MODULATION OF TUMORICIDAL ACTIVITIES OF DENDRITIC CELLS TO ENHANCE ANTIGEN UPTAKE AND CROSS-PRESENTATION

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Abstract

Dendritic cells (DCs) are professional antigen-presenting cells that are integral to the induction of primary, antigen-specific T cell responses. In the cancer setting, DCs mediate cross-priming of tumor-reactive T cells by presenting tumor antigens acquired from viable or dead cancer cells. Due to their unique functional properties, DCs have been utilized as both vectors and targets for immunological intervention in numerous diseases and are optimal candidates for vaccination protocols in cancer. In addition to their antigen presentation function(s), recent evidence suggests that DCs may also perform an innate immune effector function, with human DCs reported to mediate direct tumoricidal activity *in vitro*. However, the mechanism(s) by which DCs directly kill tumor cells remain unclear. The goal of this study is to further characterize the mechanism(s) associated with murine DC tumoricidal function and to determine whether and how this function may be enhanced to promote anti-tumor immune responses that translate into therapeutic effectiveness.

One way we sought to enhance this DC effector function was through the genetic engineering of DCs themselves. After transduction with mIL-12 and/or mIL-18 cDNA using recombinant adenoviral vectors, DCs exhibited significantly elevated tumor killing activity. This was mediated, at least in part, by TNF ligand-receptor complexes, as demonstrated by antibody blocking assays. When injected in situ, these engineered DCs exhibited prolonged survival, in

association with enhanced levels of tumor apoptosis proximal to imaged DCs and our capacity to image DC that had engulfed tumor apoptotic bodies. We also observed notable therapeutic benefits upon intratumoral delivery of these DCs in concert with an expanded in vivo repertoire of anti-tumor CD8+ T cells. In addition to DC modification, we also evaluated treatments applied to tumor cells that resulted in enhanced sensitivity to (control) DC-mediated killing. Specifically, we found that pretreatment of A20 lymphoma cells with a nitric oxide (NO) donor compound, PAPA-NO, markedly increased the sensitivity of tumor cells to consequent apoptosis mediated by DCs. This appeared to provide DCs with a preferred source of tumor antigens, with which, they were capable of activating specific T cells via a cross-presentation pathway. We have also discovered that multiple TNF family ligands participated in DC-mediated tumoricidal function and that tumor cell-expressed survivin may represent a critical downstream factor regulating the apoptotic sensitivity of tumor cells to DC-mediated apoptosis. When taken together, these studies provide novel details regarding mechanisms involved in DC anti-tumor effector function, and suggest two DC-based, combinational cancer therapies that target the effective cross-priming of therapeutic T cells. The results presented in this dissertation support an efficient model in which DCs may not only serve as the gatherers and presenters of antigens, but also the hunters as well, with tumoricidal activity mediated via TNF family ligands.

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PREFACE

I would first like to thank my mentor Dr. Walter Storkus for his guidance throughout the past four years. Dr. Storkus is not only a gifted scientist, but also an exceptional advisor. Without his continuous support and encouragement, I would have never completed this study. I would like to thank my thesis committee, Dr William Chambers, Dr. Louis Falo, Dr. Paul Robbins and Dr. Nikola Vujanovic, for their guidance and assistance during my graduate education. I am also grateful to the past and present members of the Storkus Lab, especially Dr. Tomohide Tatsumi who offered me tremendous help during my first year in the lab with both knowledge and technical expertise.

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1. INTRODUCTION

1.1. Cellular immunity in cancer

The cancer immunosurveillance hypothesis was proposed some decades ago (1, 2). Since then, the question of whether the immune system can prevent and/or regulate tumor growth has been commonly asked. Recent reports have suggested that mice lacking specific cellular populations, such as T cells, natural killer (NK) cells and/or natural killer T (NKT) cells, or specific effector molecules, such as interferon (IFN)-γ, interleukin (IL)-12, perforin, or tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) display a higher incidence of tumor development (3-7). This provides strong evidence that effective cancer immunosurveillance exists in mice. Importantly, accumulating evidence also indicates that the immune system is also actively invoked in human anti-cancer defence, for instance: (a) immunosuppressed transplant recipients display higher incidences of various non-viral tumors (8); (b) the presence of lymphocytes within the tumor can be a positive prognostic indicator of patient survival (9); and (c) cancer patients are able to develop spontaneous innate and acquired immune responses to the tumors that they bear (10). The discovery that host lymphocytes can indeed recognize and destroy tumor cells provides researchers with the opportunity to intensively test a broad array of immunotherapies in pre-clinical models and clinical trials (7). At the present time, however, we continue to gain an appreciation of the profound immune deviation that occurs both systemically and locoregionally within the tumor microenvironment of patients with cancer (11), providing additional levels of complexity to model development, therapy design and data interpretation.

Protective immune responses can be largely segregated into cellular and humoral immunity, that appear to be predicated on two dominant types of cytokines produced early during cognate

dendritic cell (DC)-T cell interaction. Type-1 cytokines (i.e. IL-2, IL-12, and IFN-γ) are involved in T helper 1 (Th1) immune responses and notably promote cell-mediated inflammatory immunity, which has been typically linked with a better clinical prognosis in the cancer setting (12). Two critical effector functions associated with the eradication of developing tumors are the production of IFN-γ and cytotoxicity mediated by Type-1 effector (mainly T, NK) cells. In contrast, Type-2 cytokines (i.e. IL-4, IL-5, and IL-10) are strong promoters of humoral (antibodies) responses to tumor antigens, that are generally poor regulators of progressive tumor growth (13, 14).

Among immune cells, DCs play a crucial instructive role early in adaptive immune responses. DCs producing IL-12p70 (i.e. DC1) prime Type-1 (cellular) immune responses, whereas DCs that fail to produce IL-12p70 (i.e. DC2) favor Type-2 (humoral) immunity. In the cancer setting, IL-12p70 produced by DCs appears to represent a particularly critical cytokine, as it promotes the activation, differentiation and functional polarization of NK, NKT, Th1, and Tc1 cells, that are integrally involved in the development and maintenance of effective Type-1 anti-tumor immunity (15, 16).

1.2. Immunobiology of dendritic cells (DCs)

DCs are bone marrow-derived antigen presenting cells (APCs) that exhibit a unique ability to induce primary immune responses, thus permitting the establishment of immunological memory (17-19). Circulating progenitor DCs enter tissues, where they differentiate to and reside as immature DCs (iDC). During tissue damage, iDC capture antigens and migrate to the tissue-draining lymphoid organs, during which time they mature, and consequently activate rare antigen-specific T cells, and thereby initiate immune responses. DCs present antigen to CD4+ T

helper cells (and to CD8+ T cells), which in turn, secrete cytokines and regulate the development and function of immune effectors, including antigen-specific CD8+ cytotoxic T cells and B cells, as well as non-antigen specific macrophages, eosinophils and NK cells (18, 20).

1.2.1. DC antigen capture, processing and presentation

In most tissues, DCs exist in an "immature" state, characterized by an optimized ability to capture exogenous antigens in their microenvironment (17). Given their expression of a wide array of cell-surface receptors, iDCs can efficiently capture bacteria, viruses, apoptotic and necrotic cell fragments, proteins and immune complexes through several pathways: such as (a) macropinocytosis; (b) receptor-mediated endocytosis via C-type lectin receptors, such as the macrophage mannose receptor and DEC-205, which bind to bacterial carbohydrates (21, 22); and via FcγRII (CD32), FcγRI (CD64), FcεRI and the C3bi complement receptors (CD11b), which increase the efficiency of immune complex endocytosis (23, 24); (c) phagocytosis of particles such as latex beads, apoptotic and necrotic fragments (involving CD36 and αvβ3 or αvβ5 integrins) (25-27); and (d) internalization of heat-shock protein gp96 and hsp70 via toll-like receptor 2/4 or CD9 (28-30).

Captured antigens are processed into peptides that may be loaded into Major Histocompatibility Complex (MHC) class I and II molecules. These peptide-MHC complexes are consequently transported to the cell surface, allowing for recognition by antigen-specific CD8+ and CD4+ T cells, respectively (18). Endogenously synthesized proteins of either self- or pathogen-origin typically enter the cytosolic pathway in which proteins are degraded into peptides by the (immuno)proteasome and then transported by the transporters of antigen-processing (TAP) molecules into the endoplasmic reticulum for loading into nascent MHC class

I molecules. In contrast, exogenously acquired proteins are generally degraded in endosomes/lysosomes, where the peptides are loaded onto MHC class II molecules following degradation of the MHC II-associated invariant chain in the MIIC/endosomal/lysosomal compartments of the cell (31-33).

Besides these two typical antigen-processing pathways, an exogenous pathway for processing MHC class I-restricted antigens within DCs has also been reported (Figure 2). Bevan et al. (34) showed some years ago that protein antigens synthesized in one cell could be captured as exogenous antigens by APCs, processed into the MHC class I-presentation pathway, and then used to prime CTL-mediated immunity. This was termed "cross-priming". Subsequently, the antigen-processing associated with cross-priming was defined as "cross-presentation" (34, 35). Cross-presentation has been previously used in two contexts in the literature. In the first case, it simply refers to the processing of exogenous antigens into the MHC class I pathway. The second definition allows for the capture and re-presentation of cell-associated antigen in either the MHC class I or MHC class II pathways (32). Cross-presentation as described in this thesis refers to the latter definition.

