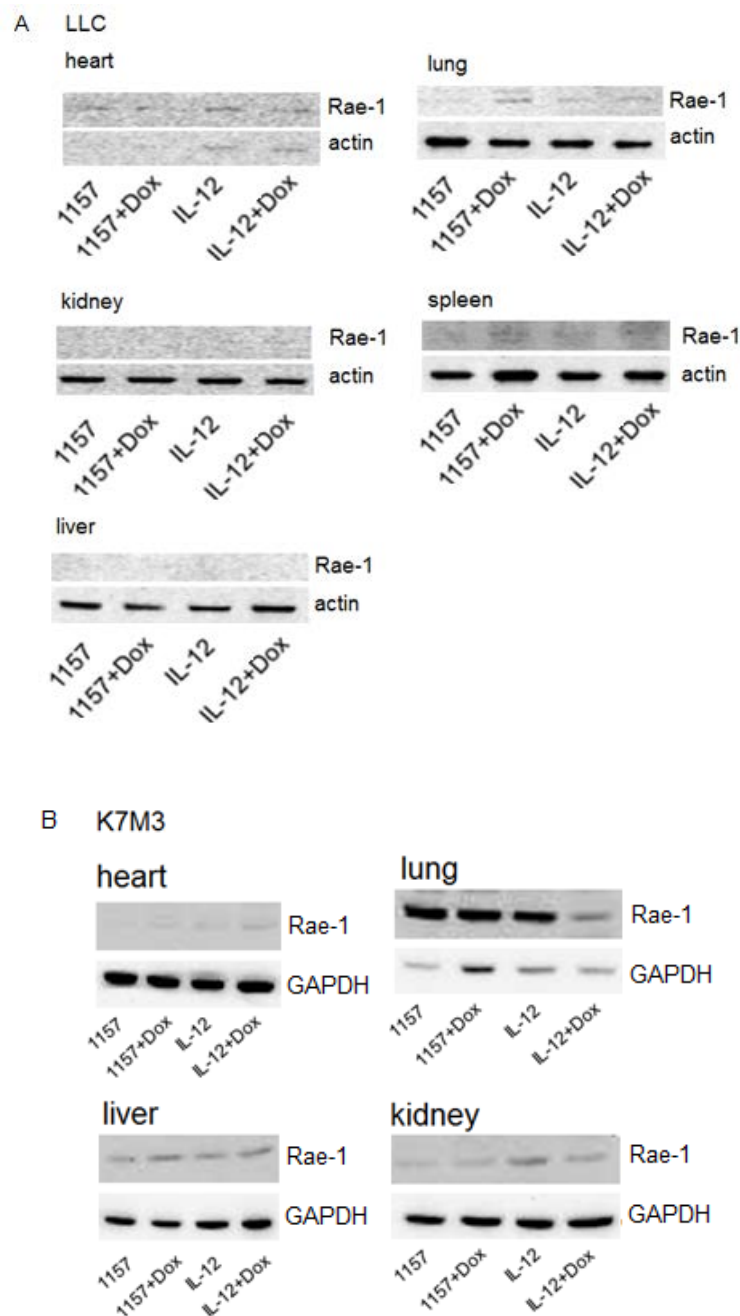


Figure 17 *Rae-1 expression levels in normal tissues after different treatments.* LLC (**A**) and K7M3 (**B**) tumor-bearing mice were given 4 standard treatments as described in Material and Methods. Western blotting assay was performed to determine Rae-1 level in the normal tissues after different treatments. Our result represents three independent experiments.

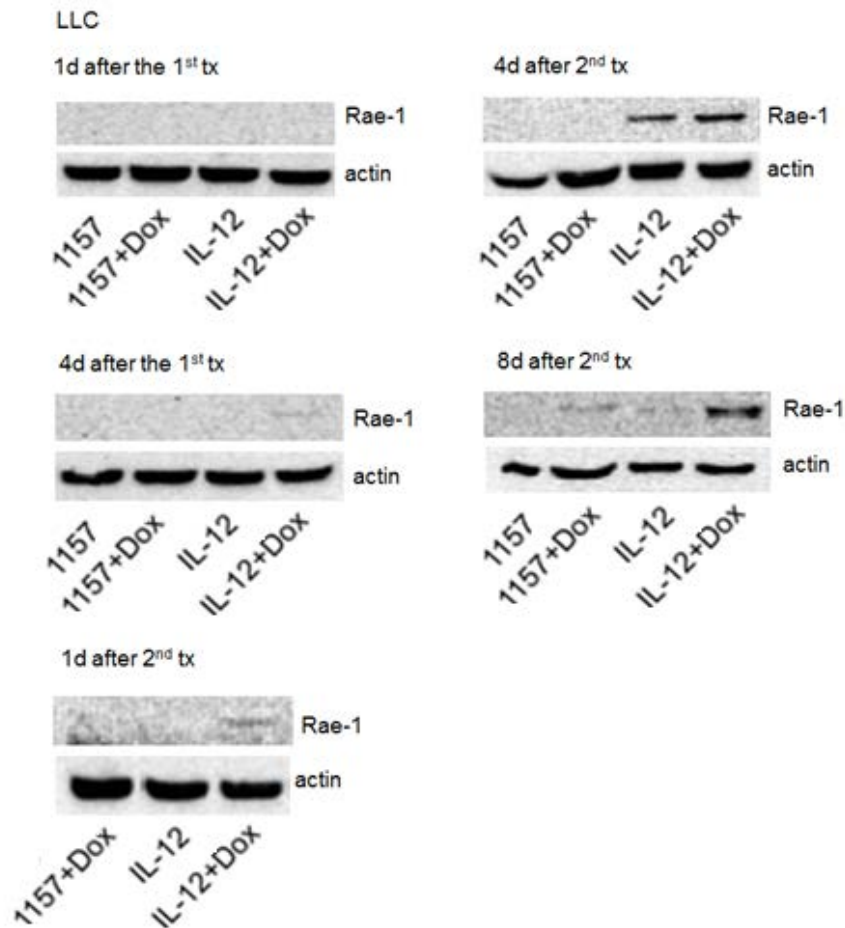


Figure 18 *Duration of Rae-1 induction after each treatment.* LLC tumor-bearing mice were given 4 standard treatments as described in Material and Methods. 1 day and 4 days after the first treatment, 1 day, 4 days and 8 days after the second treatment, tumors were collected and processed. Western blotting assay was performed to determine Rae-1 level in tumors after different treatments. Actin was also tested as the loading control. Our result represents three independent experiments.

Summary

Others have shown NKG2D ligands can be induced by various chemotherapeutic agents on tumor cells *in vitro*, this hypothesis was confirmed in two tumor cell lines, CT26 and K7M3 (Figure 14A, 14B). However, the same chemotherapeutic agents failed to induce Rae-1 *in vivo* (Figure 14C).

To induce Rae-1 *in vivo*, we administered different combinations of cytokines plus chemotherapeutic agents to different tumor model bearing mice (Figure 15). We found IL-12 plus doxorubicin could induce Rae-1 at the highest level in all the tested tumor models (Figure 15).

To demonstrate Rae-1 induction in tumors by the co-administration is not tumor model specific, we administered the treatment on mice carrying different types of tumors such as 4T-1 breast carcinoma, CT26 colon cancer, LLC lewis lung carcinoma, B16F10 melanoma and K7M3 osteosarcoma (Figure 16). We also conducted independent western blotting assay, flow cytometry analysis and immunohistochemistry assay to examine Rae-1 expression in tumors (Figure 16). In all the tumor models we tested, Rae-1 was dramatically increased by the co-administration (Figure 16). To exclude the possibility that the treatment might also increase Rae-1 on normal tissues, we examined Rae-1 expression on normal tissues receiving the 4 standard treatments (Figure 17). We did not detect Rae-1 induction in normal tissues after any treatment (Figure 17).

To test the dynamic change of Rae-1 levels in tumors after the co-administration of IL-12 plus doxorubicin, we examined Rae-1 expression at different time points after each treatment (Figure 18). We noticed IL-12 could only induce modest level of Rae-1 up to 4 days after the second treatment, but the co-administration induces a high level of Rae-1 expression for at least 8 days post treatment (Figure 18).

Taken together, we discovered an optimal strategy to increase NKG2D ligand Rae-1 in tumors *in vivo*. By applying the co-administration of IL-12 and doxorubicin on tumor-bearing mice, NKG2D ligand Rae-1 was induced in different tumor models and multiple mouse strains *in vivo*. The Rae-1 induction is tumor specific, and no other normal tissues were affected. In comparison with doxorubicin or IL-12 single treatment, the co-administration could cause a high level and long term induction of Rae-1 in tumors.

Chapter 6 Tumor cells express increasing level of Rae-1 by interacting with CD8⁺T cells

Rationale and hypothesis

We have found that IL-12 plus doxorubicin induces the expression of NKG2D ligand Rae-1, but the mechanism was unclear. According to current textbooks, NKG2D ligand induction is triggered by the activation of stress signaling. The stress signals include virus infection, heat shock, oxidative stress, DNA damaging agents and cell toxic chemicals (145). When cells are undergoing stress, protein kinases ATM (ataxia-telangiectasia, mutated)/ATR (ATM and Rad3-related), as sensor of DNA damage, are activated to phosphorylate checkpoint kinase 1 and 2 (chk1 and chk2) which induce NKG2D ligand expression to transmit the “non-self” signals to immune cells (59). Based on this well-defined mechanism, it is possible that the Rae-1 induction in tumors by IL-12 plus doxorubicin was through stress signaling activation. In chapter 4, we demonstrated that IL-12 plus doxorubicin facilitated the infiltration of NKG2D⁺CD8⁺T cells in tumors. Therefore, CD8⁺T cells infiltration may regulate Rae-1 induction in tumors. In this chapter, our first goal is to decipher which mechanism is true for IL-12 plus doxorubicin-mediated Rae-1 induction.

If we demonstrate that the interaction between tumor cells and activated CD8⁺T cells accounts for Rae-1 induction, our next goal is to identify the receptor through which tumor cells communicate with immune cells and transduce the signals to induce Rae-1. Antigen presenting cells (APC) and a few tumor cells display multiple receptors which could communicate with immune cells to either activate or inhibit immune cell response. These receptors are categorized into two groups: B7 family including CD80, CD86, PD-L1, PD-L2, B7H3 and B7H4, and TNF family such as CD40, CD70, 41BBL and OX40L (75). B7

family receptors on APC or tumor cells can be recognized by CD28 family receptors on immune cells. Among B7 family receptors, CD80 and CD86 play dual roles to activate immune cells by interacting with CD28, or to regulate immune response by interacting with CTLA4 (77). Other B7 family receptors PD-L1, PD-L2, while binding to PD1 on immune cells, protect tumor cells from being attacked by immune cells (146). B7H3 could activate antitumor immune response (147), however the function of B7H4 remains controversial. These receptors are expressed on tumor cells and interplay with immune cells, therefore, the critical receptor that is involved in Rae-1 induction on tumor cells is more than likely one of these receptors.

Results

Rae-1 induction in tumors by IL-12 plus doxorubicin is not through stress-induced signaling pathway

To investigate the mechanism by which Rae-1 is induced by the co-administration of IL-12 and doxorubicin, we examined the activation of stress signaling pathway after each treatment (Figure 19).

CT26 tumors that received the 4 standard treatments were processed to extract the total proteins. Western blotting assay was performed to examine the expression of proteins in stress-induced signaling pathway including phosphorylated ATR, phosphorylated ATM, total ATM and downstream phosphorylated chk1, phosphorylated chk2 and total chk2 (Figure 19). Our result showed no detectable phosphorylated ATM, phosphorylated ATR, phosphorylated chk1 or phosphorylated chk2 in any of the samples, suggesting that the stress signaling was not activated by any of the treatment (Figure 19). This means Rae-1 induction in tumors by the co-administration of IL-12 and doxorubicin is not due to the activation of stress signaling pathway.

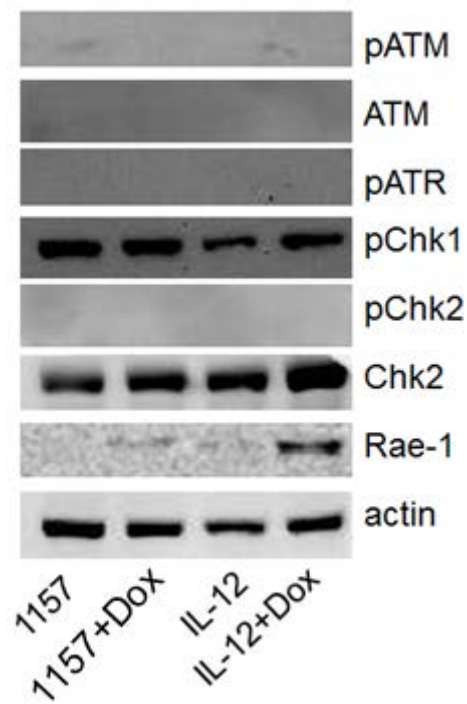


Figure 19 *Rae-1 induction in tumors is not due to the stress signaling activation.* CT26 tumor-bearing BALB/C mice received the 4 standard treatments as described in the Material and Methods (Chapter 2). Tumors were collected and processed after the second treatment. The stress signaling factors (pATM, pATR, pChk1 and pChk2) were examined via western blotting. The result represents three independent experiments.

Activated T cell and tumor cell contact-dependent Rae-1 induction

As Rae-1 induction by the co-administration of IL-12 plus doxorubicin was not through the stress signaling-mediated mechanism, there must be an alternative mechanism. This alternative mechanism could be either through the direct effect from IL-12 plus doxorubicin or the tumor infiltrating lymphocytes. To test whether the direct effect of IL-12 plus doxorubicin accounts for Rae-1 induction, we treated CT26 and K7M3 tumor cells with low dose of IFN γ (50ng/ml) and doxorubicin (40nM). The doses were calculated based on their concentration in blood after the co-administration (124) (Figure 20A, 20B). The reason we treated tumor cells with IFN γ instead of IL-12 was because most tumor cells lack IL-12 receptor to transduce the signaling. It is unlikely that IL-12 directly acts on tumor cells to augment doxorubicin-mediated Rae-1 induction. IFN γ is the hallmark effector molecule produced by the IL-12 signaling *in vivo*. Unexpectedly, no Rae-1 induction on tumor cells was detected after the IFN γ plus doxorubicin treatment *in vitro* (Figure 20A, 20B).

In our previous publication as well as some of the experiments in chapter 4, we have demonstrated that the co-administration could facilitate NKG2D⁺CD8⁺T cells infiltration into tumors (Figure 12). Therefore, we hypothesized the cytokines secreted by tumor infiltrating immune cells could induce NKG2D ligands on tumor cells. If this working hypothesis is true, we should be able to observe Rae-1 induction on tumor cells after treating tumor cells with T cell condition medium. To test this hypothesis, we indirectly co-incubated CT26 tumor cells with IL-12 plus doxorubicin-treated splenocytes via boyden chamber system (Figure 20C). Tumor cells were plated at the bottom chamber, while the activated splenocytes were seeded in the top chamber. The tumor cells and splenocytes did not have direct interaction, but the cytokines secreted by the immune cells could freely flow into the tumor cell culture medium. Twenty four hours after the co-incubation, we

performed flow cytometry to test Rae-1 level on tumor cells. We did not observe Rae-1 induction on the co-incubated tumor cells (Figure 20C). This result suggested that Rae-1 induction on tumor cells was not simply induced by the cytokines released by tumor infiltrating immune cells.

We then investigated whether direct contact between tumor cells and immune cells could induce Rae-1 on tumor cells (Figure 21). Splenocytes were collected from IL-12 plus doxorubicin treated BALB/C mice, and co-incubated with CT26 (Figure 21A) and K7M3 (Figure 21B) tumor cells overnight. We observed a substantial increase of Rae-1 expression on tumor cells after the activated splenocytes co-incubation (Figure 21). This result from the *in vitro* model reveals a novel Rae-1 induction mechanism: activated splenocyte contact-dependent Rae-1 induction on tumor cells.

To determine whether this novel mechanism of Rae-1 induction also applies in human tumor cells, we activated human PBMCs (peripheral blood mononuclear cells) and co-incubated with human colon cancer cells HCT116 (Figure 22A) and human osteosarcoma cells CCH.OS.D (Figure 22B). We observed NKG2D ligand MICA and pan-NKG2D ligands induction on HCT116 cells, and ULBP2 and pan-NKG2D ligands induction on CCH.OS.D cells (Figure 22).

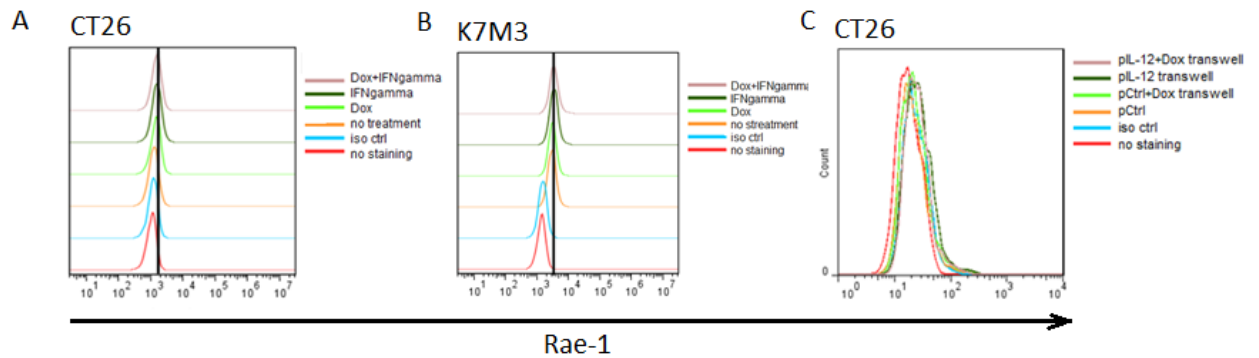


Figure 20 *In vitro*, *Rae-1* cannot be increased by *IFN* γ plus doxorubicin nor cytokines from stimulated splenocytes. CT26 cells (A) and K7M3 cells (B) were treated with *IFN* γ alone (100ng/ml), doxorubicin (40nM) alone or *IFN* γ plus doxorubicin. *Rae-1* level was tested after different treatments via flowcytometry. (C) CT26 cells were co-incubated with splenocytes received one of the 4 standard treatments in the boyden chamber transwell system. The tumor cells were plated at the bottom chamber, while the immune cells were cultured on the top chamber at the ratio of tumor cells : splenocytes = 1:5 in RPMI tissue culture medium. 24 hours after the co-incubation, tumor cells were harvested to test *Rae-1* level by using flow cytometry. All the results represents three independent replicates.

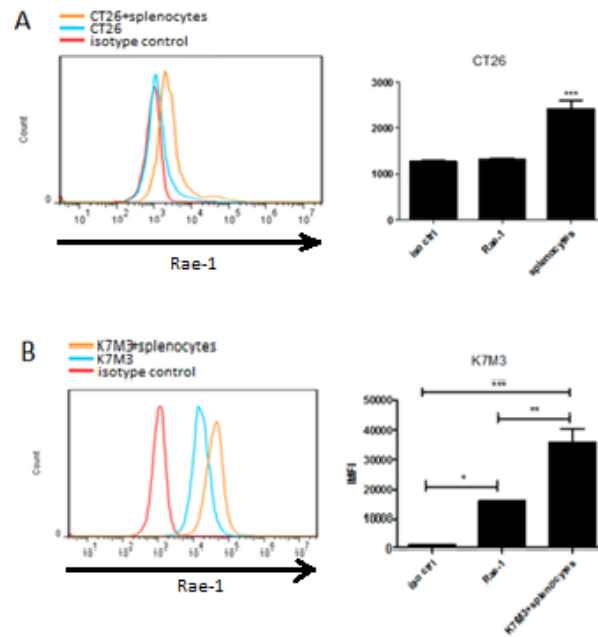


Figure 21 *Rae-1* induction on tumor cells after co-incubation with stimulated splenocytes. CT26 cells (**A**) or K7M3 cells (**B**) were co-incubated with the splenocytes that received the co-administration at the ratio of tumor cells: splenocytes=1:5. Rae-1 level was tested comparing to isotype control via flow cytometry. The graphs at the right side represent the statistical analysis of three replicates base on the pan-NKG2D ligands MFI value in the flow cytometry analysis. Our result is representative of three replicates. *, $p<0.05$; **, $p<0.01$, ***, $p<0.001$.

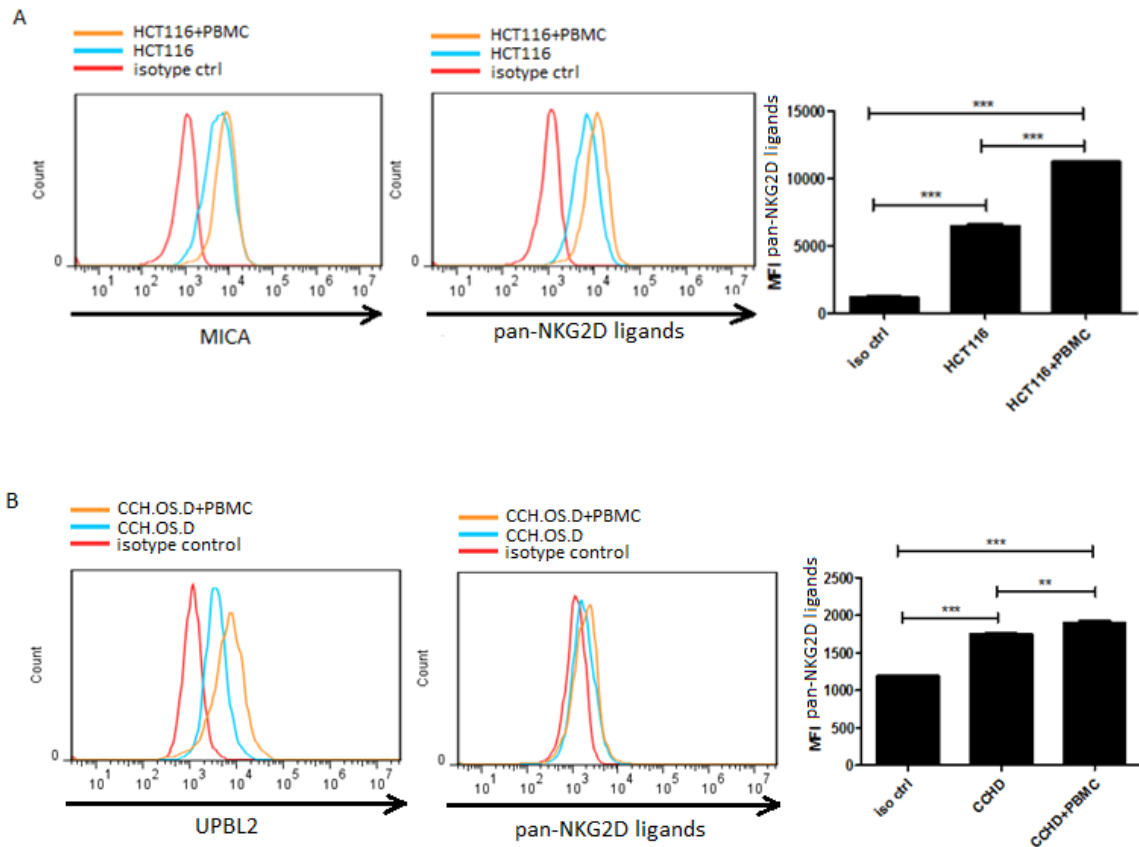


Figure 22 *NKG2D ligands on human tumor cells after incubating with stimulated PBMC.* HCT116 cells (**A**) and CCH.OS.D cells (**B**) were co-incubated with stimulated PBMC at the ratio of tumor cells : PBMC = 1:5. Rae-1 level was tested comparing to isotype control via flow cytometry. The graphs at the right side represent the statistical analysis of from three replicates base on the pan-NKG2D ligands MFI value in the flow cytometry analysis. Our result is representative of three replicates. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Rae-1 induction on tumor cell surface requires direct interaction with CD8⁺T cells

To study which immune cell subpopulation in the splenocytes contributes to Rae-1 induction in tumors, we depleted different subpopulations of immune cells by using the depleting antibodies. If a specific type of immune cells is critical, then depleting that immune cell population should abolish Rae-1 induction after IL-12 plus doxorubicin treatment. CT26 tumor-bearing mice were given 50μg of control IgG, CD4⁺T cell depleting antibody (clone GK1.5) (Figure 23A), CD8⁺T cell depleting antibody (clone 2.43) (Figure 23B) or NK cell depleting antibody (anti-Asialo GM1) (Figure 23C) in PBS twice weekly along with a total of two times of IL-12 plus doxorubicin treatment. Four days after the second co-administration, splenocytes were collected to assure the depletion of each respective immune cell subpopulation was completed using flow cytometry (Figure 23A-C). Tumors were collected and processed to extract the total proteins for western blotting assay. Our results showed that only after depleting CD8⁺T cells, Rae-1 expression levels were dramatically reduced in tumors (Figure 23D), which implies that IL-12 plus doxorubicin-induced Rae-1 relies on CD8⁺T cells.

To validate the importance of CD8⁺T cells in Rae-1 induction, we tested Rae-1 induction in tumor-bearing nude mice after IL-12 plus doxorubicin treatment. These immune deficient mice lack mature T cells; the co-administration therefore should not be able to induce Rae-1 to the same level as in immunocompetent mice. Four days after the second treatment, we processed the tumors to extract the total proteins for western blotting assay to detect Rae-1. The tumor sample from wild-type mice treated by co-administration was used as the positive control, and tumor sample from wild-type mice treated by control DNA was used as the negative control. As we expected, the Rae-1 induction by co-administration was dramatically impaired in the T cell deficient mice, suggesting the critical role of T cells on Rae-1 induction in tumors (Figure 24).

To validate the roles of different subpopulations of immune cells on Rae-1 induction *in vitro*, we depleted different subpopulations (NK cells, CD4⁺T cells and CD8⁺T cells) of immune cells by using the depleting antibodies on BALB/C mice twice weekly in combination with twice of the co-administrations of IL-12 plus doxorubicin (Figure 25). The same as the *in vivo* depleting experiment, if CD8⁺T cells play an important role, the splenocytes lacking CD8⁺T cells should fail to induce Rae-1 on tumor cells. After two weeks of the depleting antibody treatment, we collected the splenocytes and confirmed the depleting efficiency of each immune cell subpopulation via flow cytometry. CT26 tumor cells were co-incubated with specific immune cell-depleted splenocytes. Twenty four hours after the co-incubation, we performed flow cytometry to test Rae-1 expression level on the tumor cells. We found that compared with the control IgG treated splenocytes, only the CD8⁺T cell-depleted splenocytes failed to induce Rae-1 on tumor cells (Figure 25). Consistent with what we indicated in the *in vivo* experiments, CD8⁺T cells are needed for Rae-1 induction on tumor cells *in vitro* as well. This result further validated our *in vitro* model which accurately recapitulates the *in vivo* observation.

To show direct evidence that supports CD8⁺T cells are important for Rae-1 induction *in vitro*, we collected splenocytes after the co-administration and enriched different subpopulations of immune cells by using the negative selection immune cell enrichment kits from STEMCELL Technologies. The enrichment efficiency was confirmed by using flow cytometry. NK cells were enriched from 5% in total splenocytes to over 90% (Figure 26A, 27A), CD4⁺T cells from 30% to over 90% (Figure 26B, 27B), and CD8⁺T cells from 20% to over 90% (Figure 26C, 27C). CT26 (Figure 26D) and K7M3 (Figure 27D) tumor cells were co-incubated with enriched immune cells. Twenty four hours after the co-incubation, Rae-1 expression level was examined via flow cytometry. We discovered that only CD8⁺T cells

could significantly induce Rae-1 levels on tumor cells in both CT26 and K7M3 cell lines (Figure 26D, 27D).

Here, we demonstrated that the co-administration of IL-12 plus doxorubicin failed to increase Rae-1 in T cell deficient mice. Both *in vivo* and *in vitro*, CD8⁺T cell depletion abrogated Rae-1 induction in tumors (Figure 23, 25). Only the enriched CD8⁺T cells substantially increased Rae-1 *in vitro* (Figure 26, 27). In conclusion, CD8⁺T cells are required for Rae-1 induction by the co-administration of IL-12 plus doxorubicin.