1.2.2 DC maturation

Maturation is a terminal differentiation process that transforms DCs from cells specialized in antigen capture into cells specialized for T cell stimulation (36). DC maturation can be induced following interaction with diverse stimuli. Bacterial products such as lipopolysaccharide (LPS) or uptake of apoptotic bodies can induce DC maturation. Signals also act on receptors that trigger intracellular signaling, including receptors for host-derived inflammatory molecules such as CD40 ligand (CD40L), tumor necrosis factor α (TNFα), IL-1, and IFNα (31).

Upon maturation, the expression pattern of many DC surface markers and secreted factors changes dramatically. Mature DCs (mDC) lose some of their antigen processing capacity and, instead, acquire numerous appendages/processes (dendrites), allowing mDCs to increase their motility and migrate toward the regional lymph nodes (LN) (17). MDCs also increase their level of membrane-localized MHC class II complexes and exhibit significantly higher levels of accessory/costimulatory molecule expression than iDCs, including CD40, CD80, CD83 and CD86. Furthermore, they also display a distinct cytokine and growth factor profile, perhaps best characterized by their ability to produce increased levels of IL-12p70 in response to environmental stimuli (24, 37).

1.2.3. DC Interaction with lymphocytes

One of the primary events after maturation is the programmed up-regulation in expression of the chemokine receptor CCR7 on the DC surface, providing DCs the capacity to respond to the secondary LN chemokines ELC/MIPβ and SLC/6Ckine (38). These ligands are chemoattractants for DCs and are specifically expressed in the T-cell-rich areas of LNs, where mature DC home to and become interdigitating DC (39).

Initial DC-T cell interactions are mediated, in part, via adhesion molecules, such as DC-SIGN/ICAM-3, ICAM-1/LFA-1 and LFA-3/CD2 (36, 40). Following recognition of MHC-peptide complexes on DCs by antigen-specific TCRs (signal 1) and engagement of T cell expressed CD28 molecules by DC expressed B7-1 and B7-2 co-stimulatory molecules (signal 2), DCs become competent to stimulate the activation, expansion and differentiation of T effector/memory cells. Naïve CD8+ T cells can be primed directly by DCs, even in the absence of T cell help (41, 42). In the case of CD4+ T helper cells, activation (by DC) in the presence of

IL-12p70 promotes the functional development of IFNγ-producing Th1-type T cells that, in turn, support the differentiation and maintenance of CD8+ cytotoxic T lymphocytes (CTLs). When activated by DCs in the presence of IL-4, however, T cells differentiate into Th2-type effector T cells that secrete IL-4, IL-5 and IL-13. These cytokines activate eosinophils, and serve as B cell growth and differentiation factors, favoring the development of humoral immunity (15, 17).

1.2.4 DC tumoricidal function

In addition to their capacity to mediate antigen presentation, several recent reports suggest that DCs may also directly 'kill' target cells. Initial results published in 1996 suggested that CD8+ DCs express FasL and are well equipped to kill activated, Fas-expressing CD4+ T cells (43). These data indicated that DCs expressing FasL could be functionally specialized to mediate the apoptotic death of Fas+ target cells. Accordingly, it was recently reported that DCs also exhibit direct cytotoxic activity against a wide range of tumor cell lines *in vitro*. After stimulation with either IFN-α or IFN-γ, human CD11c+ blood DCs acquire the ability to kill tumor cell targets (44). Human CD34+ stem cell-derived DCs and human CD14+ monocyte-derived DCs, stimulated with IFNβ, also exhibit cytotolytic reactivity against certain types of tumor cells (45). Such tumoricidal activity appears to be principally mediated by DC-expressed TRAIL molecules. Others have argued that human DCs induce tumor-specific apoptosis via a Fas/FasL-dependent pathway (46), or by secretion of soluble factors (47). In rat, a subset of DCs exhibit cytotoxic activity against selected tumor cells, via both Ca^{2+}- dependent, and -independent mechanisms (48). Moreover, our group has recently reported that human monocyte derived and fresh blood iDCs directly and effectively induce apoptosis via caspase 8 and caspase 9 pathways in a large variety of cultured and fresh cancer cells, without damaging normal cells,

and that blocking antibodies specific to TNF family ligands, TNF, FasL, LT-α1β2, and TRAIL, inhibit the killing of cancer cells by DCs (49, 50). Despite these findings, even now, the detailed mechanism(s) by which DCs kill cancer cells remains incompletely understood.

1.3. Apoptosis

Apoptosis, or programmed cell death, is an essential physiological process that plays a critical role in embryonic development, immune system function and the maintenance of tissue homeostasis (51). However, apoptosis is also involved in a wide range of pathological conditions, including cancer. Defects in the apoptosis pathways can eventually lead to expansion of a population of neoplastic cells. Resistance to apoptosis can also augment the escape of tumor cells from surveillance by the immune system. The goal of cancer therapy is to selectively promote the death of cancer cells without causing unacceptable levels of collateral damage to normal cells/tissues (52-54).

1.3.1. Apoptosis pathways

Upon initiation of apoptosis, cells may undergo specific morphological and biochemical changes, including cell shrinkage, chromatin condensation, and internucleosomal cleavage of genomic DNA (51). At the molecular level, apoptosis is mainly orchestrated by the activation of the aspartate-specific cysteine protease (caspase) cascade. To keep the apoptotic program under control, caspases are initially synthesized as inactive precursor proteins (zymogens) that are cleaved by an upstream protease, or by an increase in the local concentration that leads to cleavage-independent activation, to produce a mature protein with full enzymatic activity (55).

In principle, there are two pathways that initiate apoptosis: one is the "extrinsic pathway" mediated by death receptors on the cell surface; the other is the "intrinsic pathway" involving mitochondria (54). In a simplified description of the extrinsic pathway, binding of ligands such as FasL, TNF, or TRAIL to their corresponding receptors (i.e. Fas, TNF receptor or TRAIL receptor, respectively) results in the formation of the death induced signaling complex (DISC). The DISC contains the adaptor proteins that allow for the recruitment of pro-caspase-8, which leads to its autoactivation. Active caspase-8, often referred to as an initiator caspase, subsequently cleaves and activates pro-caspase-3. Activation of caspase-3, the effector caspase, leads to the ultimate demise of the cell (54-57).

Alternatively, the intrinsic pathway is triggered by various extracellular and intracellular stresses, such as growth factor withdrawal, hypoxia and DNA damage. These stresses induce a series of biochemical events that result in the translocation of a pro-apoptotic Bcl-2 family member, such as Bax, into the mitochondria, and the release of cytochrome *c* from mitochondria into the cytosol. This may be accompanied by loss of mitochondrial membrane potential and destabilization of the outer mitochondrial membrane. In the cytosol, cytochrome *c* forms a complex with apoptotic protease activating factor 1 (APAF1), ATP and inactive initiator caspase pro-caspase-9. Within this complex — known as the "apoptosome" — caspase-9 is activated, which allows for the consequent activation of caspase-3, eventually resulting in apoptosis (55-57).

Despite these simplified schemes, there is undoubtedly crosstalk between these two pathways. For example, cleavage of the Bcl-2 family member bid by caspase-8 induces translocation of bid to the mitochondria and triggers the intrinsic apoptotic pathway. This is proposed to serve as a potentiating loop to amplify the apoptotic signal (58).

1.3.2. Regulators of apoptosis

The apoptotic self-destruction machinery is tightly-controlled, with various proteins regulating the apoptotic process at different levels. FLIPs (FADD-like interleukin-1 β-converting enzyme-like protease (FLICE/caspase-8)-inhibitory proteins) interfere with the initiation of apoptosis directly at the level of death receptor (57). In addition, two other major families of apoptosis regulators have been identified to date: the Bcl-2 family and the inhibitor of apoptosis proteins (IAP) (59).

The members of the Bcl-2 family are an important class of proteins that regulate apoptosis at the mitochondrial level. According to their function, these proteins can be divided into anti-apoptotic (Bcl-2, Bcl-xL, Bcl-w, Mcl-1) and pro-apoptotic proteins (Bax, Bak, Bid, Bad). Most anti-apoptotic members contain the Bcl-2 homology (BH) domain 1, 2 and 4, whereas the BH3 domain seems to be crucial for apoptosis induction (57). Once activated, the pro-apoptotic proteins, Bax and Bak, are able to perturb the membrane permeability of mitochondria where they localize. This results in the release of apoptogenic proteins, such as cytochrome *c*, from the mitochondria into the cytosol (60). These effects are counterbalanced by the anti-apoptotic activity of survival members, such as Bcl-2, Bcl-xL, and the ratio between anti-apoptotic and pro-apoptotic proteins helps to determine overall cellular susceptibility to death stimulations (60, 61). The protective function of Bcl-2 and Bcl-xL appears to be mediated by formation of inactivating heterodimers with Bax/Bak, thus preventing the release of apoptogenic factors that would otherwise occur in response to apoptotic stimuli (62).

In addition to proteins such as Bcl-2 that inhibit cell death upstream of the mitochondria, and prior to activation of the caspases, there is another family of proteins termed IAPs that act

after caspases become activated by binding to them and preventing them from cleaving their substrates (63). They might also function as ubiquitin ligases, promoting the degradation of bound caspases (57). Eight human IAP family members, also known as baculovirus IAP repeat-containing (BIRC) proteins, have been identified thus far, these include: c-IAP1, c-IAP2, XIAP, NIAP, survivin, apollon, ML-IAP and ILP2 (64).

Being a novel member of the IAP family, survivin has recently attracted attention from both basic and translational researchers. At 16.5kDa, survivin is the smallest member in the mammalian IAP family (65). A number of distinct features enable this protein to become an attractive candidate target in cancer therapy design. Survivin is ubiquitously expressed during embryonic and fetal development, but is undetectable in most normal adult tissues. In contrast, dramatic overexpression has been shown for virtually all cancers thus far evaluated, including carcinomas of the lung, colon, breast, pancreas, stomach, liver, ovaries, as well as, haematopoietic malignancies (65, 66). Several lines of evidence also suggest that survivin is a reliable marker of aggressive and unfavorable disease, and is associated with an abbreviated overall survival of cancer patients (66-68). When taken together, these features indicate that survivin may serve as a ubiquitously-expressed tumor-associated antigen for both diagnostic and therapeutic purposes (65).

Another unique property of the survivin protein is its duality of function. One question that has frequently been asked is whether survivin is predominantly an apoptosis inhibitor, a mitotic regulator, or whether it equally participates in both processes (66). Despite controversy in the reports published, a role for survivin in apoptosis inhibition is now well-established. In cell culture systems, overexpression of survivin inhibits apoptosis initiated via the extrinsic or intrinsic apoptotic pathways (65). Molecular antagonists of survivin cause caspase-dependent

cell death and lead to an enhancement of sensitivity to apoptotic stimuli (69, 70). Although not conclusively determined, the mechanism by which survivin exerts its inhibitory function(s) on apoptosis circuitry appears to be through its interaction and inhibition of caspases -3, -7, or -9 (65, 71). In addition to its involvement in cell death regulation, survivin also appears to play an important role in cellular mitosis. The kinetics of survivin expression display a clear cell-cycle dependency (72). During mitosis, survivin localizes to various components of the mitotic apparatus, such as centrosomes, microtubules of the metaphase and anaphase spindle, and the remnants of the mitotic apparatus — midbodies (66). Intriguingly, a very recent report showed that survivin is required for both proliferation and inhibition of apoptosis of expanding T cells and that these two processes are so inter-dependent that they cannot be functionally- or temporally-dissociated from one another (73, 74).

1.3.3. TNF family ligands and their receptors

TNF ligand superfamily members induce pleiotropic biological responses, including cell activation, proliferation, differentiation, and death (75). The ability to induce cell death is a unique feature of the TNF ligand family and has been well established for TNF-α, FasL and TRAIL (75, 76). TNF-α, FasL and TRAIL are synthesized as type II transmembrane proteins that contain a short cytoplasmic segment and a relatively long homotrimeric extracellular region, which are not only expressed as membrane-bound molecules, but also secreted as soluble proteins after proteolytic cleavage (77).

In parallel with the elucidation of the TNF ligand superfamily, a large family of complementary molecules, named the TNF receptor superfamily, has also been identified. All members of this family are type I membrane-bound proteins containing characteristic cysteine-

rich repeats in their extracellular domains (77). Functional TNF receptors usually exist as trimeric complexes that are stabilized by intra-cysteine disulfide bonds. Although sequence homology in the cytoplasmic domains does not exceed 25%, the death domain is shared by the apoptosis-inducing receptors TNF receptor 1 (TNFR1), Fas, death receptor 3 (DR3), DR4 and DR5 (77-80).

TNF family ligand/receptor complexes play important roles in both innate and adaptive immune responses. TNF-α and FasL are expressed on activated CTLs and NK cells, and exhibit potent cytotoxic activity, inducing apoptosis in susceptible cells within hours (77, 81-83). Numerous lines of evidence have shown a pivotal role of FasL in regulating normal B and T cell function, suppression of autoimmunity, control of infection, and immune surveillance (84-87). In particular, expression of FasL on DCs appears to be involved in maintaining lymphocyte homeostasis (88, 89). Among the known apoptosis-inducing molecules, TRAIL has received a significant degree of attention in the past few years. One of TRAIL's unique characteristics is the induction of apoptotic cell death of a variety of tumor cells or transformed cells, but not normal cells (90). TRAIL is expressed on the surface of activated T lymphocytes, NK cells and monocytes, and has been shown to participate in tumoricidal activity *in vitro* and *in vivo* (91-94).

1.4. Cancer immunotherapy

When cells become cancerous, they may produce new, uncommon antigens. The immune system may recognize these antigens as "non-self" and destroy cancer cells expressing the antigens. However, it has been well-characterized that immune function is frequently impaired in cancer patients, and that tumor cells may evolve a variety of mechanisms in order to circumvent anti-tumor immune responses (95). Tumor escape strategies include the: (a) absence or down-

modulation of MHC molecule expression on tumor cells; (b) reduced or deficient expression of co-stimulatory molecules and adhesion molecules on cancer cells; (c) production of immunosuppressive soluble factors (e.g. IL-10, TGF-β); (d) expression of FasL and/or absence of Fas receptor (95-97). In addition, the failure of host defense mechanisms (i.e. impaired T, NK, or APC cell function) may also be related to the ability of tumor cells to progress(95, 98).

It is now known that tumor cells may be eliminated by the immune system through both cellular and humoral responses. Cellular cytotoxicity is believed to play a critical role in anti-cancer immunity (95, 99). Cancer immunotherapy is an approach to the treatment of cancer, which is designed to induce or up-regulate T cell-mediated tumor-specific immune responses (100). In this regard, numerous strategies have been developed, which include non-specific activation of the immune system with microbial components or cytokines, antigen-specific immunotherapy with passive transfer of antibodies and/or T cells, and antigen-specific active immunotherapy (i.e. vaccination) (37). Among these modalities, the most attractive strategy may be vaccination, which is expected to induce both therapeutic T-cell immunity (by stimulating tumor-specific effector T cells) and protective T-cell immunity (by stimulating tumor-specific memory T cells that can prevent patient relapse) (37, 101).

1.4.1. DC-based cancer therapy

Many recent vaccine designs have targeted the optimization of antigen delivery to DC *in situ*, due to the unique immunostimulatory properties of these APCs, including their high degree of antigen capture and presenting capacities that support the extremely efficient induction and maintenance of specific cell-mediated immune responses (31, 102). Recently, a novel approach to vaccination against cancer was reported, which exploited DCs and their “natural adjuvant”

qualities, by actively immunizing cancer patients with a sample of their own DCs that had been pulsed *ex vivo* with tumor antigens (103). The rationale for using DCs as a component of an anti-tumor vaccine is compelling: DCs are not only capable of internalizing and processing tumor epitopes, of migrating to regional LNs and optimally-presenting these epitopes to T cells, but also of secreting cytokines that promote the amplification of T-cell responses and the development of immunologic memory (36, 37). The recent discovery that DCs are functionally compromised in patients with cancer provides another reason for the use of *ex vivo* generated DCs in cancer vaccines, where their function and numbers may be selected and regulated (104). Moreover, *ex vivo* differentiated autologous DCs obtained either from peripheral blood monocytes or bone marrow stores of patients with cancer appear fully-functional, and may be readily used in vaccines (24).

The choice of tumor antigen to be employed in DC-based vaccines is an important consideration. MHC class I-restricted peptide antigens are frequently used, including altered or enhanced peptides (i.e., altered/agonist peptide ligands; APL) that boost immunity to less immunogenic self-antigens or that improve antigen presentation or T-cell receptor affinity (31, 105). Pulsing synthetic peptides derived from known tumor-antigens such as MAGE-1, MART-1, gp100 or MUC1 onto human DCs has been shown to represent an effective therapeutic vaccine in the clinical setting (106-109). The disadvantages of using defined tumor antigens are that loaded peptides only reside on the DC surface (in the context of specific MHC allelic proteins) for a short period of time and most antigenic peptides are only applicable for patients who express a defined, specific Human Leukocyte Antigen (HLA) type that is capable of presenting a given epitope to T cells (36, 110). In response to these vaccines that serve a sub-population of patients, DC/whole protein-based strategies that do not require prior knowledge of

the patient's MHC haplotype have been developed. DCs can be loaded with purified or recombinant proteins, transduced with non-replicating recombinant viral vectors, or transfected with RNA encoding tumor-associated antigens (31, 36). Each of these approaches allows the host's MHC molecules to select epitopes from the target antigen's complete amino acid sequence.

The route of DC administration can clearly affect the consequent tissue localization of the injected cells and their "immunogenicity"; and therefore, this variable has been intensively investigated. Typically, "therapeutic" DCs are administered either intradermally, intravenously, or, in special circumstances, intraperitoneally (24). However, most clinical protocols require very high numbers of DCs for vaccination, since only a very small amount of the injected DCs actually reach the tissue-draining LNs, as has been demonstrated in animal models (111). The delivery of DCs directly into tumors represents an attractive alternative approach in stimulating improved anti-tumor T cells responses *in situ*, via cross-priming. This is additionally supported by reports that have demonstrated that human DCs mediate tumoricidal activity against cancer cells via TNF-ligand/TNF-receptor interactions, which might lead to subsequent uptake and cross-presentation of dying tumor cell-associated epitopes *in situ* (44, 45, 50). A clinical trial for patients with colon, pancreatic, and primary liver carcinomas based on ultrasound-guided intratumoral injection of DCs expressing recombinant IL-12 is currently ongoing (112).