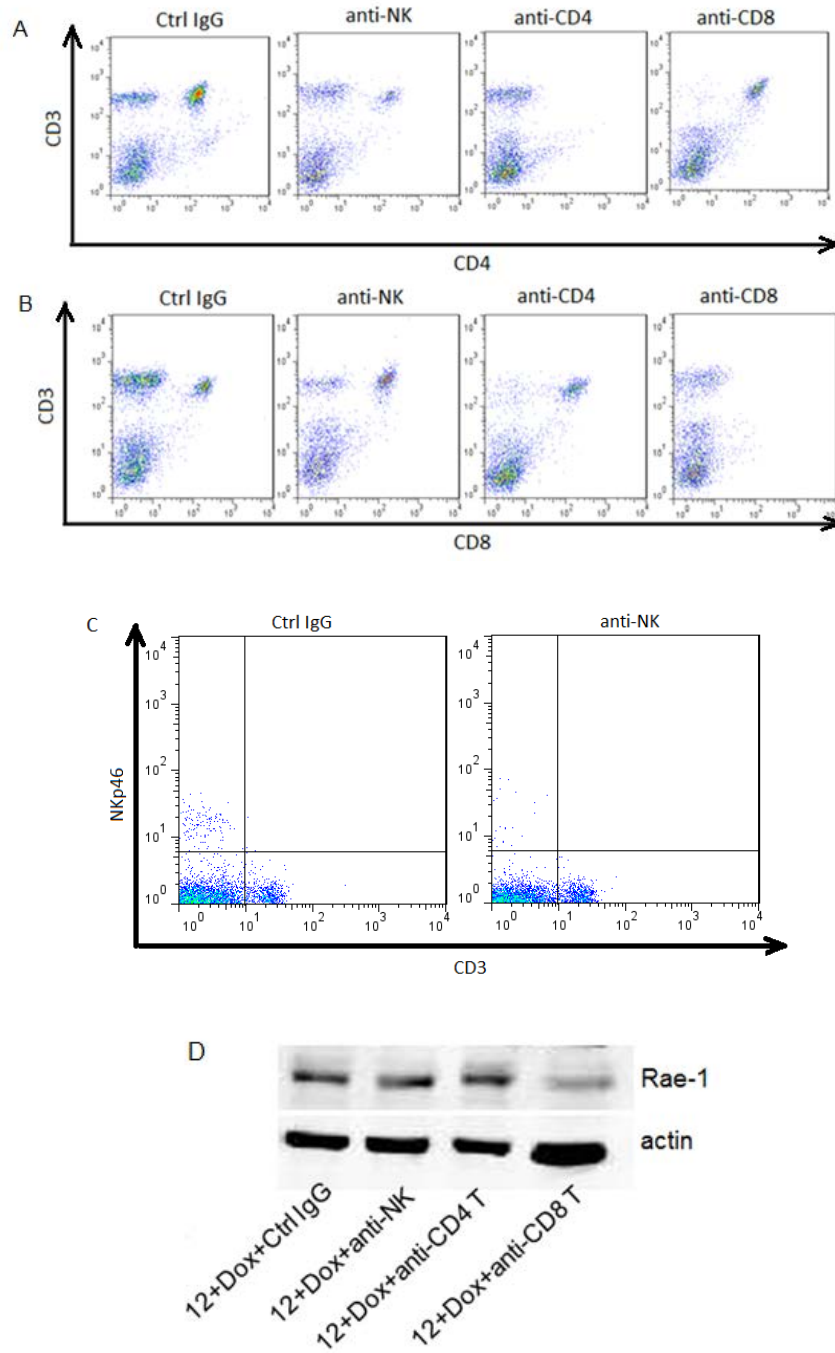


Figure 23 *Rae-1* levels in tumors from specific immune cell-depleted mice after the co-administration. Tumor-bearing mice were treated with CD4 T cell depleted antibody (clone GK1.5), CD8⁺T cell depleted antibody (clone 2.43), NK cell depleted antibody (anti-Asialo GM1) or control IgG as described in the Material and Methods. All the tumor-bearing were also given the co-administration of IL-12 plus doxorubicin. Tumors and spleens were collected and processed. CD3CD4 (A), CD3CD8 (B) and NKp46 (C) were examined on splenocytes by using flow cytometry assay to determine the immune cell depletion efficiency. (D) *Rae-1* was detected in tumors after different treatments. Actin level was also detected as the loading control. All results represent three independent replicates.

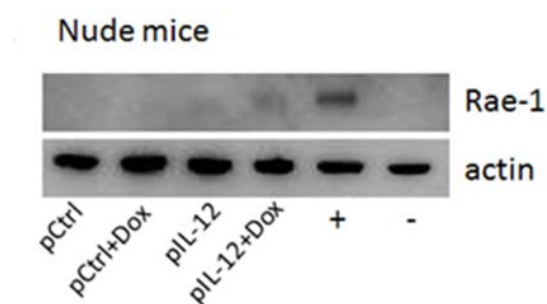


Figure 24 *Rae-1 levels in tumors from immune deficient mice after different treatments.* Tumor-bearing nude micewere given the 4 standard treatments as described in the Material and Methods (Chapter 2). Tumors were collected on day 19 and processed. Western blotting assay was performed to test Rae-1 level in each tumor sample comparing to the positive control (the same tumor model in wild type mice after the co-administration) and negative control (the same tumor model in wild type mice after the control DNA treatment). The result represents three independent replicates.

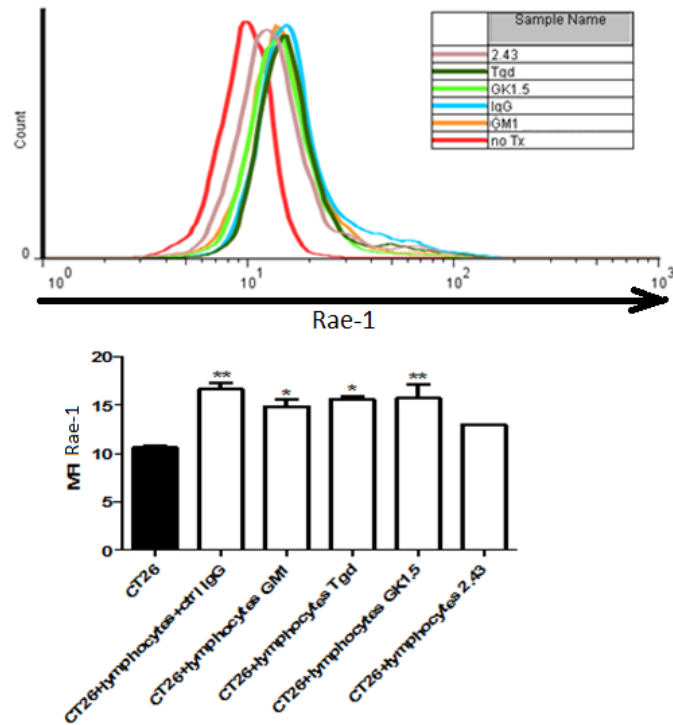
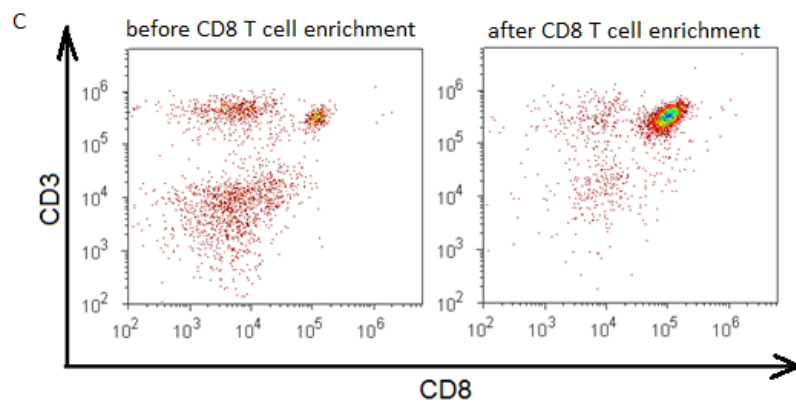
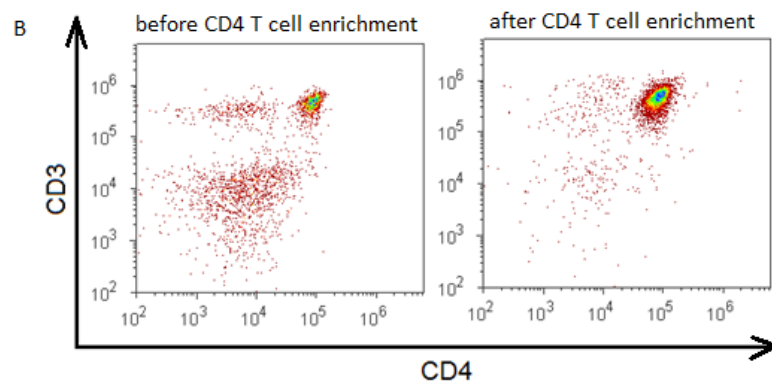
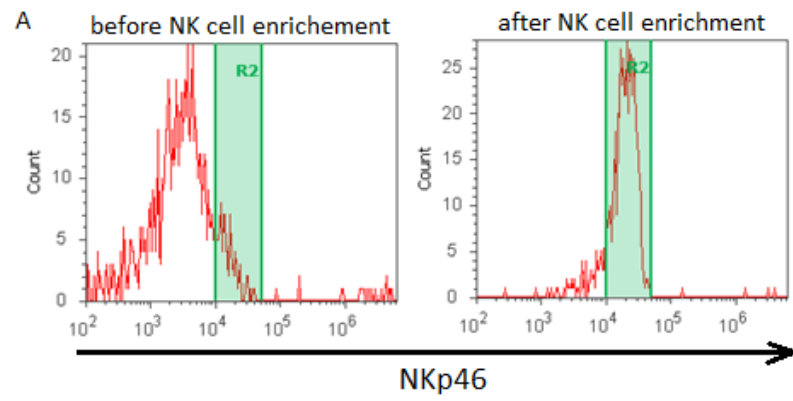


Figure 25 *Rae-1 levels on tumor cells after co-incubation with specific immune cell depleted, the co-administration treated splenocytes.* Splenocytes were collected from BALB/C mice with CD8⁺T cell (2.43), $\gamma\delta$ T cells (Tgd), CD4 T cell (GK1.5), NK cells (GM1) depletion or no depletion (IgG) as well as the co-administration treatment. CT26 cells were co-incubated with the certain immune cell depleted splenocytes. Flow cytometry assay was performed to determine Rae-1 level change on tumor cells. The lower panel shows the graph of the statistical analysis among different treatments. The result represents three replicates. One way ANOVA and Dunnett analysis was selected for the statistics. Every other group was compared with the wild type CT26 cells. *, $p < 0.05$. **, $p < 0.01$.



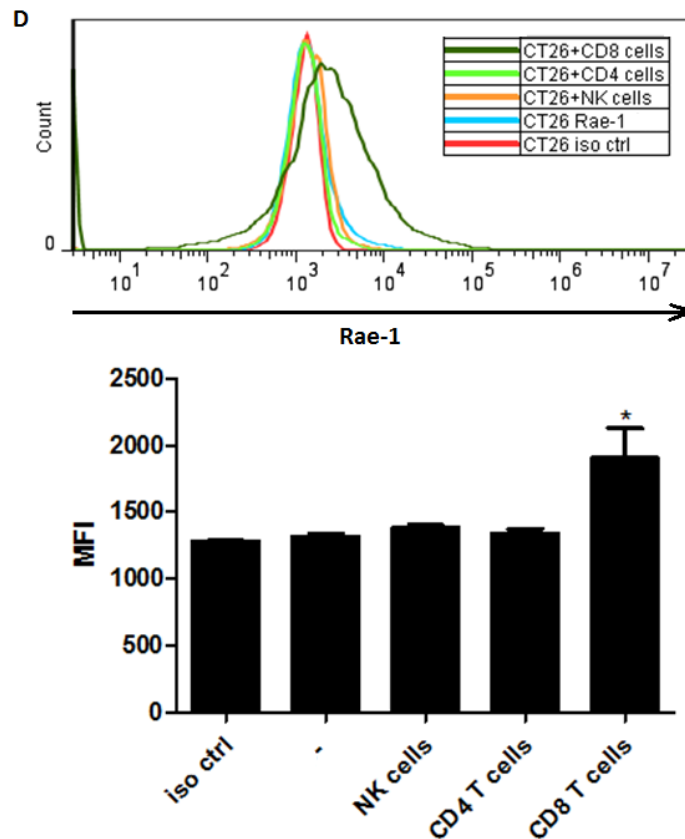
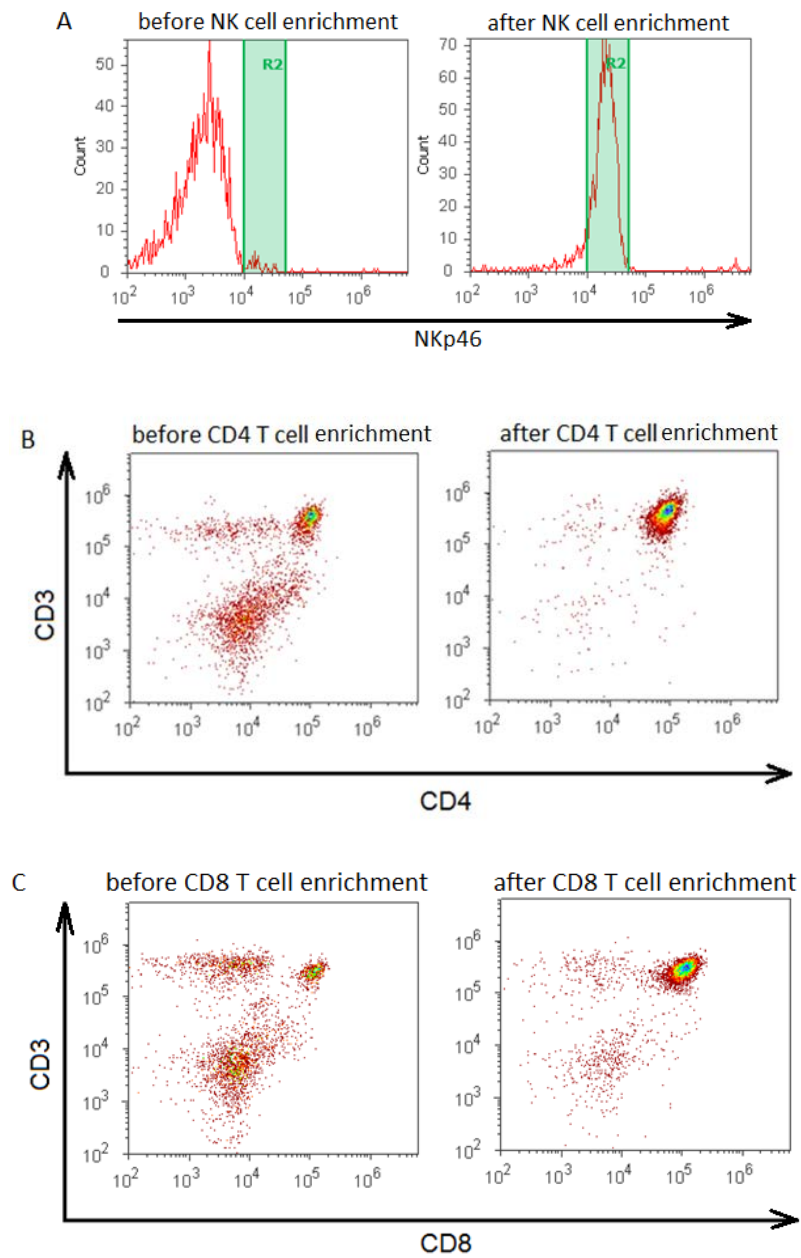


Figure 26 *Rae-1* induction on CT26 tumor cells after co-incubation with different sub populations of immune cells. NK cells (A), CD4 T cells (B) and CD8⁺T cells (C) were enriched by using the corresponding immune cell enrichment kits as described in Material and Methods (Chapter 2). The immune cell enrichment efficiency was tested via flow cytometry assay. (D) CT26 cells were co-incubated with enriched NK cells, CD4 T cells or CD8⁺T cells. Rae-1 level was examined on tumor cells after co-incubation with different sub population of immune cells comparing to the wild type tumor cells via flow cytometry assay. The lower panel shows the statistical analysis of Rae-1 in different treatment groups based on the Rae-1 MFI value in flow cytometry assay. This result represents three independent replicates. *, p<0.05.