1.4.2. Cytokine as immune response modifiers

Cytokines are (glyco)proteins secreted by immune cells as soluble immune messengers (113). A diverse array of cytokines are currently being evaluated in cancer therapies designed to enhance specific immunity, including: TNF, IL-4, IL-6 and granulocyte/macrophage colony-

stimulating factor (GM-CSF), among others. Of some concern, where evaluated, these agents have been found to be either ineffective or to provide controversial results (100, 113). Other major cytokines that are currently the focus of intense research include: IL-2, IFN-α, and IL-12p70, which have demonstrated repeated clinical efficacy in the treatment of haematologic malignancies or immunogenic solid cancers.

IL-2 is a T cell growth factor and an activator of T cells and NK cells. In 1992, high-dose (HD) bolus interleukin-2 (IL-2) received US Food and Drug Administration (FDA) approval for metastatic renal cell carcinoma, based on data that revealed durable responses in a small percentage of patients. However, this regimen is associated with significant toxicity and cost, which has limited its application to highly-selected patients treated at specialized centers (114). Several investigators have evaluated regimens with lower doses of IL-2 in an attempt to decrease toxicity. These regimens were reported to produce response rates and survival comparable to HD IL-2 with much less toxicity, but possibly fewer durable responses noted (114, 115).

Interferon (IFN) was first isolated in 1957 and called IFN because it "interfered" with viral infection. The role of IFN in cancer was later well-documented and was indicated for the treatment of multiple malignancies, including leukemia, renal cell carcinoma, and melanoma (116, 117). Currently, IFN-α_{2b} is approved by the FDA for the treatment of cancer and the application in clinical trials showed reproducible efficacy. While the toxicity of high-dose IFN-α is high, the majority of patients complete treatment with dose modification and nearly all toxicity is rapidly reversible (118).

IL-12 (i.e IL-12p70) is a very exciting cytokine. It has strong inflammatory properties that mediate the activation and attraction of innate immune cells, resulting in the recruitment of specific immune cells (such as T cells). IL-12 also enhances the maturation and antigen

presention capacity of DCs and promotes T helper cell differentiation towards the Th1-type necessary for cellular anti-tumor immune responses. Moreover, it stimulates the differentiation and lytic capacity of antigen-specific cytotoxic T lymphocytes (CTL) and promotes immune memory (119-121). Recombinant human IL-12p70 has been studied in patients with various types of cancer. Although the clinical development of recombinant IL-12 (rIL-12) as a single agent for systemic cancer therapy has been hindered by its significant systemic toxicities and disappointing anti-tumor effectiveness as a single modality (122, 123), IL-12 remains a very promising immunotherapeutic agent, because recent cancer vaccination studies in animal models and humans have demonstrated its powerful adjuvant properties (124-126).

In the immune system, cytokines function in regimented cascades. This may explain in part, why clinical trials of individual cytokines are rarely successful, since they tend not to work individually, but rather, cooperatively (100). IL-18 (also known as interferon-γ inducing factor; IGIF) is a proinflammatory cytokine that belongs to the IL-1 family. It works synergistically with IL-12p70 to enhance IFNγ production by Th1/Tc1 cells (127, 128). Therefore, combined application of both cytokines may further improve the therapeutic effectiveness of these drugs, as well as reduce the dose of individual cytokines required clinically.

1.4.3. Genetically-modified recombinant tumor vaccine

In general, first-generation cancer vaccines were composed of whole cancer cells or tumor-cell lysates mixed with non-specific adjuvants (24), while second-generation employed genetically-modified tumor cells, antigen presenting cells (DCs) or recombinant tumor antigens in the absence or presence of adjuvants (95). In particular, a large number of cellular gene-modified vaccines have been developed, most of which have involved the transfection of cancer

cells or APCs with genes encoding specific tumor antigens, co-stimulatory molecules, HLA proteins, as well as cytokines (95), prior to delivery of these modified cell products into patients.

Gene delivery systems, which form the technological basis for genetically-modified tumor vaccines may be divided into two major groups: nonviral and viral (95). Adenoviral vectors are a commonly used viral gene delivery system. There are a number of reasons that make them preferred for gene delivery when compared to other vectors. Most adenoviruses cause mild diseases in immunocompetent human adults and by deletion of crucial regions (i.e. E1, E3 genes) of the viral genome the vectors can be rendered replication-defective, which increases the safety of these agents. Adenoviruses exhibit a broad target cell tropism, infecting a variety of dividing and non-dividing cell types. They can be grown to high titers in tissue culture. They can be applied systemically as well as across mucosal surfaces and their relative thermostability profile facilitates and expands their clinical utility (129).

1.4.4. Combinational therapies

Just as cancer chemotherapy began with the use of a single agent and has evolved into combination therapy, immunotherapeutic agents have been combined with each other and/or with drugs that exert anti-cancer effects. To enhance the anti-tumor immunity elicited by DC-based vaccination, systemic administration of Th1-biased cytokines, such as IL-2 and IL-12 have been applied, resulting in improved therapeutic efficacy (130, 131). Alternatively, the administration of cytokine gene-engineered DCs has been demonstrated to enhance the magnitude and diversity of tumor antigen-specific CTL reactivity (132, 133). Furthermore, the efficacy of a vaccine designed to promote specific CTLs may be improved by treatment of tumor lesions with agents that sensitize target tumor cells to CTL-mediated apoptosis (134-136).

Hence, a combination of sensitization approaches with a more conventional therapy or vaccine may yield enhanced therapeutic effectiveness.

Scope of this thesis

Several lines of evidence have recently suggested that, in addition to their professional antigen presentation function, DCs may also directly mediate the apoptotic death of tumor cells. Attempts to define the means to either enhance DC tumoricidal function or increase the sensitivity of tumor cells to this activity would be expected to result in the formulation of novel DC-based cancer vaccines and therapies.

In the first phase of the study, we transduced bone marrow-derived DCs with mIL-12 and/or mIL-18 and tested whether this modification would enhance DC-mediated cytotoxicity against tumor cells, as well as, induce therapeutic immunity. Our data clearly showed that mIL-12 and/or mIL-18 gene-engineered DCs exhibited elevated tumoricidal activity *in vitro*. This was also confirmed by *in situ* confocal imaging in which cytokine gene-transduced DCs were localized in, or in proximity to, apoptotic regions of the tumor, while control nontransduced DCs were rarely detected in injected tissues. As expected, intratumoral delivery of these DCs induced more profound and poly-specific anti-tumor immune responses.

We then sought to further evaluate the mechanism(s) by which DC mediated their tumoricidal activity, and whether this activity could be enhanced by pre-conditioning tumor cells. The Fas-FasL pathway was demonstrated to play a dominant role in DC-mediated apoptosis of A20 lymphoma cells. Using a pharmacological agent PAPA-NO (an NO donor compound), we successfully showed that treated lymphoma cells were sensitized to DC-induced apoptosis, and that subsequently DC Ag uptake and cross-presentation to specific T cells were also markedly enhanced as a consequence. These findings argue for the development of combinational therapies integrating autologous DC injection into tumor lesions in concert with

conditioning regimens that sensitize tumor cells to DC-mediated killing, in order to promote effective cross-priming of therapeutic anti-tumor T cells.

2. Preface Chapter 2

DC-based vaccines and therapies represent attractive strategies in the treatment of cancer. In the current study, murine bone marrow (BM) derived-DCs transduced with the mIL-12 and/or mIL-18 genes were used to vaccinate tumor-bearing mice, and the anti-tumor immune responses and tumor growth were evaluated. In addition to expressing higher levels of MHC class I/II and costimulatory molecules, DCs engineered to secrete mIL-12 and/or mIL-18 displayed a dramatic increase in their tumoricidal activity against tumor cells *in vitro*. This enhanced lytic capacity occurred in concert with elevated expression of multiple TNF family ligands on the cell surface of DCs. Blocking experiments using antagonist Abs to these ligands suggested a dominant role of TNFα, and possibly a minor role of TRAIL in DC-mediated killing of tumor cells. Using a confocal imaging technique, we confirmed that the cytokine engineering of DCs promoted DC survival, tumor apoptosis, and antigen uptake *in situ*. Perhaps most importantly, intratumoral delivery of these engineered DCs significantly inhibited the growth of tumors *in vivo* when compared to delivery of control DC. Evaluation of the reactivity pattern of T cells in treated mice revealed that the repertoire of natural CMS4-derived peptide epitopes recognized by CD8+ T cells was dramatically expanded, in association with the observed improvement in disease course. This suggests that cytokine gene-engineered DCs are more efficient in cross-presenting tumor-derived peptide epitopes to specific T cells *in vivo*, yielding a poly-specific immune response that is more effective in regulating tumor progression.

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2.1. Abstract

Dendritic cells (DCs) were adenovirally-engineered to constitutively and durably secrete the potent Th1-biasing cytokines interleukin-12 (IL-12, AdIL12DC) and/or interleukin-18 (IL-18, AdIL18DC) and evaluated for their ability to promote therapeutic anti-tumor immunity in murine sarcoma models. Injection of either AdIL12DCs or AdIL-18DCs into day 7 CMS4 or MethA tumors resulted in tumor rejection or slowed tumor growth when compared with control cohorts. Importantly, intratumoral injection with DCs engineered to secrete both IL-12 and IL-18 (AdIL12/IL18DC) reduced tumor size and caused complete tumor rejection better than any other treatment group analyzed. This strategy was also effective in promoting the regression of contralateral, untreated tumors. Both CD4+ and CD8+ T cells were required for tumor rejection. CD8+ splenic T cells from mice treated with AdIL12/IL18DC displayed the broadest repertoire of Tc1-type reactivity to acid-eluted, tumor-derived peptides among all treatment cohorts. This apparent enhancement in cross-presentation of tumor-associated epitopes *in vivo* may result from the increased capacity of engineered DCs to kill tumor cells, survive tumor-induced apoptosis and to present immunogenic MHC/tumor peptide complexes to T cells after intratumoral injection. In support of this hypothesis, cytokine gene engineered DCs expressed higher levels of MHC and costimulatory molecules, as well as FasL and membrane-bound TNF-α, with the latter markers associated with elevated tumoricidal activity *in vitro*. Cytokine gene-engineered DCs appeared to have a survival advantage *in situ* when injected into tumor lesions, to be found in approximation with regions of tumor apoptosis, and to have the capacity to ingest apoptotic tumor bodies. These results support the ability of combined cytokine gene transfer to enhance multiple effector functions mediated by intralesionally injected DCs that may concertedly promote crosspriming and the accelerated immune-mediated rejection of tumors.

2.2. Introduction

Dendritic cells (DCs) effectively elicit primary, and boost secondary, immune responses to self and foreign antigens (19, 137). Since these specialized APCs can induce the generation of both antigen-specific cytotoxic T lymphocytes (CTL) and T helper cells, DC-based vaccines are attractive strategies for the treatment of cancer. In this regard, DCs pulsed with tumor-associated antigens in various forms, including whole cell lysates (138, 139), peptides (140, 141), proteins (142), RNA (143) or DNA (144, 145), have proven effective in eliciting protective and therapeutic anti-tumor immunity in murine models. The results of several DC-based tumor vaccine trials have also recently been reported in the setting of B-cell lymphoma, melanoma, prostate cancer, and renal cell carcinoma, among others (103, 146-148). Although tumor-specific T cells were promoted by vaccination in most patients, objective clinical responses have thus far only been observed in only a minority of treated individuals. These modest current clinical successes for DC-based cancer vaccines would be expected to improve if study designs were modified for optimal DC promotion of Th1-type immunity in cancer-bearing hosts.

Interleukin-12 (IL-12) exhibits a number of immunologically important activities, including the ability to enhance NK and CTL activities (149-151), and to polarize CD4+ T cell responses by supporting Th1/Tc1-type, and suppressing or repolarizing Th2-type immunity (152, 153). We and others have reported potent anti-tumor effects associated with IL-12 gene therapy using IL-12 gene modified tumor cells (154-156) and DCs (133), or systemic administration of IL-12 protein (130, 157) in murine tumor models. Based on these results, phase I/II clinical trials of IL-12 gene therapy have been performed, with significant, but transient objective clinical responses reported to date (158).

Interleukin-18 (IL-18) is a member of the IL-1 family of proinflammatory cytokines, produced by activated macrophages and DCs, that also appears to play an important role in

driving Th1/Tc1-dominated immune responses (159-161). Recently, IL-18 has also demonstrated potential as a biologic "adjuvant" in murine tumor models, with systemic administration of recombinant IL-18 or direct intratumoral injection of IL-18 adenoviral vector inducing significant anti-tumor effects in multiple murine tumor models (162-164). Indeed, we have recently reported that intratumoral delivery of IL-18 gene transduced DCs can elicit anti-tumor Th1-type immunity in association with enhanced therapeutic efficacy in the CMS4 tumor model (165).

IL-12 acts synergistically with IL-18 by enhancing IFN-γ production from Th1/Tc1-type T cells (127, 128), thereby providing a strong rationale for the use of these factors in combined cytokine gene therapy (CGT) approaches. While the coordinate administration of these two cytokines (as recombinant proteins) in murine tumor models has resulted in more potent anti-tumor responses than that observed for the single agents, coadministration has also been associated with lethal organ damage and septic shock-like toxicities that appear attributable to the extremely high systemic levels of IFN-γ evoked by this strategy (163). To overcome such systemic toxicities, we examined the effectiveness of therapies based on the injection of genetically-transduced DCs in order to provide paracrine secretion of IL-12 and/or IL-18 in the tumor-associated microenvironment. We demonstrate that intratumoral delivery of DCs genetically modified to secrete both IL-12 and IL-18 safely induce accelerated tumor rejection, in association with stronger Type-1 immunity and a more diverse "therapeutic" repertoire of tumor-reactive, Tc1-type T cells *in situ*.

2.3. Materials and methods

2.3.1. Mice

Six-to-eight week old female BALB/c mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and maintained in micro-isolator cages. We generated the BALB/c.EGFP Transgenic (Tg) mice by 8 cycles of backcrossing C57BL/6-TgN(ACTbEGFP)1Osb mice (Jackson) onto the BALB/c background within the Central Animal Facility at the University of Pittsburgh. Animals were handled under aseptic conditions per an Institutional Animal Care and Use Committee (IACUC)-approved protocol and in accordance with recommendations for the proper care and use of laboratory animals.

2.3.2. Cell lines and cultures

CMS4 and Meth A are chemically induced BALB/c sarcomas and have been described previously (166). Cell lines were maintained in complete media (CM, RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100U/ml penicillin, 100 μg/ml streptomycin and 10mM L-glutamine: all reagents from GIBCO/Life Technologies, Grand Island, New York) in a humidified incubator at 5% CO_2 and 37°C.

2.3.3. Generation of DCs *in vitro* from BM

The procedure used in this study was as described by Son et.al. (167). Briefly, BALB/c or BALB/c.EGFP Tg bone marrow was cultured in CM supplemented with 1000U/ml of rmGM-CSF and rmIL-4 (Schering-Plough, Kenilworth, NJ) at 37^{o}C in a humidified, 5% CO_2 incubator for 7 days. DCs were then isolated at the interface of 14.5% (w/v) metrizamide (Sigma, St

Louis, MO) in CM discontinuous gradients by centrifugation. DCs typically represented >90% of the harvested population of cells based on morphology and expression of the CD11b, CD11c, CD40, CD54, CD80, CD86, class I and class II MHC antigens (data not shown).

2.3.4. Viral vectors

The mock adenoviral vector Adψ5 and the adenovirus encoding mouse IL-18 gene were used as previously described (165). The Ad.mIL-12 was produced and provided by the University of Pittsburgh Cancer Institute's Vector Core Facility as previously reported (168).

2.3.5. Mouse IL-18 and IL-12 production from adenoviral transduced DCs

Five million (day 7 cultured) DCs were infected with recombinant adenoviruses encoding mouse IL-18 (AdIL18), mouse IL-12 (AdIL12), both AdIL18 and AdIL12 or mock vector (AdΨ5), as previously reported (165). After 48 hr, adenoviral infected DCs were harvested and analyzed for phenotype and function. Culture supernatants were also collected for measurement of mouse IL-18 and mouse IL-12 production using species specific IL-18 and IL-12p70 Enzyme Linked Immuno Sorbent Assay (ELISA) kits (BD-Pharmingen, San Diego, CA), with lower levels of detection of 31.5 and 62.5 pg/ml, respectively.

2.3.6. Flow cytometry

For phenotypic analyses of adenovirally infected DCs, PE- or FITC-conjugated monoclonal antibodies against mouse cell surface molecules [CD11b, CD11c, CD40, CD54, CD80, CD86, H-2K^d, I-A^d (all from BD-Pharmingen)], and appropriate isotype controls were used, and flow cytometric analyses were performed using a FACscan (Becton Dickinson, San Jose, CA) flow

cytometer. Cell surface expression of TNF family ligands was assessed using a previously described, highly sensitive, three-step flow cytometry technique (82). First, adenovirally-infected DCs were stained with anti-mouse TNF-related apoptosis-inducing ligand (TRAIL) antibody (e-Bioscience, San Diego, CA), anti-mouse Fas ligand (FasL) antibody (MBL, Medical&Biological Laboratories, Nagoya, Japan), anti-mouse TNF-α antibody (Endogen, Woburn, MA) or appropriate isotype-matched controls. Second, the DCs were labeled with biotin-conjugated secondary antibodies (Vector Laboratories, Burlingame, CA) and, third, were labeled with PE-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA). The results of flow cytometric analyses of TNF family ligand expression are reported in arbitrary mean fluorescence intensity (MFI) units.