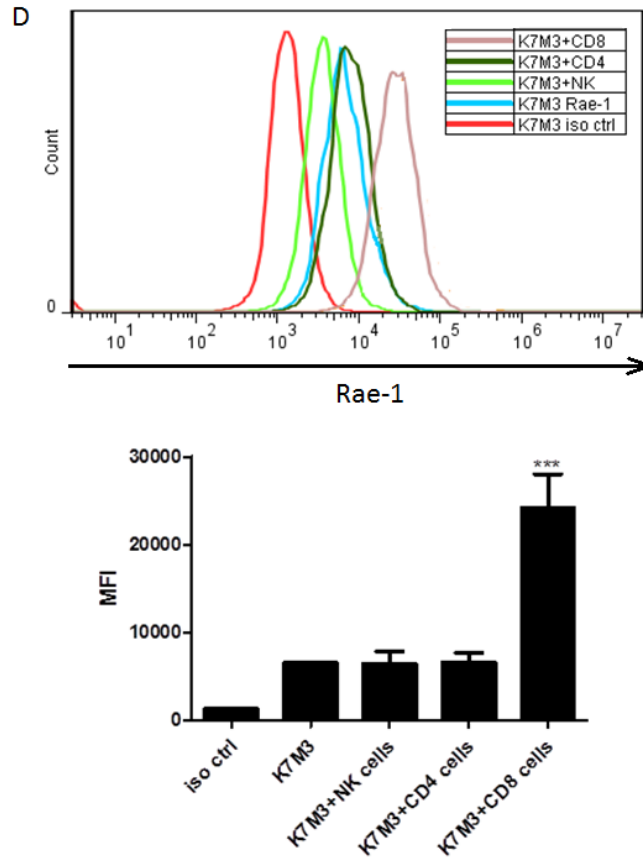
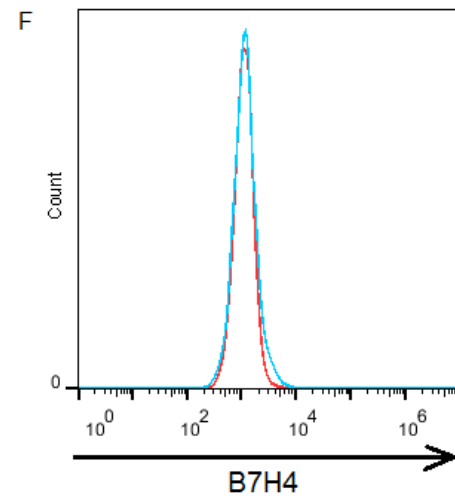
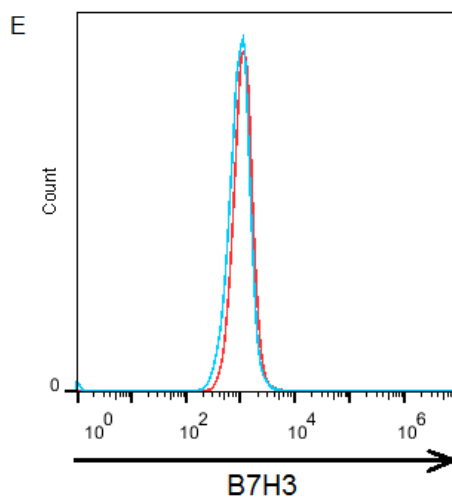
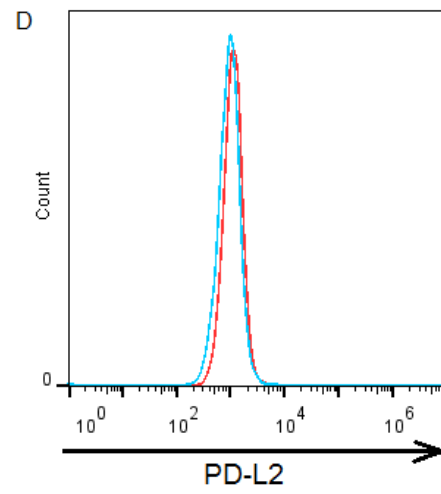
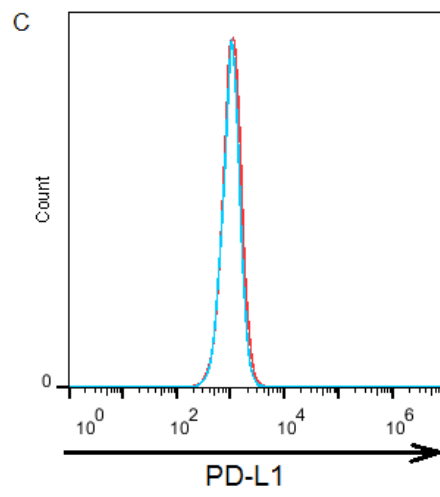
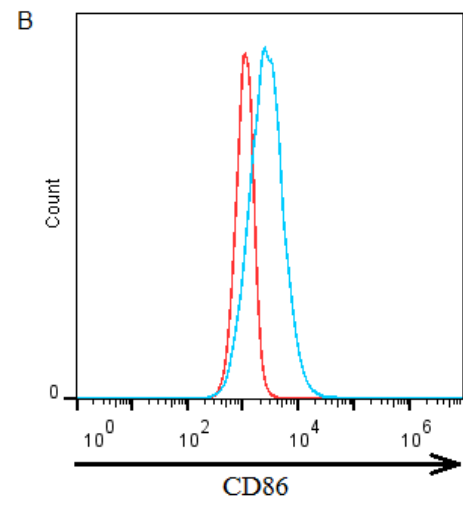
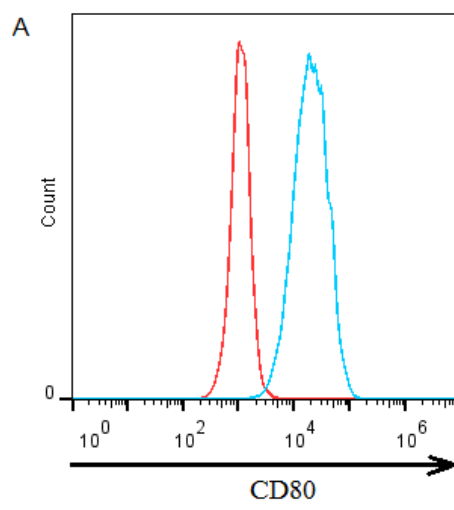


Figure 27 *Rae-1* induction on K7M3 tumor cells after co-incubation with different sub populations of immune cells. NK cells (A), CD4 T cells (B) and CD8⁺T cells (C) were enriched by using the corresponding immune cell enrichment kits as described in Material and Methods (Chapter 2). The immune cell enrichment efficiency was tested via flow cytometry assay. (D) K7M3 cells were co-incubated with enriched NK cells, CD4 T cells or CD8⁺T cells. Rae-1 level was examined on tumor cells after co-incubation with different sub population of immune cells comparing to the wild type tumor cells via flow cytometry assay. The lower panel shows the statistical analysis of Rae-1 in different treatment groups based on the Rae-1 MFI value in flow cytometry assay. This result represents three independent replicates. ***, $p < 0.001$.

The expression levels of B7 and TNF family receptors among multiple tumor cells lines

To determine which receptor in the B7 family (CD80, CD86, PD-L1, PD-L2, B7H3 and B7H4) and in the TNF family (CD40, OX40L, 41BBL and CD70) involves in the activated splenocytes-mediated Rae-1 induction, the expression levels of each receptor was examined on tumor cells (Figure 28). The highly expressed receptor is more likely to interact with splenocytes and transduce the signals to induce the Rae-1. The expressing levels of each receptor were analyzed via flow cytometry. On CT26 cells, CD80 had the highest expressing level among all the receptors (Figure 28A), and CD86 was also stained positive (Figure 28B). However, other receptors including PD-L1, PD-L2, B7H3 and B7H4 in B7 family (Figure 28 C-F), as well as CD40, OX40L, 41BBL and CD70 in TNF family were negative (Figure 28 G-J). On K7M3 cells, CD80 also showed positive (Figure 29A), but CD86 was not expressed (Figure 29B). Therefore, CD80 seemed to be the most possible receptor leading to Rae-1 induction. To confirm this hypothesis, we tested the CD80 expression levels on LM8 (murine osteosarcoma lung metastasis cell line) cells in which the activated splenocytes failed to induce Rae-1 expression (Figure 30A). As we expected, LM8 is a CD80 negative cell line (Figure 30B). Our results reveal that CD80 is the best candidate of the critical receptor which communicates with activated splenocytes and in turn transmits the signal to induce Rae-1 expression.



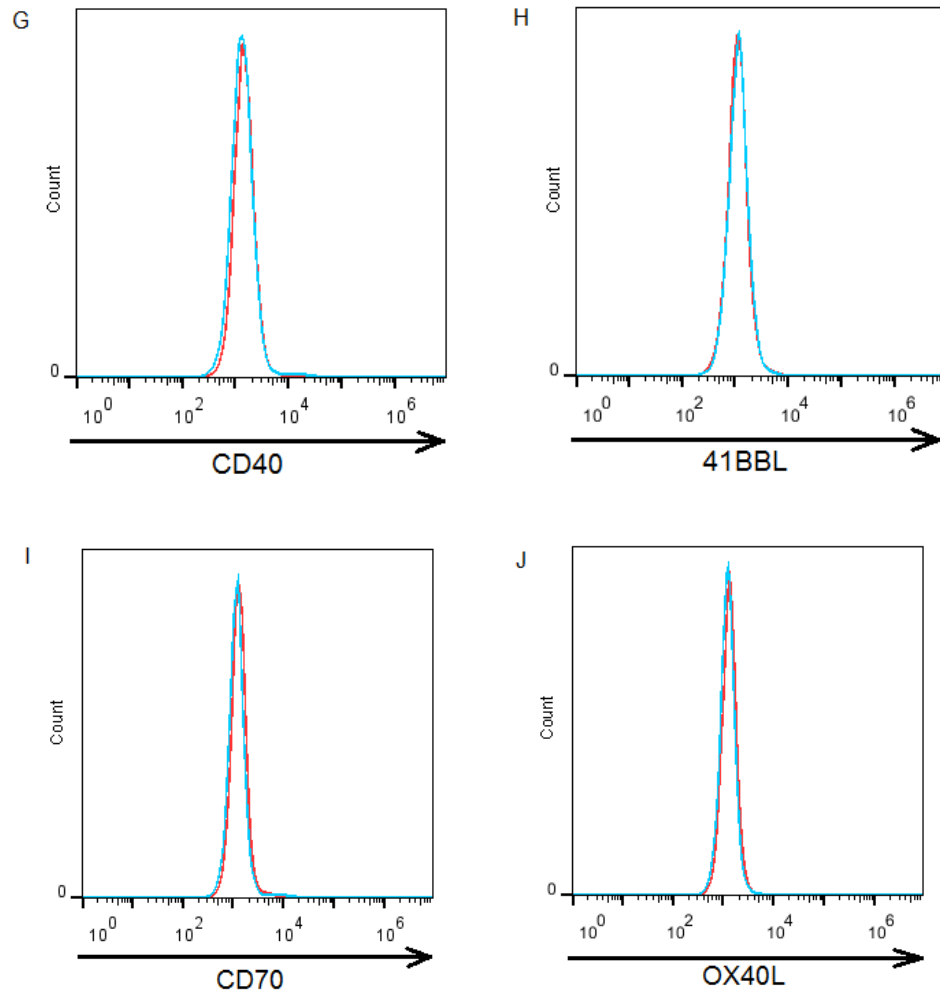


Figure 28 *B7 family and TNF family receptor expression levels on CT26 cells.* CD80 (A), CD86 (B), PD-L1 (C), PD-L2 (D), B7H3 (E), B7H4 (F), CD40 (G), 41BBL (H), CD70 (I) and OX40L (J) expressing levels were tested on CT26 cells comparing to corresponding isotype controls (red) via flowcytometry.

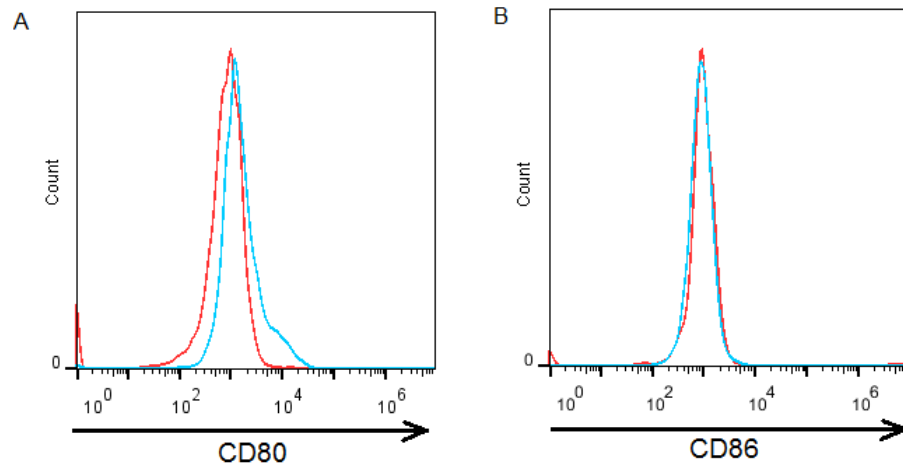


Figure 29 *CD80 and CD86 expression levels on K7M3 cells.* CD80 (A) and CD86 (B) expressing levels were tested on K7M3 cells comparing to the isotype control (red) via flow cytometry.

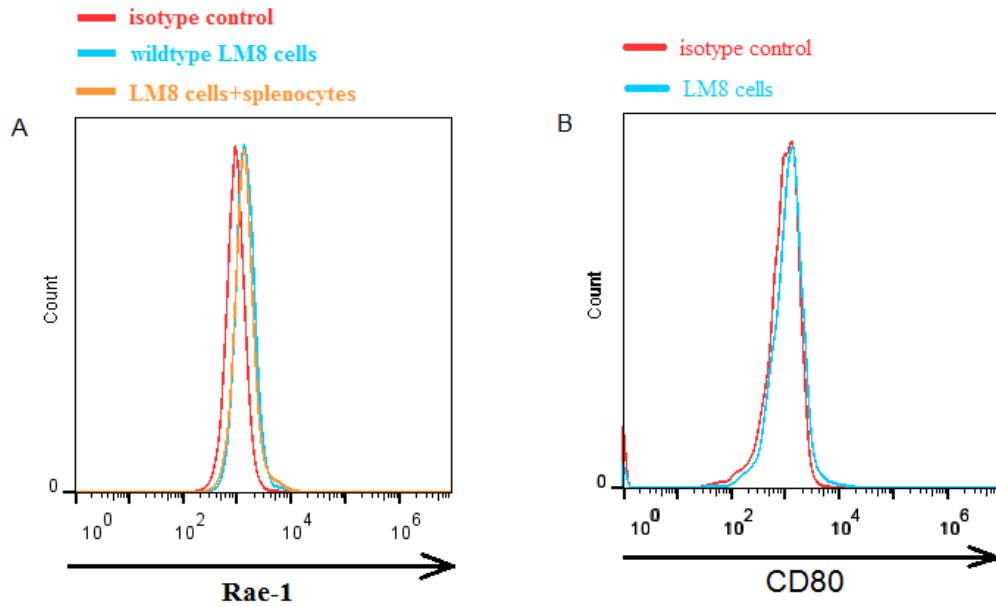


Figure 30 *CD80 expression level on LM8 cells.* (A) Rae-1 expression on LM8 cells. LM8 cells were co-incubated in the presence or absence of activated splenocytes. Rae-1 expression level was determined by using flow cytometry. (B) CD80 expressing level (blue) was tested on LM8 cells comparing with isotype control (red) via flow cytometry.

CD80 overexpression in LM8 cells can facilitate the activated lymphocyte-mediated Rae-1 induction

To investigate whether CD80 is sufficient to facilitate the activated splenocytes-mediated Rae-1 induction in tumor cells, we exogenously introduced CD80 into LM8 cells. LM8 cells were transfected with either *CD80* plasmid DNA or control vector by using electroporation (Figure 31). Since LM8 is a CD80 negative cells line, splenocytes co-incubation fails to induce Rae-1 on LM8 cells (Figure 30A). After CD80 overexpression in LM8 cells, the activated splenocytes mediated Rae-1 induction is expected. CD80 expression level was examined after CD80 encoding DNA or control vector transfection to confirm the transfection efficiency (Figure 31A). In presence or absence of activated splenocytes co-incubation, we compared Rae-1 expression levels in control vector- versus CD80 encoding DNA-transfected LM8 cells. As expected, the activated splenocytes-mediated Rae-1 induction was observed in the CD80 encoding DNA transfected LM8 cells, but not in the control vector-transfected LM8 cells (Figure 31B). We concluded that CD80 holds key for activated splenocytes-mediated Rae-1 induction, suggesting that CD8⁺T cells binding to CD80 is sufficient to induce Rae-1 in tumor cells.

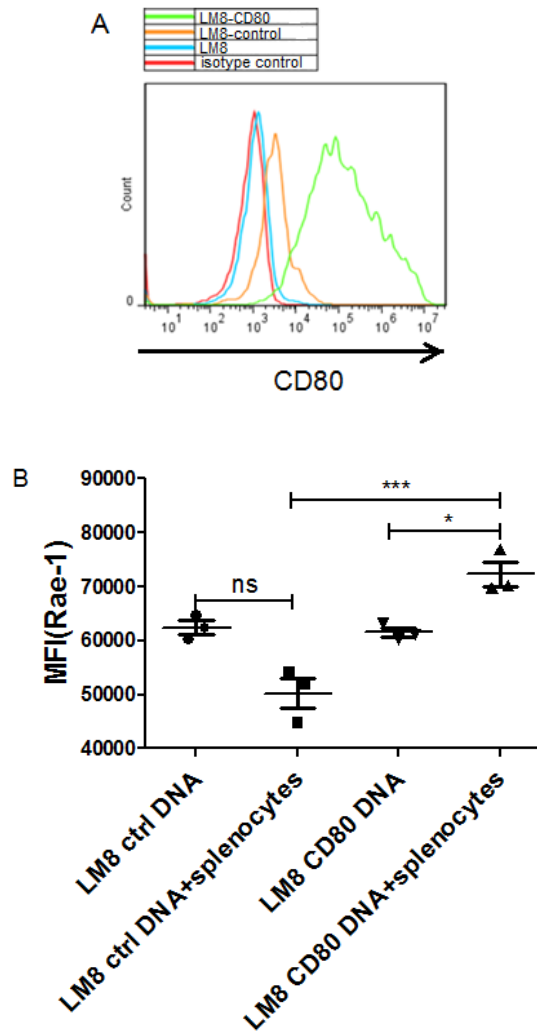


Figure 31 *CD80 overexpression facilitated the splenocytes co-incubation induced Rae-1.* (A) LM8 cells were transfected with control DNA or CD80 DNA. The transfection efficiency was determined by using flow cytometry. (B) Rae-1 level was examined on control DNA or CD80 transfected LM8 cells with or without the splenocytes co-incubation. The result represents three independent replicates. ***, $p < 0.001$. *, $p < 0.05$.

CD80 reduction in CT26 cells can abolish activated splenocytes-mediated Rae-1 induction

To validate activated splenocytes-mediated Rae-1 induction relies on CD80, CD80 expression was reduced by CD80 siRNA transfection in CD80 high CT26 cells (Figure 32). Reduction of CD80 expression was confirmed after the CD80 or sham siRNA transfection (Figure 32A). In the presence or absence of activated splenocytes co-incubation, Rae-1 induction was compared between sham siRNA and CD80 siRNA transfected CT26 cells. After reducing the level of CD80 expression in CT26 cells, a significant decrease in Rae-1 induction was observed, suggesting that CD80 is required for activated splenocytes-mediated Rae-1 induction (Figure 32B).

Our direct incubation, CD80 overexpression and CD80 reduction experiments clearly suggest that Rae-1 induction is triggered by activated splenocytes binding to CD80 on tumor cells.

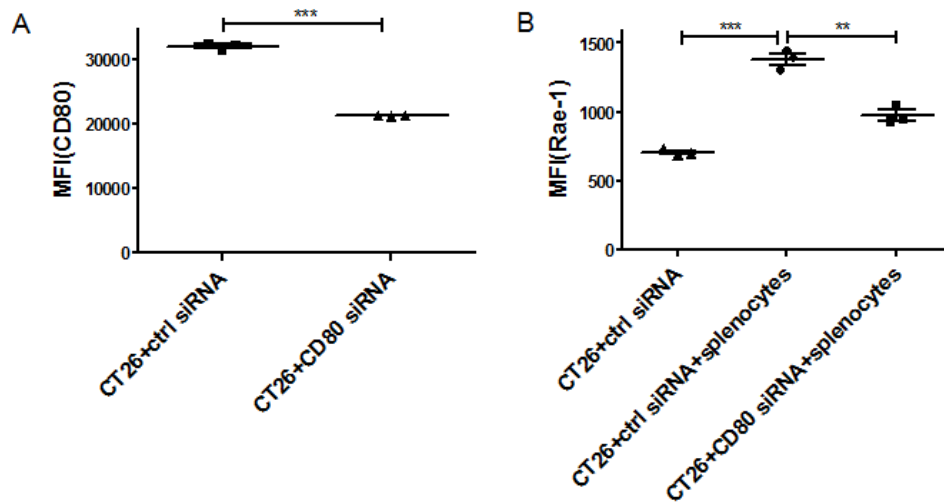


Figure 32 *Reduction of CD80 abolished the splenocytes co-incubation induced Rae-1.* (A) Control siRNA or CD80 siRNA were introduced into CT26 cells. Flow cytometry was performed to determine the CD80 reduction. (B) CT26 cells transfected with control siRNA or CD80 siRNA were co-incubated with splenocytes. Rae-1 level was determined by using flow cytometry. The results represent three replicates. **, $p < 0.01$. ***, $p < 0.001$.

Summary

In this chapter, we illustrated the mechanism through which Rae-1 was induced in tumors by the co-administration of IL-12 DNA plus doxorubicin. The conventional view is that the activation of stress signaling pathway induces Rae-1. Our study found that stress-signaling mediated Rae-1 only operates *in vitro*, but not work *in vivo* (21). A novel CD8⁺T cell-dependent mechanism accounts for IL-12 plus doxorubicin-mediated Rae-1 induction. This mechanism is validated by T cell depletion study (Figure 23) and a T cell deficient model (Figure 23). A newly established *in vitro* model is confirmed in two different tumor cell lines (Figure 21).

In this chapter, not only did we clarify the novel mechanism of Rae-1 induction, we also explained why the co-administration caused impressive improvement of antitumor therapeutic effect. The reason is the co-administration of IL-12 DNA plus doxorubicin could promote NKG2D⁺CD8⁺T cells infiltration into tumor microenvironment, and then the infiltrating CD8⁺T cells facilitate Rae-1 induction on tumor cells via the direct contact with tumor cells (Figure 26-27). The increasing levels of Rae-1 on tumor cells in turn activate the NKG2D receptor function dependent antitumor immune response. Our next question is through what receptor(s) CD8⁺T cells and tumor cells could communicate and induce Rae-1 on tumor cells.

The examination of B7 and TNF family receptors on tumor cells showed CD80 is only receptor that is expressed on both CT26 and K7M3 cells (Figure 28-29). More importantly, there is no CD80 expression on LM8 cells which fail to induce Rae-1 after co-incubating with activated splenocytes (Figure 30). CD80, therefore, becomes the most likely receptor which contributes to the splenocytes-mediated Rae-1 induction.

To validate the critical role of CD80 on Rae-1 induction, we transiently transfected CD80 into LM8 cells by using electroporation. We found that the splenocytes failed to

induce Rae-1 on the control vector transfected LM8 cells, but dramatically induced Rae-1 on the CD80 encoding DNA transfected LM8 cells (Figure 31), suggesting CD80 is crucial and sufficient for Rae-1 induction. To test the role of CD80 on splenocytes-mediated Rae-1 induction, we also reduced the level of CD80 expression on CT26 cells by transfecting CD80 siRNA. We found that CD80 reduction partially abolished Rae-1 induction (Figure 32), suggesting that CD80 is required by the activated splenocytes-mediated Rae-1 induction.

Chapter 8

Discussion

IL-12 is a cytokine produced mainly by antigen presenting cells. Several known mechanisms account for IL-12-mediated antitumor efficacy, including induction of IFN- γ , activation of NK cells, promotion of T_H1 response, and stimulation of CD8⁺T cell antitumor response (148, 149). Likewise, doxorubicin has been shown to act through several mechanisms, including disruption of DNA synthesis, initiation of DNA damage, and compromising the cell membrane, leading to apoptosis (116, 150). Not only can chemotherapeutic agents directly inhibit tumor cell proliferation and survival, some chemotherapeutic agents also prevent immunosuppressive effects by either alone or properly combining them with other treatments (151). Moreover, chemotherapeutic agents were found to promote antitumor immuno-surveillance via facilitating immune cells infiltration into tumor microenvironment and sensitizing tumor cells to antitumor immune response (126). However, the underlying mechanism of therapeutic compound-enhanced immune cell infiltration is not clear. In this study, we hypothesized that doxorubicin may enhance IL-12-mediated NKG2D expression which may promote immune cell localizing in tumors. This hypothesis is supported by others' observation that IL-12 induces modest level of NKG2D expression (65). This hypothesis is also supported by our observation that IL-12 plus doxorubicin increases the number of tumor infiltrating CD8⁺T cells (124), and leads to NKG2D function-dependent antitumor effect (unpublished data).

In chapter 3, we discovered that doxorubicin, which has potent effects against multiple types of tumors, was able to effectively augment IL-12-mediated increase of NKG2D positive splenocytes (Figure 8). We further demonstrated that the subset of splenocytes with

induced NKG2D receptor is CD8⁺T cells, but not NK cells or CD4⁺T cells (Figure 9-11). Different from the old notion in which NKG2D receptor is mainly expressed on NK cells and activates NK cell function, we observed a substantial NKG2D induction on CD8⁺T cells instead of NK cells after the co-administration (Figure 9). This discovery may disturb the traditional view that the NKG2D receptor is expressed mainly on NK cells, but it is not exclusive. For example, there are reports of NKG2D expression on CD8⁺T cells. One study found that mouse NKG2D was expressed only on mature CD8⁺T cells (38). Furthermore, induction of NKG2D and activation of its signaling pathway on CD8⁺T cells could trigger T cell-mediated NK cell-like cytolytic activity (38). This observation was validated by both NK and T cell-depletion studies (Figure 10-11). We found in CD8⁺T cell-depleted tumor-bearing mice, the co-administration failed to induce NKG2D receptor on lymphocytes, but in NK cell depleted tumor-bearing mice, just like in wild-type mice, NKG2D receptor was induced on lymphocytes and CD8⁺T cells (Figure 10-11). These results clearly show that CD8⁺T cells but not NK cells are critical for NKG2D induction by the co-administration of IL-12 and doxorubicin.

Our study via different approaches shows that NKG2D high expressing cells efficiently infiltrate into tumors. A substantial increase of NKG2D receptor in tumors was detected after the co-administration by using northern blot (Figure 12A), suggesting that IL-12 plus doxorubicin could promote NKG2D positive immune cells infiltrating into tumors. In the immunocytochemistry assay, we observed a dramatic promotion of NKG2D and CD8⁺T positive immune cells in the tumor sections receiving IL-12 plus doxorubicin treatment (Figure 12B), but we did not observe any NKG2D⁺CD4⁺T cells or NK cells in tumor sections received any treatment (Figure 12C, 12D). To better demonstrate our discovery, we performed co-localization staining on tumor sections from NK cell depleted mice and CD8⁺T cell depleted mice. Not surprisingly, NK cell depletion did not affect the NKG2D⁺

CD8⁺T cell infiltrating into tumors after the co-administration (Figure 13A). However, CD8⁺T cell depletion totally abolished the co-administration-promoted NKG2D positive immune cell infiltrating into tumors (Figure 13B). Our results suggest that upregulation of NKG2D in CD8⁺T cells by doxorubicin plus IL-12 appears to be critical for the increased infiltration of immune cells into tumors.