2.3.7. MTT assays

To evaluate the cytotoxicity of control or genetically-engineered DCs against tumor cells, 24 hr MTT assays were performed as previously described (49). For blocking the interaction between the TNF family and its ligands, DCs were preincubated for 60 minutes with antagonist antibodies against TRAIL, Fas ligand or TNF-α (final concentration 20 μg/ml). Effector DCs and targets were then mixed in a 5:1 (DC:T) ratio, and cytotoxicity assays were performed as described above (49).

2.3.8. Animal experiments

BALB/c mice were injected subcutaneously with $2x10^5$ CMS4 or 5×10^5 MethA cells in the right flank on day 0. On day 7, tumor size reached approximately 20-30mm^2. On day 7 and 14, BALB/c mice were treated with intratumoral immunization of $1x10^6$ adenoviral transduced

DCs in a total volume of 100 µ l of phosphate-buffered saline (PBS). Tumor size was assessed every 3 or 4 days and recorded in mm^2 by determining the product of the largest perpendicular diameters measured by vernier calipers. Data are reported as the average tumor area ± SD. To assess the impact of systemic immunity from vaccination, we examined the growth of contralateral, untreated tumors. For the latter models, BALB/c mice were injected subcutaneously with $2x10^5$ CMS4 cells in both flanks on day 0. On day 7 and 14, $1x10^6$ AdIL-12 and AdIL-18 co-infected DCs (AdIL12/IL18DC) were injected in the tumor on the right flank and both tumors were measured every 3 or 4 days.

To assess the fate and function of injected DCs, we generated day 7 BM-derived DCs from BALB/c.EGFP Tg mice and infected them with the Adψ5, AdIL-12, AdIL-18 or AdIL12 + AdIL-18 viruses as indicated above. Forty-eight hours later, 1 x 10^6 control or virally-infected DCs were harvested, washed in PBS and injected into day 7 CMS4 tumors established in syngeneic BALB/c mice. After 1 additional day, tumors were resected, fixed for 1h in 2% paraformaldehyde (in PBS) and then cryoprotected in 30% sucrose in PBS, prior to being shock frozen in liquid nitrogen-cooled isopentane. Five micron frozen sections were then generated and incubated in a reaction mix containing 1 mM Cy3-conjugated UTP, 250 U/ml terminal transferase in 200 mM potassium cacodylate, 25 mM Tris, 20 mM cobalt chloride (Boehringer Mannheim, Indianapolis, IN). After a 45-min incubation at 37°C the reaction was terminated by washing with PBS and counterstained with 2 mg/ml Hoechst 33258 (Sigma Chemical Co., St. Louis, MO) for 3 minutes. The washed sections were then mounted in Gelvatol (Monsanto) and observed using an Olympus BX51 microscope equipped with a cooled CCD color camera. Images of TUNEL, EGFP, and Hoescht stained nuclei were collected.

2.3.9. T-cell depletion experiments

On days -1, 6, 11, 16 after tumor inoculation, mice were injected i.p. with 100 μl of PBS (control) or ascitic fluid of anti-CD4 (GK1.5 hybridoma, American-Type Culture Collection, ATCC, Rockville, MD), anti-CD8 (53-6.72 hybridoma, ATCC), or isotype control antibody (H22-15-5 hybridoma, ATCC). The efficiency of specific subset depletions were validated by flow cytometry analysis of splenocytes using PE-conjugated anti-CD4 and anti-CD8 mAbs (Pharmingen). In all cases, 99% of the targeted cell subset was specifically depleted (data not shown).

2.3.10. Splenic CD8 + T-cell responses against CMS4 tumors and eluted naturally processed peptides derived from CMS4 cells

Peptides were acid-eluted from viable CMS4 cells and separated on reverse-phase high-performance liquid chromatography (RP-HPLC), as previously described (169). Individual HPLC fractions were lyophilized to remove organic solvent and then reconstituted in 200 μ l of PBS and stored at -20°C until use. Pooled CD8+ T cells were isolated to a purity of > 95% from the spleens of two treated mice/group seven days after the second DC injection (i.e. day 21 post-tumor inoculation) using magnetic bead cell sorting (MACS; Miltenyi Biotec, Auburm, CA) and then co-cultured ($1x10^5$/well) with 1 x 10^4 irradiated (10,000 rads) CMS4 cells or syngeneic DCs ($2x10^4$/well) and HPLC-fractionated peptides in 96-well tissue culture plates. After a 48hr incubation, culture supernatants were collected and analyzed for IFN-γ release using a commercial ELISA (BD-Pharmingen) with a lower limit of detection of 31.5pg/ml. Data are reported as the mean +/- SD of triplicate determinations.

2.3.11. Statistical analyses

All experiments with three or more groups in which treatment was applied as a completely randomly design, were first analyzed by a one way or two way factorial analysis of variance. If the resulting p value was less than 0.05, specific pairwise contrasts were tested with a t test with Welch's correction for unequal variance as needed. Data were checked for distributional properties and appropriate transformations were applied. Cytotoxicity was determined in repeat experiments in which results were expressed as a percentage of target cells killed. These data were arcsin transformed and analyzed by a two way mixed model analysis of variance using experiments as a random effect. Tests for between-group differences were subsequently stratified by experiments. Analyses of IFN-γ production from splenocyte-derived T cell and expression of TNF family ligands were conducted with the exact Kruskal-Wallis test. If the p value for the Kruskal-Wallis test was <0.05, a priori contrasts were evaluated with the Wilcoxon test. The analysis of therapeutic single tumor inoculation murine treatment models was conducted with mixed linear models. Data were log transformed, within-mouse covariance was estimated, and fixed effects of treatment were adjusted for random mouse effects. Raw p values for comparing pairs of groups at a single time were adjusted by bootstrap resampling. The growth of bilateral inoculated tumors was analyzed by a two-way fractional fixed effects model. Tumor rejection rates were fit to a generalized linear model (with binomial link) that incorporated treatment group, day of observation, and their interaction.

2.4. Results

2.4.1. Cytokine production by, and phenotype of, adenovirally infected DCs.

We initially validated IL-12 and IL-18 production from adenovirally infected DCs (Table 1). As expected, DCs infected to produce both IL-12 and IL-18 (AdIL12/IL18DC) secreted significant quantities of both murine IL-12 and IL-18, respectively (Table 1). The culture medium of mIL-18 cDNA transfected DCs (AdIL18DC) contained mIL-18, but also a significantly elevated quantity of IL-12p70. In contrast, the culture medium of IL-12 transfected DCs (AdIL12DC) contained significant quantities of IL-12, but no detectable IL-18. Finally, the supernatants derived from control Adψ5DC did not contain detectable levels of either IL-12 or IL-18. Analyses of the supernatants harvested from engineered DCs over time indicated that DCs secreted peak levels of IL-12 and/or IL-18 two days after infection with AdIL12 or AdIL18, but that they continued to produce statistically elevated levels of these cytokines for up to 10 days post-transfection (data not shown).

A flow cytometric examination of the impact of IL12 and/or IL18 cDNA insertion on DC phenotype was then performed 48h after adenoviral infection. AdIL12/IL18DCs displayed significantly elevated levels of the MHC class I/II and CD86 costimulatory molecules and AdIL18DCs exhibited elevated levels of the MHC class I/II, CD80 and CD86 molecules, when compared to either Adψ5 control virus infected DCs or non-infected DCs (DC), with these two control groups yielding indistinguishable results (Table 2). In contrast, among the markers analyzed, AdIL12DC expressed increased levels of MHC class I and class II, but not costimulatory, molecules when compared to control DCs (Table 2).

2.4.2. Increased expression of TNF family ligands on adenoviral infected DCs.

Human DCs express several TNF family ligands, including FasL, TRAIL, TNF-α and LT-α1β2, and can implement these in mediating the apoptotic death of tumor cells *in vitro* and *in vivo* (49, 50). Our pilot studies indicated that mouse DCs have similar properties, and that Th1-biasing cytokine (such as IL-12 or IL-18) gene transfer into DCs might enhance the anti-tumor effector function of DCs (133, 164). Based on these findings and considerations, we evaluated the impact of cytokine gene insertion on DC expression of FasL, TRAIL and TNF-α. As shown in Figure 5, non-infected DCs express a basal level of TRAIL, FasL and TNF-α on their cell surface; with the expression level of TRAIL being very low, while FasL and TNF-α are expressed at comparatively moderate levels. While Adψ5 infection of DCs did not result in any modulation of TRAIL, FasL or TNF-α expression, both FasL and TNF-α were increased in intensity on the cell membrane of AdIL12/IL18DC, AdIL18DC and AdIL12DC when compared to Adψ5 or non-infected DCs ($p=0.0286$). TRAIL expression by AdIL12/IL18DC was also significantly elevated vs. non-infected DCs ($p=0.0286$), while AdIL12- or AdIL18-infection of DCs did not result in statistically significant alternation in expression of TRAIL.

2.4.3. Cytokine gene-engineered DCs exhibit improved tumoricidal activity *in vitro* and enhanced survival/effector function *in vivo*.