We noticed that the IL-12 plus doxorubicin-mediated NKG2D induction on lymphocytes or CD8⁺T cells is just 3-5 times higher than IL-12 or doxorubicin treatment alone (Figure 8), but the co-administration promoted NKG2D⁺ CD8⁺T cell infiltration is more than 10 times higher than the single treatment alone (Figure 12A, 12B). The discrepancy is very likely caused by the duration of NKG2D expression by the co-administration of IL-12 plus doxorubicin. The persistent expression of NKG2D receptor on CD8⁺T cells by the co-administration gave rise to immune cell infiltration into the tumors.

Results from others showed that IL-12 alone could induce modest level of NKG2D receptor on lymphocytes (65), but our discovery suggests that doxorubicin is not only a cytotoxic chemical compound but also, at low doses, a booster of immune response. This discovery illustrates that chemotherapy-induced promotion of antitumor immune response and regulation of T-cell localization in tumors could be achieved through induction of NKG2D expression on CD8⁺T cells. Moreover, there is currently no established biomarker for chemotherapeutic agents-induced immune response. From a broad point of view, this discovery shows that measuring NKG2D expression in circulating T cells may be a valuable screening method to discover new immune stimulatory medicines.

Currently, there are few reliable markers to predict antitumor immune responses. We believe that increased expression of NKG2D in CD8⁺T cells may be established as an antitumor immune response endpoint, because one approach to eradicating tumor cells is to

create an environment in which effector immune cells constitutively express the NKG2D receptor, thereby triggering NKG2D-dependent tumor cell death (28). Others found that a modest dose of doxorubicin had the potential to boost immune response and potentiate the IL-2 response against tumor cells (152-154). In fact, another report demonstrated that the doxorubicin-mediated therapeutic effect against cancer requires CD8⁺T cells and IFN- γ (155). Although the mechanism was unknown in both cases, we speculate that the immune response may be boosted by upregulating NKG2D through a combination of doxorubicin plus IL-2 or doxorubicin plus IFN- γ . Of course, IL-2 and IFN- γ are different from the IL-12 that we used in this study, but they are all T_H1 cytokines which induce signals in T cells.

One significant question is how these NKG2D⁺ CD8⁺T cells infiltrated into tumors. In tumor immunosurveillance, immune cells could be bound to tumor cells by NKG2D-NKG2D ligand interaction. So next, we investigated whether the co-administration could also increase NKG2D ligands level on tumor cells, and therefore facilitating NKG2D⁺ CD8⁺T cells infiltrating into tumors.

Others have shown that chemotherapeutic agents induce NKG2D ligands on tumor cells *in vitro* (58), our results support this possibilities because different levels of NKG2D ligand Rae-1 induction were found in tumor cells after the treatment of various chemotherapeutic agents (Figure 14A, 14B). However, Rae-1 induction was not observed in tumors from K7M3 tumor-bearing mice which received the same chemotherapy treatment as we used *in vitro* (Figure 14C). It means that unlike the *in vitro* cell treatment, systemic injection of chemotherapeutic agents *in vivo* failed to induce Rae-1 in tumors. This result agrees with our previous observations, in which we found although NKG2D receptor was induced by doxorubicin, there was no tumor infiltrating NKG2D⁺ CD8⁺T cells detected, suggesting that the substantial increase of NKG2D⁺ CD8⁺T cells infiltrating into tumors after the co-administration is very likely due to Rae-1 induction in tumors. The co-

administration of IL-12 plus doxorubicin could best induce Rae-1 in tumors comparing to other combinations of cytokines and chemotherapeutic agents (Figure 15). This result is not surprising, because we have demonstrated in Chapter 3-4 that the co-administration could increase NKG2D positive CD8⁺T cells and dramatically promote NKG2D⁺ CD8⁺T cell infiltrating into tumors (Figure 12). The tumor cells with increasing levels of Rae-1 could attract more NKG2D⁺ CD8⁺T cells infiltrating into tumor microenvironment. The increase of Rae-1 on tumor cells and NKG2D receptor on CD8⁺T cells enhanced the NKG2D-NKG2D ligand interaction-mediated antitumor immune response, which absolutely explained why the co-administration could induce much better antitumor therapeutic effect against tumors.

We also noticed in previous study that the co-administration had long term effect to inhibit primary tumor growth as well as to prevent metastatic tumor occurrence. This led us to test whether the co-administration mediated Rae-1 induction in tumors was also a long term effect. A time course study was performed to determine Rae-1 levels in tumors at different time points (Figure 18). We found that Rae-1 induction in tumors by the co-administration started after the second treatment. Both IL-12 alone and the co-administration could induce Rae-1 4 days after the second treatment, but the co-administration could induce Rae-1 at a higher level for at least 8 days after the second treatment. Only the co-administration could maintain Rae-1 induction in tumors for this long. Such long term induction of Rae-1 effectively reduces the dose and frequency of cancer treatment so as to reduce toxicity (Figure 18).

To understand whether the Rae-1 induction by the co-administration was tumor model specific, we tested our 4 standard treatments (control DNA, control DNA plus doxorubicin, IL-12 DNA or IL-12 DNA plus doxorubicin) on several tumor models in both BALB/C and C57BL/6 mice. Rae-1 expression levels were examined by using western blotting, flow

cytometry and immunohistochemistry assays (Figure 16). We clearly showed that the co-administration could dramatically increase Rae-1 in all the tested tumor models by different assays (Figure 16). Another concern was whether Rae-1 induction by the co-administration also occurred in normal organs. If so, the NKG2D⁺ CD8⁺T cells could also infiltrate into normal organs to kill normal cells, which would cause severe toxicity to patients. Therefore, it was important for us to determine whether the Rae-1 induction was tumor specific. We examined Rae-1 expression levels in hearts, livers, spleens, lungs and kidneys (Figure 17). Fortunately, we did not detect any Rae-1 induction after the co-administration in the normal organs (Figure 17), meaning that the Rae-1 induction after IL-12 plus doxorubicin treatment was tumor specific. It makes the NKG2D⁺ CD8⁺T cells more efficiently infiltrate into tumors to recognize the Rae-1 positive tumor cells with very limited side effects and toxicity to the normal organs.

In Chapter 6, we studied the mechanism by which Rae-1 is induced by the co-administration *in vivo*. The traditional mechanism for Rae-1 induction is through the DNA damage-induced stress signaling (58). We wanted to determine whether the co-administration mediated Rae-1 induction is also through the stress signaling or through a novel mechanism. Firstly, we ruled out the possibility that the Rae-1 induction was through the DNA damage-induced stress signaling ATM/ATR-chk1/chk2 (Figure 19), which means the Rae-1 induction by the co-administration was through a novel mechanism. Since our treatment could promote immune cell infiltrating into tumors to enhance tumor immunosurveillance, the possible novel mechanism should be immune cell dependent. Our *in vitro* result showed that the Rae-1 induction was not due to the cytokines released from activated splenocytes (Figure 20). Another hypothesis was Rae-1 induction was induced by the direct communication between the tumor cells and the immune cells in tumor microenvironment. Rae-1 expression levels were examined after the direct incubation of

tumor cells with activated splenocytes, and a dramatic induction of Rae-1 was observed on both murine tumor cell lines we tested (Figure 21). Moreover, we demonstrated that human NKG2D ligands could also be induced after co-incubating human colon tumor cells or osteosarcoma cells with stimulated human PBMCs (Figure 22). This result on human tumor cells suggests that the IL-12 plus doxorubicin co-administration may yield the same therapeutic effect in human cancer patients as in tumor-bearing mice. Results from others indicated that high levels of NKG2D ligands in tumors associated with long term survival in colorectal cancer patients (66), neuroblastoma patients (Oncogenomics) and Triche Rhabdomyosarcoma patients (Oncogenomics). Here, we proposed a novel mechanism of Rae-1 induction on tumor cells by direct contact of tumor cells with activated splenocytes. This novel mechanism explained how Rae-1 could be induced in tumors by the co-administration, and also showed the great potential to be applied in cancer treatment.

Moreover, we found that the co-administration failed to increase Rae-1 in the T cell deficient mice while substantially inducing Rae-1 in the wild-type mice bearing the same tumor model (Figure 24). As the nude mice lack mature T cells, our result suggests that the Rae-1 induction by the co-administration required mature T cells (Figure 24). *In vivo*, we also performed the co-administration on tumor-bearing mice with certain immune cell (CD4⁺T cells, CD8⁺T cells or NK cells) depleted, and tested Rae-1 expression levels in tumors (Figure 23). We found Rae-1 was dramatically decreased only in CD8⁺T cell depleted mice, but not in other immune cell depleted mice or the control antibody treated mice. *In vitro*, consistent with the result from the *in vivo* experiment, only the CD8⁺T cell-depleted splenocytes failed to induce Rae-1 on tumor cells (Figure 25), meaning that CD8⁺T cells were the crucial subpopulation of immune cells that interact with tumor cells and increase Rae-1 *in vivo* and *in vitro*. Among various enriched immune cell subpopulations, only CD8⁺T cells dramatically induced Rae-1 on tumor cells (Figure 26, 27). NK cells or

CD4⁺T cells failed to change Rae-1 expressing level. Interestingly, in Chapter 3 and 4, we found the co-administration could induce NKG2D receptor only on CD8⁺T cells and then facilitate those CD8⁺T cells to localize in tumor sites (Figure 9, 12). In Chapter 6, we discovered Rae-1 induction by the co-administration was also dependent on CD8⁺T cells (Figure 23, 24).

Not only did we clarify that the novel mechanism of Rae-1 induction requires the physical interaction between immune cells and tumor cells, we also investigated which tumor cell receptor contacts with CD8⁺T cells and transmit the signals to induce Rae-1 in tumor cells. CD8⁺T cells were shown to play the absolutely critical role in the immune cell interaction-mediated Rae-1 induction (Figure 26, 27). Hence, the Rae-1 induction should be through receptors that interact with CD8⁺T cells. Although the co-stimulatory receptors which interact with immune cells and regulate immune response are primarily expressed on antigen presenting cells, we found that some of the receptors are also express on tumor cells. Therefore, we hypothesized that the immune cell-tumor cell interaction-dependent Rae-1 induction was through the co-stimulatory receptors. After the examination of various co-stimulatory receptors on multiple tumor cell lines, we found CD80 is the only receptor that is expressed on both CT26 and K7M3 cells (Figure 28, 29), but not on LM8 cells (Figure 30B). Since the activated splenocytes-mediated Rae-1 induction occurs on CT26 and K7M3 cells (Figure 21), but not LM8 cells (Figure 30A), we hypothesized CD80 plays the critical role in activated splenocytes-mediated Rae-1 induction.

Supporting this hypothesis, overexpression of CD80 in LM8 cells promoted the activated splenocytes-mediated Rae-1 induction (Figure 31B), while the reduction of CD80 from CT26 cells abolished Rae-1 induction (Figure 32B). We demonstrated that the tumor cell and immune cell co-incubation induced Rae-1 induction is through tumor cell receptor CD80. Besides what previous publications had indicated that CD80 and immune cell

interaction could activate immune cells, we found that the immune cell-tumor cell interaction through CD80 also transduces signals to induce Rae-1 in tumor cells.

Taken together, our results from the NKG2D receptor study and the Rae-1 study elucidated the picture of the mechanism (Figure 33): the co-administration augments NKG2D positive CD8⁺T cells and promotes these CD8⁺T cells infiltrating into tumors to contact with tumor cells through CD80, which transduces the signals to induce Rae-1 on tumor cells. Rae-1 induction on tumor cells and NKG2D induction on CD8⁺T cells enhances their interaction, which triggers CD8⁺T cells- mediated NKG2D dependent antitumor cytotoxicity.

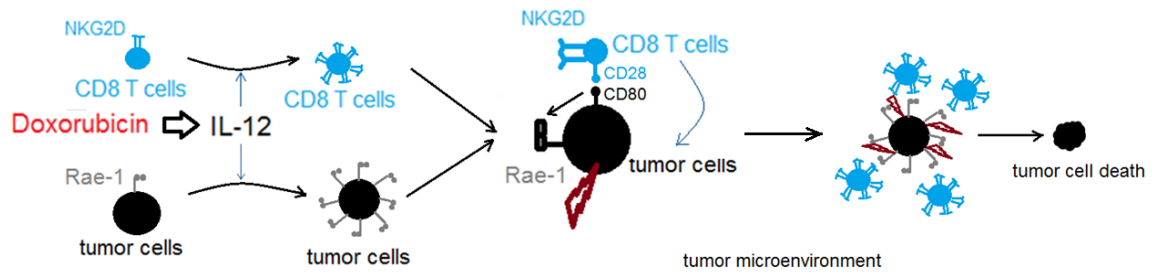


Figure 33 *The mechanism of IL-12 plus doxorubicin induced tumor inhibitory effect.* IL-12 plus doxorubicin treatment could effectively inhibit tumor progression by simultaneously inducing NKG2D receptor on CD8⁺T cells and NKG2D ligand on tumors.

Chapter 8

Conclusion

In summary, we have shown *in vivo* evidence that IL-12 plus doxorubicin induces CD8⁺T cell-specific NKG2D upregulation (Figure 8-11). This upregulation facilitates the infiltration of these NKG2D-expressing CD8⁺T cells into the tumor site (Figure 12-13). The interaction between CD8⁺T cells and tumor cells was through CD80 to induce NKG2D ligand Rae-1 levels in the tumor cells (Figure 26, 27, 31, 32). The Rae-1 induction is due to the direct contact to CD80 in tumor cells. Simultaneous induction of NKG2D receptor on CD8⁺T cells and NKG2D ligand on tumor cells after IL-12 plus doxorubicin treatment seems to be the real mechanism to boost the antitumor immune response. Therefore, the improved therapeutic effect against primary and metastatic tumors by the IL-12 plus doxorubicin treatment is largely dependent on the NKG2D function *in vitro* and *in vivo*.