We next examined the tumoricidal activity of adenovirally-infected DCs against CMS4 cancer cells using 24hr MTT assays. Non-infected DCs (DC) or Adψ5 DC displayed approximately the same levels of killing activity (Figure 6A), suggesting that adenoviral infection alone did not modulate this DC function. In contrast, AdIL12DC and AdIL12/IL18 (but not AdIL18DC) were significantly better killers of CMS4 target cells on a cell-per-cell

basis, than Adψ 5DC or non-infected DCs (all $p<0.01$). Combined infection of DCs with both AdIL-12 and AdIL-18 yielded the highest level of cytotoxicity observed for any DC effector cell group tested in these analyses, with this increased cytotoxicity significantly better than the AdIL-18DC, Adψ5DC or control DCs cohorts (all $p \leq 0.05$), but not the AdIL12DC group ($p = 0.71$). We observed a similar order: AdIL12/IL18DC > AdIL12DC > AdIL18DC > Adψ5DC or DC at various times in a 42 day experiment evaluating the *in vitro* tumoricidal activity of DC effector cells against the MethA sarcoma (data not shown).

To determine the role of TNF family ligands in DC-mediated cytotoxicity, we performed 24 hr MTT assays in the presence of antagonist antibodies (Figure 6B). AdIL12/IL18DC-mediated cytotoxicity against CMS4 cells was significantly decreased when anti-TRAIL or anti-TNF−α antibodies were applied ($p<0.0001$), while blocking anti-FasL antibody had little effect. When all three blocking antibodies were added, DC-mediated cytotoxicity was decreased by approximately 50% ($p<0.0001$). These results demonstrate that AdIL-12 and/or AdIL-18 infection enhances the cytotoxic activity of DCs against CMS4 cells, with both TRAIL and TNF−α playing significant roles in this effector function.

To assess the impact of cytokine gene engineering on DC stability and function within the tumor microenvironment *in situ*, we first generated BM-derived DCs from BALB/c.EGFP transgenic (EGFP Tg) mice, infected these DCs with Adψ5, AdIL-12 and/or AdIL-18 and injected 1×10^6 of these engineered (or control uninfected) DCs into the lesions of syngeneic BALB/c mice bearing day 7 CMS4 tumors. Twenty-four hours later, tumors were resected, fixed, sectioned and counterstained for TUNEL+ apoptotic cells. As shown in Figure 7 (panels A-C), tumors injected with PBS, uninfected DCs or Adψ5DCs failed to contain either EGFP+ (51) DCs and a limited number of TUNEL+ (red) apoptotic tumor cells. In marked contrast, we

were able to detect EGFP+ DCs in tumors if these DCs had been infected with AdIL-12 and/or AdIL-18 prior to injection (Figure 7, panels D-F). These viable EGFP+ DCs were typically localized in, or proximal to, regions of increased tumor apoptosis, with some injected DCs containing apoptotic tumor bodies (Figure 7D).

2.4.4. Intratumoral delivery of DCs engineered to secrete IL-12 and/or IL-18 is therapeutic in the CMS4 tumor model.

Based on our observation that cytokine gene engineered DCs exhibit increased survival and tumoricidal activity, and hypothesizing that this might yield a mechanism that would enhance the cross-priming of anti-tumor T cells *in vivo*, we next examined whether IL-12 gene and/or IL-18 gene transfer enhanced the therapeutic potential of DCs delivered intratumorally in the CMS4 tumor model. BALB/c mice were injected subcutaneously with $2x10^5$ CMS4 cells. On day 7, these tumors exhibited a mean tumor area of 20-30 mm^2. Tumor-bearing mice were then treated by intratumoral injection of $1x10^6$ AdIL12/IL18DC, AdIL12DC, AdIL18DC, Adψ5DC or PBS. As shown in Figure 8A, the growth of CMS4 tumors in mice treated with AdIL12/IL18DC, AdIL12DC or AdIL18DC was significantly inhibited when compared with tumors in the mice treated with the control protocols ($p< 0.05$ on days 14 through 42 vs. Adψ5DC or PBS). Both AdIL12/IL18DC (at all time points after day 7) and AdIL12DC (from day 21 through 42) produced significantly greater anti-tumor effects than AdIL18DC, with AdIL12/IL18DC yielding greater therapeutic benefit than AdIL12DCs from day 10 through day 24 (all $p<0.05$). Although intratumoral injection of Adψ5 infected DCs did not lead to complete regression of established tumors in any instance, tumor growth was significantly inhibited ($p< 0.05$ from day 28 through day 42) when compared to mice treated with PBS only. As shown in Figure 8B, the highest

observed rate of tumor rejection occurred in mice treated with AdIL12/IL18DC, with all mice rejecting their tumors by day 24 (95% confidence interval for the percentage of rejected tumors = 54% to 100%). Eighty-three percent (i.e. 10 of 12) of mice treated with AdIL12 DC and 33% (i.e. 4 of 12) of mice treated with AdIL18DC also ultimately rejected their tumors. These results demonstrated that either IL-12 or IL-18 gene transfer can enhance the therapeutic effects of DC-based therapy against established CMS4 tumors, and that injection of DCs engineered to secrete both IL-12 and IL-18 accelerates the therapeutic effectiveness of this treatment strategy. We obtained similar results in a homologous treatment model for the MethA sarcoma, with AdIL-12/IL-18DC-based therapies proving statistically superior ($p < 0.05$) to AdIL-12DC-, AdIL18DC-, or Adψ5DC-based therapies until day 28 (Figure 8C).

To prove that the therapeutic benefit of our AdIL12/IL-18DC-based regimen in the CMS4 tumor model was T cell-dependent, we performed T cell subset depletion studies (Figure 8D). Both CD4+ and CD8+ T cell depletions significantly inhibited the therapeutic efficacy of intratumoral injections with AdIL12/IL18DCs ($p < 0.05$ at all time points after day 7).

2.4.5. DCs are required for the observed efficacy of AdIL-12/IL-18DC-based intratumoral therapy.

To prove that DCs play a requisite role in the observed therapeutic benefit associated with combined IL-12 + IL-18 cytokine gene therapy, we performed additional control experiments in the CMS4 tumor model. Mice bearing established day 7 CMS4 tumors were injected with PBS, AdIL12/IL18DCs or AdIL-12 (5×10^7 pfu) + AdIL-18 (2×10^8 pfu) adenoviruses. The amount of each adenovirus injected was equivalent to the total amount of each virus used to generate the AdIL12/IL18DCs applied in the comparitor cohort. As shown in Figure 9, AdIL12/IL18DCs,

but not the combined adenoviruses promoted the rapid rejection of CMS4 tumors after they were administered intratumorally.

2.4.6. Intratumoral injection of AdIL12/IL18DC enhances Tc1-type anti-tumor T cell responses.

Based on our *in vivo* depletion data, we next evaluated whether the therapeutic benefits observed in our DC-based treatment regimens were associated with the degree of anti-CMS4 effector CD8+ T cell generation in treated animals. CD8+ T cells were MACS-isolated from splenocytes 14 days after the final intratumoral injections of DCs and then co-cultured with irradiated CMS4 tumor cells for 5 additional days. CD8+ T cells obtained from mice treated with the AdIL12/IL18DC regimen produced substantially elevated levels of the Th1/Tc1-associated cytokine IFN-γ vs. CD8+ T cells obtained from mice treated with any other DC-based regimen or with PBS only (Figure 10). IFN-γ production differed significantly among groups ($p=0.0011$, exact two tailed Kruskal-Wallis test). Although there were too few mice to conclude significant differences between pairs of treatments, the higher observed level of IFN-γ production by Tc1-type CD8+ T cells elicited in response to CMS4 tumor cells *in vitro* after treatment with AdIL12/IL18DC suggests there may be an association with the degree of therapeutic effects observed in this system.

2.4.7. Splenic CD8+ T cells isolated from mice treated with intratumoral injections of AdIL12/IL18DC react against an expanded array of CMS4-derived peptide epitopes.

To evaluate the repertoire of CMS4-derived peptide epitopes recognized by Tc1-type T cells in treated tumor-bearing mice, IFN-γ production was measured by ELISA after co-culture

of splenic CD8+ T cells with syngeneic DCs pulsed with peptides that had been acid-eluted from CMS4 cells and subsequently separated using reverse-phase HPLC. As shown in Figure 11, CD8+ T cells harvested from mice treated with the AdIL12/IL18DC regimen reacted against a wide range of HPLC fractions containing CMS4 peptides. CD8+ T cells isolated from animals treated with AdIL12DC or AdIL18DC injections reacted against a more limited set of HPLC fractionated CMS4 peptides, and in cases where common fractions were recognized by T cells from mice in all of the cytokine gene engineered DC treatment cohorts, the magnitude of IFN-γ production in the AdIL12/IL18DC group was typically highest. CD8+ T cells from mice treated with Adψ5DC or PBS responded poorly to CMS4 fractionated peptides. These results suggest that the therapies based on intratumoral delivery of DCs modified by IL-12 and IL-18 gene transfer can amplify and expand the repertoire of Tc1-type, anti-tumor CD8+ T cell responses in association with increased therapeutic benefit.

2.4.8. Both locoregional and systemic therapeutic anti-tumor immunity is induced by intratumoral injection with AdIL12/IL18DC.