Chapter 9

Future directions

Rae-1 regulation in tumor cells

NKG2D ligands are present on tumor cells at different levels. The regulation of NKG2D ligands expression associates with complex processes that have not been fully understood. Recent publications suggest that besides gene transcription and translation, multiple post-transcriptional and post translational modifications also contribute to NKG2D ligands expression levels (56, 156, 157). MicroRNAs targeting MICA and MICB 3'UTR have been discovered to downregulate the protein levels (158). Mult1, a murine NKG2D ligand, was expressed at high level of mRNA in certain normal cells, but absent at protein level. The mechanism is that Mult1 protein underwent ubiquitination in normal cells, which was reduced upon stress stimulation to regulate the protein levels (159). HDACs (histone deacetylase) upregulation in cancers were recently proved to associate with repression of tumor suppressor genes. HDAC inhibitors such as TSA (trichostatin A) and sodium butyrate were reported to induce NKG2D ligands on tumor cells *in vitro* (57, 160). One possible well studied mechanism involves in the remodeling of chromatin by histone acetylation which opens the chromatin structure and allows the access to transcription factors. Another novel mechanism participates in directly adding acetyl group to lysines on proteins, in our case, NKG2D ligands. Protein acetylation could also contribute to protein stabilization (161).

Our preliminary data suggested that neither the co-administration *in vivo* nor the splenocytes co-incubation *in vitro* induce Rae-1 at mRNA level in tumor cells. The

translational signaling is not dramatically activated by the treatment either. Therefore, it is very likely that epigenetic changes account for Rae-1 induction after the co-administration or splenocytes co-incubation. Firstly, we are going to determine whether Rae-1 increase is because of Rae-1 stabilization. To address this question, we will treat tumor cells with translation inhibitor in the presence or absence of splenocytes incubation. If we observe an acute decrease of Rae-1 expression on tumor cells in the absence of splenocytes, but a gradual downregulation of Rae-1 expression in the presence of splenocytes, it means the splenocytes co-incubation could stabilize Rae-1 after shutting down the translation signaling and therefore to increase Rae-1 level. Secondly, we will be interested in studying which post-translational modification could contribute to Rae-1 stabilization. Since results from others suggested ubiquitination and acetylation occur in some other NKG2D ligands to regulate the expression levels, it will be exciting to investigate whether our treatment could inhibit ubiquitination-mediated degradation or enhance acetylation-mediated stabilization. Last but not least, we are going to investigate what is the intracellular mediator which is recruited to CD80 after CD8⁺T cells binding to tumor cells, and initiates the regulation of Rae-1 post-translational modification.

Adoptive T cell transfer

Adoptive cell transfer has been successfully applied in clinical trials in cancer treatment. Several trials have shown the impressive effect of adoptive T or NK cell transfer on extending patients' survival. For example, 113 stage II and III NSCLC patients received TIL plus IL-2 or control treatment, and the patients' median survival was increased 8 months by the TIL plus IL-2 treatment ($n < 0.05$) (162). In another trial, 88 melanoma patients were given TIL plus IL-2 or IL-2 alone. The TIL treatment dramatically decreased the relapse rate ($p = 0.028$), and increased the survival time ($p = 0.039$) (162). Other cancers including gastric cancer and renal cancer also respond to the adoptive cell transfer treatment

(162). The adoptive cell transfer strategy has experienced different steps, from TIL transfer in which the TILs are isolated from patients, *ex vivo* expanded in the presence of IL-2, and reinfused back into the patient. The expanded tumor targeting lymphocytes are expected to migrate into tumors with antitumor cytotoxicity. A more effective strategy is to engineer tumor-targeting molecules on lymphocytes before expanding the cells. Therefore, the reprogrammed lymphocytes are directed to eradicate the target tumor cells. A promising approach is the chimeric antigen receptor (CAR) expressing T cell transfer in which single chain monoclonal antibody (e.g. anti-CD19 CAR) is graft onto T cell by retrovirus or sleeping beauty transposon-transposase system (163). However, in some cases, introducing an exogenous molecule onto lymphocytes may cause cell apoptosis.

Our study suggests that the co-administration of IL-12 plus doxorubicin could promote NKG2D on CD8⁺T cells and induce NKG2D ligands on tumor cells. These findings led us to believe that a novel strategy of adoptive cell transfer could rely on inducible receptors. We treat lymphocytes with IL-12 plus doxorubicin, which should induce NKG2D expression on CD8⁺ T cells. The NKG2D⁺ CD8⁺ T cells will be isolated and expanded. Then, we will treat the host also with the co-administration to induce NKG2D ligand expression in tumors. With induced NKG2D ligand in tumors, NKG2D⁺ CD8⁺ T cells are expected to effectively infiltrate into tumors and kill NKG2D ligand positive tumor cells. The advantage of our adoptive cell transfer strategy is that we induce NKG2D receptor on immune cells instead of using any gene delivery method that could be risky to the host. Moreover, we induce NKG2D ligands on tumor cells, which facilitates the transferred immune cells more efficiently targeting to tumors.

Chapter 10

Translational implications

A novel function of chemotherapeutic agents has been identified to facilitate immune effector cells infiltrating into tumors and sensitize tumor cells to immune cell attack. Immune response becomes a potential predictor of the therapeutic outcomes in cancer patients after chemotherapy. However, the mechanism by which chemotherapy promotes immune responses is largely unknown. We discovered that a chemotherapeutic agent doxorubicin effectively promoted IL-12-induced NKG2D expression in CD8⁺T cells. NKG2D induction in CD8⁺T cells in turn regulated tumor-specific localization of CD8⁺T cells and associated with NKG2D-dependent inhibition of tumor growth and metastasis *in vivo*. This discovery is significant in chemo-immune therapy because our discovery illustrates at a molecular level how chemotherapy promotes an antitumor immune response and directs T cell to localize into tumors. Our study also suggested that increased expression of NKG2D will possibly serve as an established antitumor immune response endpoint of chemotherapy in cancer patients.

Besides, our findings of the simultaneous induction of NKG2D on CT8⁺T cells and NKG2D ligand on tumor cells after IL-12 plus doxorubicin treatment could contribute to inducible adoptive T cell transfer in cancer treatment. Current adoptive cell transfer treatment started from collecting tumor infiltrating immune cells, expanding these cells *ex vivo*, and eventually reintroducing these immune cells back into the patient to boost the tumor targeting immune response. An improved strategy is to reprogram immune cells from healthy donors with CAR (chimeric antigen receptor), which is usually a single chain antibody targeting to a specific molecule on tumor cells, and then introduce the expanded immune cells to cancer patients. The problems of current strategy include: Firstly, the gene

delivery system such as retrovirus system could be dangerous to patients. Secondly, some proteins (e.g. NKG2D) after overexpression induce cell apoptosis. Our inducible system could avoid above problems, because the IL-12 plus doxorubicin treatment simultaneously induces NKG2D receptor and ligands. NKG2D receptor can be induced by IL-12 plus doxorubicin treatment, therefore the induction of NKG2D does not need any gene delivery method. Then, NKG2D ligands can also be induced in tumors by the co-administration. Adoptive transfer of NKG2D⁺ CD8⁺T cells to patients who received the IL-12 plus doxorubicin treatment should direct the CD8⁺T cells specifically targeting to tumors. The NKG2D ligand induction in tumors should greatly improve the efficiency of antitumor immune response.

Our IL-12 plus doxorubicin treatment could also combine with other treatment to enhance the antitumor efficacy. Publications from others already suggested that high levels of NKG2D ligands in tumors benefit cancer patients with longer survival time (62, 66). Our findings suggest that IL-12 plus doxorubicin treatment substantially induces a long term of Rae-1 expression. Therefore, together with other agents, it is expected to be a promising antitumor strategy that associates with substantially improved clinical outcomes.

Appendix

The selection of Rae-1 monoclonal antibody

Rationale

In order to study Rae-1 expression in tumors after each treatment, we need a Rae-1 antibody that can recognize Rae-1 protein in various assays. The commercially available Rae-1 antibodies are mainly for flow cytometry assay. To develop a better monoclonal antibody that specifically recognizes Rae-1 in common used experiments, in collaboration with our core facility, we applied a novel strategy of antigen preparation and animal immunization in the process of generating Rae-1 monoclonal antibody. Since it is the first time to develop Rae-1 antibody in this way, the efficiency of the antibody was unpredictable. The selection of the most efficient clone of Rae-1 monoclonal antibody becomes really important for the later study.

Results

CT26-Rae-1 and CT26-GFP stable cell lines

We stably transfected full length Rae-1 beta in retrovirus vector pBMNGreen into murine cells CT26, the vector transfected cells as control, and immunized animals with the antigen expressing cells. Transfection was confirmed under fluorescence microscope and flow cytometry assay (Figure A1). The details of Rae-1 monoclonal antibody development were described in chapter 2 Materials and Methods.

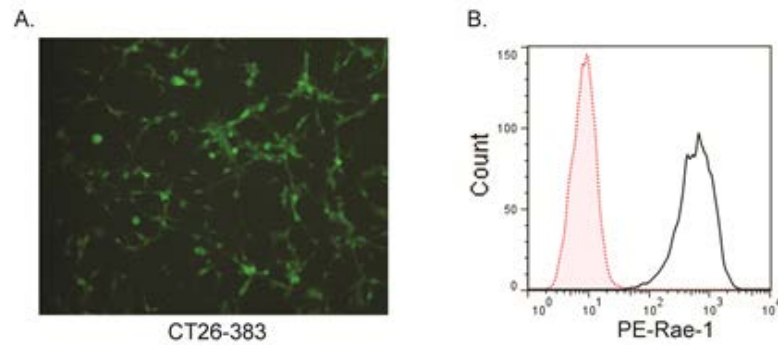


Figure A1 *Rae-1* overexpression in CT26 cells. (A) GFP expression in *Rae-1* stable cell line. CT26 cells were transfected with *Rae-1* in retrovirus vector pBMNGreen fusion with GFP. *Rae-1* stable transfected CT26 cells were observed under fluorescence microscopy to detect GFP expression. (B) *Rae-1* expression in *Rae-1* stable cell line. *Rae-1* stable transfected CT26 cells were stained with PE-*Rae-1* antibody or isotype control antibody. *Rae-1* level was determined by using flow cytometry.

Screening of the effective anti-Rae-1 subclones

We received anti-Rae-1 serum from 60 different subclones. To do the first round screening for the effective antibodies, every 6 antibodies were mixed and stained Rae-1 positive YAC-1 cells in flow cytometry assay (Figure A2). The Rae-1 expression on YAC-1 cells was firstly confirmed by using flow cytometry stained with a commercial purchased Rae-1 antibody (Figure A2A). We found antibodies in group 4 are effective to recognize Rae-1 in flow cytometry assay (Figure A2B). The second round of screening was done to test each anti-Rae-1 serum subclone in group 4 comparing with the commercial purchased Rae-1 antibody (Figure A2C). We found two subclones 52A and 60A which stain positive on YAC-1 cells. 52A seemed almost as good as the commercial antibody, and 60A also recognized Rae-1 in flow cytometry assay.

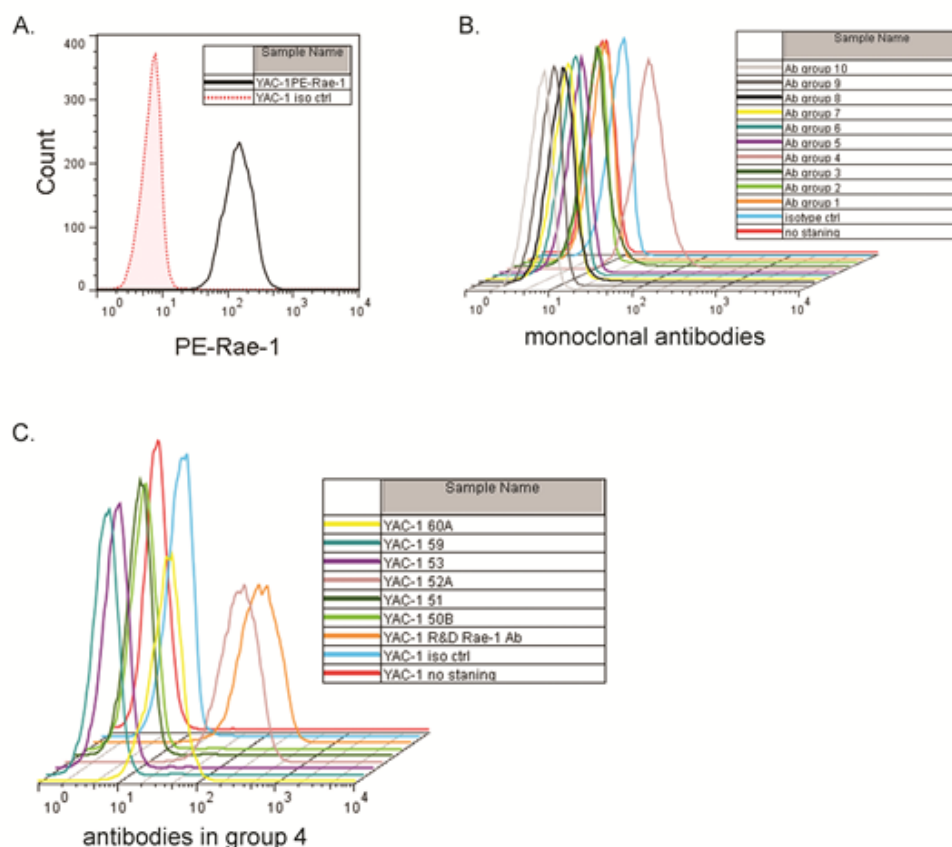


Figure A2 Screening of effective anti-Rae-1 monoclonal antibody. (A) Validation of Rae-1 expression on YAC-1 cells. YAC-1 cells were stained with PE-Rae-1 or isotype control antibody, and Rae-1 expression on YAC-1 cells was confirmed using flow cytometry. (B) First round screening to determine the best group of Rae-1 antibodies. YAC-1 cells were stained with 10 groups of anti-Rae-1 serum mixture. Isotype control antibody was used as the negative control, and the commercial Rae-1 antibody was used as the positive control. The efficiency of anti-Rae-1 serum was determined by using flow cytometry. (C) Second round screening to determine the clones of anti-Rae-1 serum that specifically recognizes Rae-1. YAC-1 cells were stained with each clone of anti-Rae-1 serum in group 4. Isotype control antibody was used as the negative control, and the commercial Rae-1 antibody was used as the positive control. The efficiency of anti-Rae-1 serum was determined by using flow cytometry.

Clone 52A and 60A anti-Rae-1 efficiency determined by ELISA, flow cytometry and immunofluorescence assays

The Rae-1 targeting ability of purified anti-Rae-1 serum from subclone 52A and 60A was tested by using ELISA. The anti-Rae-1 serum from both clones was diluted into different concentrations, and the affinity to Rae-1 protein was measured by reading optical density (OD). The result from ELISA showed that the 52A (Figure A3A) and 60A (Figure A3B) serum even after 50 times and 25 times dilution, respectively, still had strong reaction with Rae-1 expressing cells, suggesting that both clones specifically recognize Rae-1.

To validate the efficiency of Rae-1 monoclonal antibody clone 52A, we stained several Rae-1 negative and positive cell lines, and tested by using flow cytometry. Like the result from commercial Rae-1 antibody, we confirmed Rae-1 positive cell lines K7M3 (Figure A4A), LLC (Figure A4B) and TC1 (Figure A4C), and Rae-1 negative cell line CT26 (Figure A4D) and B16F10 (Figure A4E).

We tested the Rae-1 monoclonal antibody on immunofluorescence assay. Rae-1 overexpressing cells were stained with Rae-1 monoclonal antibody (clone 52A) or isotype control antibody, followed by FITC anti-mouse IgG and DAPI. The slides were observed by using a fluorescence microscopy with appropriate filter (Figure A5). Our Rae-1 monoclonal antibody (clone 52A) could specifically recognize Rae-1 protein on tumor cell surface.

Besides, we also compared our antibody (clone 52A) and commercial Rae-1 antibodies on western blot, immunoprecipitation and immunohistochemistry assays. We observed similar results by using our antibody and the commercial Rae-1 antibodies (data not shown). Therefore, instead of using several commercial Rae-1 antibodies for different assays, in our study, we used our Rae-1 monoclonal antibody (clone 52A).

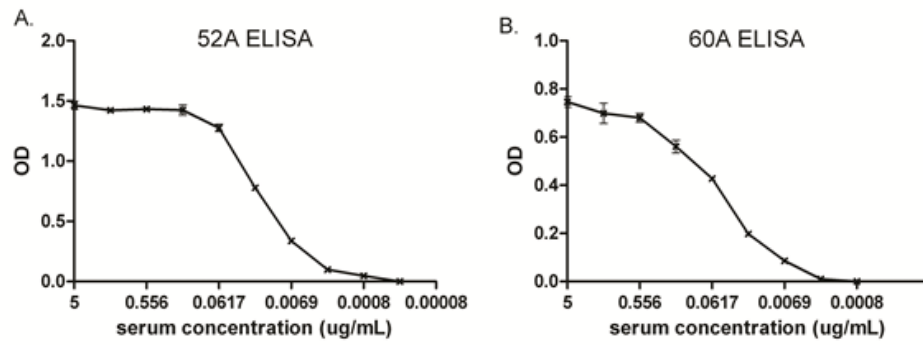


Figure A3 The efficiency of Anti-Rae-1 serum determined by ELISA. Anti-Rae-1 serum clones 52A and 60A were diluted into various concentrations. Their affinity to Rae-1 was determined by using ELISA.

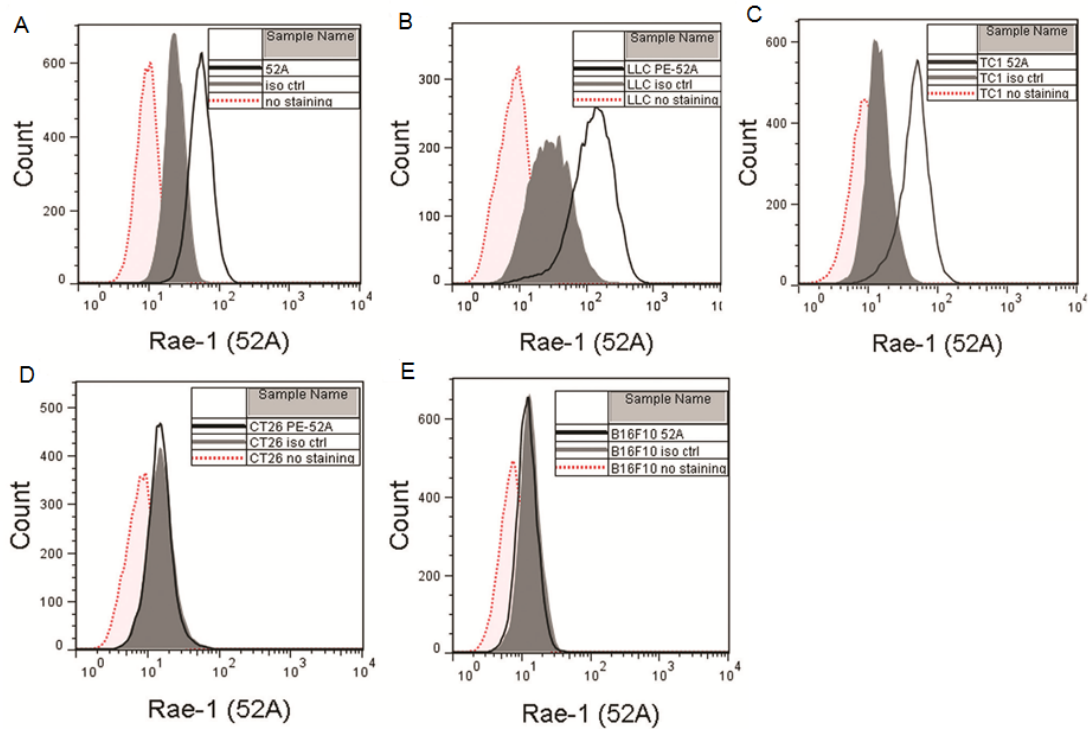


Figure A4 Rae-1 levels on multiple cell lines determined by 52A. K7M3 cells (A), LLC cells (B), TC1 cells (C), CT26 cells (D) and B16F10 cells (E) were stained with anti-Rae-1 antibody (clone 52A) or isotype control antibody, and FITC anti-mouse IgG. Rae-1 level was tested by using flow cytometry.

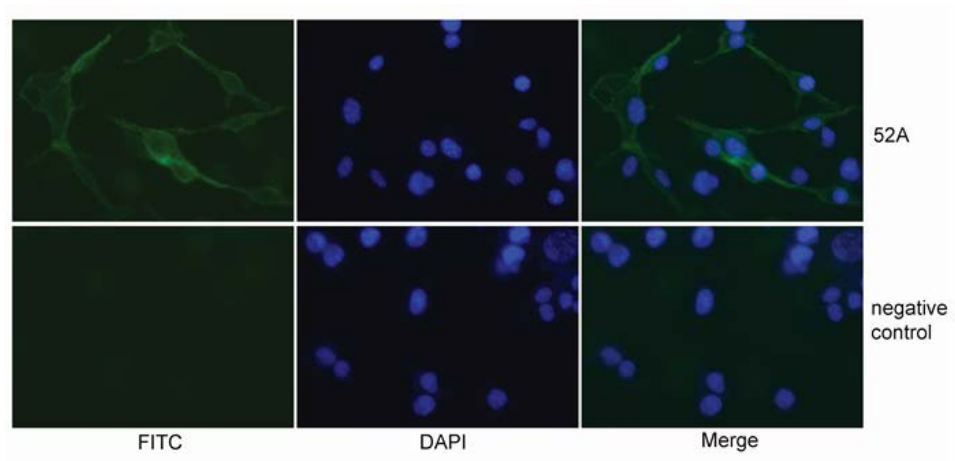


Figure A5 *Rae-1* expression determined by immunofluorescence. Rae-1 overexpressing CT26 cells were stained with Rae-1 antibody (clone 52A) or isotype control antibody, followed by FITC anti-mouse IgG and DAPI. Rae-1 expression was observed using fluorescence microscopy with appropriate filter.

Summary

We developed anti-Rae-1 monoclonal antibody by immunizing mice with a Rae-1 overexpressing stable cell line. The subclones of anti-Rae-1 serum were screened two rounds by testing a Rae-1 positive cell line YAC-1. Two clones of anti-Rae-1 serum specifically recognized Rae-1 on YAC-1 cells, and one clone was comparable to the commercial Rae-1 antibody. We also validated the efficiency of anti-Rae-1 monoclonal antibody (clone 52A) via ELISA, flow cytometry and immunofluorescence assays. In ELISA assay, our Rae-1 monoclonal antibody still had strong reaction with Rae-1 overexpressing cells after 50 times dilution. In flow cytometry assay, the Rae-1 antibody identified Rae-1 positive cell lines K7M3, LLC and TC1. In immunofluorescence assay, Rae-1 overexpressing cells were recognized by the monoclonal antibody. The monoclonal antibody was also proved to be comparable with commercial Rae-1 antibodies on western blot, immunoprecipitation and immunohistochemistry. This monoclonal antibody was used in all the Rae-1 studies.

Rae-1 expression induces tumor rejection

Rationale

NKG2D ligand overexpression-induced tumor rejection was reported in others' study in which Rae-1 and H60 transfected EL4 and B16 tumor cells failed to develop tumors in mice (61), while wild-type tumors grew rapidly. Results from others also suggested that the expression levels of NKG2D ligands in tumors correlated with survival time (66).

Colorectal cancer patients with high level of NKG2D ligands expression seemed to have higher 10 year survival rate than the patients carrying the same grade of cancer but with low level of NKG2D ligands expression (66).

CT26 is the main tumor model we used to study IL-12 plus doxorubicin-induced Rae-1. However, there is no study showing that the Rae-1 expression in CT26 tumors could benefit the tumor-bearing animals with lower tumor burden and longer survival time. We hypothesized that Rae-1 overexpressing in CT26 cells inhibit tumor progression and extend animal survival time.

Results

Rae-1 suppresses CT26 tumor growth and extends survival time in vivo

To compare the tumor cell growth rate of CT26-GFP and CT26-Rae-1 cells *in vitro*, 1×10^3 CT26-GFP and CT26-Rae-1 cells were cultured 24, 48, 72, 96 and 120 hours in 96-well plates (n=5). The cell viability was determined by using MTT assay. The cell growth curve of these two cell lines overlapped with each other, suggesting that these two cell lines have similar growth rate *in vitro* (Figure A6). Thus, the difference of their tumor growth rate *in vivo* all depends on Rae-1-mediated antitumor immune response.

To determine the role of Rae-1 on tumor progression and survival time *in vivo*, CT26-GFP or CT26-Rae-1 cells were introduced into BALB/C mice (n=7), and tumor volume was measured twice a week. Animals were euthanized due to their big tumor burden. The survival rate of tumor-bearing mice was estimated by Kaplan- Meier curve. We observed that Rae-1 overexpression could significantly inhibited CT26 tumor growth *in vivo* since 12 days after the inoculation (Figure A7A). CT26-Rae-1 tumor-bearing mice also showed a substantial extended survival time comparing to the CT26-GFP bearing mice (Figure A7B). Like what others have found, we concluded that high expression of Rae-1 in CT26 tumors effectively inhibited tumor progression.

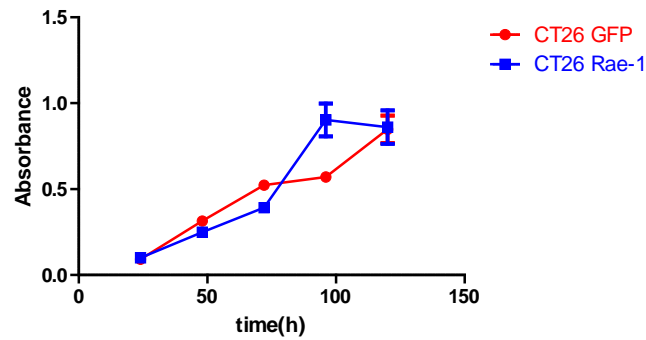


Figure A6 *The tumor cell growth rate of CT26-GFP vs. CT26-Rae-1 in vitro.* Tumor cell growth rate of CT26-GFP and CT26-Rae-1 cells were determined by using MTT assay (n=5). No significant difference was observed.

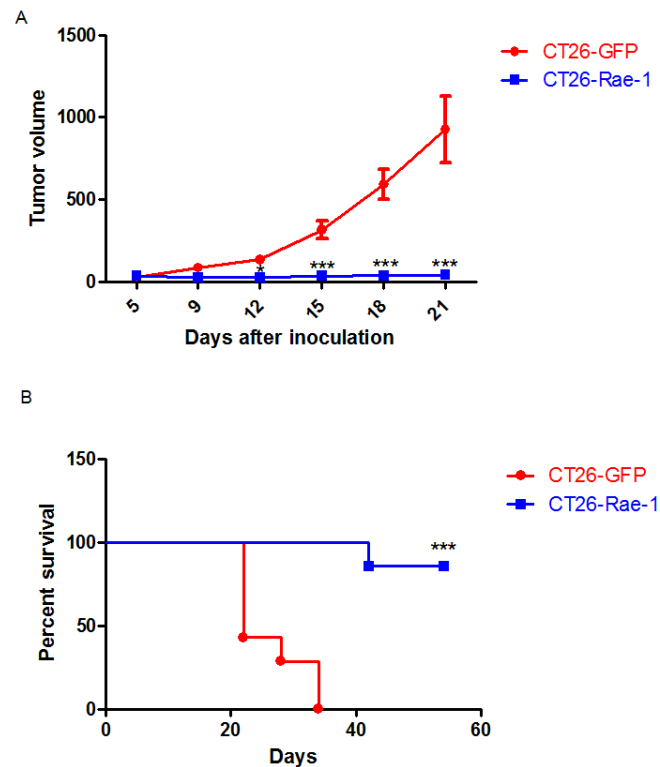


Figure A7 *Tumor volume and survival time of CT26-GFP vs. CT26-Rae-1 carrying mice in vivo.* CT26-GFP and CT26-Rae-1 cells were inoculated to BALB/C mice (n=7) via s.c. injection. **(A)** Tumor volume was measured twice a week. **(B)** Animal survival rate was determined by Kaplan-Meier curve.

Summary

Others have indicated that high expression levels of NKG2D ligands gave rise to tumor rejection and extended survival time *in vivo* in many cancers, but there was no study showing how Rae-1 could affect CT26 tumor progression. Since CT26 tumor model is throughout our Rae-1 induction study, we are interested in investigating how Rae-1 overexpression in tumors affects tumor progression.

We observed similar tumor cell growth rate of CT26-GFP and CT26-Rae-1 cells determined by MTT assay. Then, tumor volume and survival time were compared between mice carrying CT26-GFP tumors versus the ones with CT26-Rae-1 tumors. Our result clearly suggested that, like what has been found in other tumor models, Rae-1 overexpression dramatically inhibits CT26 tumor progression and extends the survival time *in vivo*.

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