Since the broadest and strongest Tc1-type, anti-tumor T cell responses were induced in AdIL12/IL18DC-treated animals, we next chose to analyze whether the treatment of a CMS4 lesion in one flank would impact the progression of contralateral, untreated CMS4 tumors. Bilateral tumors were established in BALB/c mice for 7 days. On days 7 and 14, tumors established in the right flank of these animals were injected with AdIL12/IL18DC, Adψ5DC or PBS. Tumors on the left flank remained untreated. As shown in Figure 12, both the treated and non-treated tumors in mice receiving the AdIL12/IL18DC regimen were significantly smaller on days 14 through the chosen endpoint of these experiments on day 28 (all $p<0.01$) when compared

with tumors in mice treated with either PBS or the Adψ5DC regimen, and in 2/5 cases, animals in the AdIL12/IL18DC treatment group were rendered tumor-free by day 21.

2.5. Discussion

In the current study, mice bearing established CMS4 or MethA sarcomas were treated with intratumoral injections of syngeneic DCs engineered to constitutively and durably secrete the Th1-biasing cytokines IL-12 +/- IL-18. We theorized that cytokine gene engineered DCs would survive longer and be capable of mediating tumoricidal activity, tumor apoptotic body uptake and subsequent "cross-priming" of tumor-reactive T cells in the tumor-draining LN and spleen of treated animals. Therapy-associated, cross-primed T cells would exhibit an expanded repertoire of anti-tumor specificities that could theoretically be more effective in mediating tumor regression.

Our results demonstrate that established CMS4 or MethA tumors may be therapeutically treated by intratumoral injection with either IL-12 and/or IL-18 cDNA transfected DCs (but not control DCs), suggesting that Th1-cytokine gene transfer into DCs enhances their anti-tumor efficacy in this tumor model. Importantly, tumors injected with DCs engineered to secrete both IL-12 and IL-18 regressed most acutely of all treatment groups and were statistically superior to either the AdIL12DC or AdIL18DC until day 24, when the AdIL12DC (but not the AdIL18DC) cohort was provided a comparable level of therapeutic benefit. While we would hypothesize that the superior impact of AdIL12/IL18DC therapy requires co-transfection of DCs to produce IL-12 and IL-18, we have not yet formally evaluated whether the intratumoral injection of AdL12DC and AdIL18DC (i.e. single cytokine cDNA transfectants) yields a similar favorable outcome.

Subsequent analyses revealed that the anti-tumor efficacy associated with intralesional AdIL12/IL18DC therapy; 1) requires both CD4+ and CD8+ T cells based on the results of T cell subset depletion experiments, 2) requires DCs and cannot be reproduced by simple intratumoral injection of AdIL-12 + AdIL-18 viruses, and 3) is correlated with the induction of a strong Tc1-

type, anti-tumor T cell responses. These latter Tc1-type responses were polyspecific in nature based on the ability of isolated immune CD8+ T cells to recognize and secrete IFN-γ in response to a broad array of HPLC-resolved CMS4 peptides when presented by syngeneic DCs *in vitro*. While a surprising number of HPLC fractions containing CMS4-derived peptides were recognized by CD8+ T cells from AdIL12/IL18DC treated mice when presented by DCs, these T cells did not react against all fractions, nor did they react against control, non-pulsed DCs. We are currently in the process of determining which peptide-containing fractions recognized by these "therapeutic" T cells are idiotypic to the CMS4 sarcoma and which fractions contain shared sarcoma epitopes by analyzing a corresponding peptide fractionation derived from alternate H-2^d tumors, including the MethA sarcoma, the Renca renal cell carcinoma and the TS/A mammary carcinoma.

Importantly, experiments in a bilateral tumor model suggest that treatment of a single lesion by intratumoral delivery of AdIL12/IL18DC promotes the regression of both treated and non-treated, contralateral tumors, supporting the ability of this treatment protocol to induce systemic anti-tumor immunity as noted above. It should be noted, however, that contralateral tumors regressed at a slower rate than treated lesions, suggesting that additional DC- and/or T cell-dependent effects beyond those linked to the induction of systemic anti-tumor effector T cells were in play within tumors directly injected with AdIL12/IL18DC. Induction of TNF family ligand expression could be either due to the direct effect(s) of IL-12 and IL-18 on DCs or to the indirect effects of IL-12/IL-18-induced factors secreted by NK cells or other contaminating cells in the DC preparations analyzed. Moreover, the impact of locally co-produced IL-12 and IL-18 would be expected to be multifunctional and one must consider the ability of these cytokines to inhibit angiogenesis, promote the production of IFN-γ-dependent

chemokines and lymphocytic infiltration, and maintain immune effector function(s) within the typically immunosuppressive or pro-apoptotic tumor microenvironment (170-173). Indeed, we have observed that injected cytokine gene engineered DCs exhibit improved viability within the tumor site *in vivo*, arguably allowing these cells to generated and acquire apoptotic tumor bodies and to extend the window of time during which they may productively cross-prime anti-tumor T cells *in situ*. We are currently performing extensive kinetic experiments to determine the fate and migration patterns of control vs. engineered DCs in/to draining LN after their injection into tumor lesions to partially address this issue. One would also hypothesize that given extended durability in cytokine production by injected AdIL12/IL18DC within the treated lesion, that increases in inflammatory cell infiltrates and the resistance of these cells to tumor-induced apoptosis would be likely, based on the underlying immunobiologies of IL-12 and IL-18 (15, 174, 175). Interestingly, our finding that AdIL18DCs produce not only elevated levels of IL-18, but also IL-12 may prove be important in discerning the mechanisms by which AdIL18DC-based therapy is at least partially effective in our sarcoma models. Currently, there is no literature to support or refute the ability of IL-18 to elicit IL-12 production from DCs. However, DCs isolated from mice deficient in functional p38 MAP kinase are impaired in their ability to produce IL-12 (176) and IL-18 is known to activate p38 MAP kinase in murine DCs (177). Hence, it is conceivable that IL-18 produced by AdIL-18DCs may act in an autocrine fashion to facilitate IL-18R+ DC production of IL-12p70. This assumption could be tested by demonstrating the expression of IL-18R on DCs using Abs against IL-18R. Alternatively or additionally, AdIL18DC may produce more IL-12 due to indirect effects, such as IL-18 induced production of IFN-γ from contaminant (< 5%) activated T or NK cells, with subsequent IFN-γ promotion of a more DC1-type phenotype (178).

In addition to their well-publicized roles in antigen cross-presentation, DCs injected directly into tumor lesions may mediate direct tumoricidal activity as a result of their expression of TNF family ligands, including TNF-α, lymphotoxin (LT)-α/β, TRAIL, and FasL (49, 50). Indeed, in the current study, we have not only demonstrated that DCs engineered to secrete IL-12 and/or IL-18 (but not control DCs) express significantly higher levels of MHC and costimulatory molecules, but also elevated levels of membrane-bound TNF-α and FasL. MTT assays revealed that DCs infected with AdIL-12 and/or AdIL-18, especially DCs coinfected with AdIL-12 and AdIL-18, mediated enhanced cytotoxicity against CMS4 cells *in vitro*, and that this killing was partially blocked with anti-TRAIL and anti-TNF-α antagonist monoclonal antibodies. The residual DC-mediated killing of tumor cells that cannot be blocked by the mixture of blocking anti-TNF-α, anti-FasL and anti-TRAIL Abs may be due to the influence of DC expressed LT-α/β that were not evaluated using blocking antibodies in this study. These findings suggest that IL-12 and IL-18 gene transfection into DCs can enhance the ability of these cells to directly kill cancer cells via certain TNF family ligands, and that this mechanism may be relevant to the effective generation of apoptotic tumor bodies (as evidenced in our CMS4 imaging studies) providing tumor antigen for subsequent DC cross-presentation to specific T cells *in vivo.* We are currently evaluating this issue in the CMS4 and MethA models using intratumoral injected DCs generated from *gld* (Fas-L deficient), LT-α -/-, LT-β -/-, TNF-α -/- and LT-α/β-TNF triple KO mice. Preliminary evidence continues to support a dominant role for TNF-α in DC-mediated killing of these tumors *in vitro* and *in situ*. In this context, it is important to delineate potential importance of DC membrane-associated vs. secreted TNF-α as a tumoricidal effector molecule. While rTNF-α (at doses up to 1000 U/ml) induced a maximum of approximately 30% and 10% apoptosis of CMS4 and MethA tumor cells in 24h MTT assays, respectively (data not shown),

this level of killing was far inferior to that mediated by cytokine gene modified DCs in the current report.

In spite of recent progress and some early success reported for DC-based cancer immunotherapies, there is a great need to improve this therapeutic strategy. We have shown here that combinational adenoviral-based IL-12 and IL-18 gene transfer into DCs results in an improved therapeutic reagent capable of promoting enhanced anti-tumor efficacy *in vivo* when injected directly into tumor lesions. This paracrine delivery strategy was chosen since significant toxicities have been previously reported for combined rIL-12 + rIL-18 systemic therapy in murine tumor models (163) and similar complications might be anticipated in prospective human clinical trials. While serum IFN-γ levels became transiently elevated in mice 2 days (i.e. at the peak of transfected DC production of IL-12/IL-18 *in vitro*) after each intratumoral injection of AdIL-12/IL-18DC (when compared to Adψ5DC or control PBS injected mice), this only approached a maximal level of 400 pg/ml of serum (data not shown). This degree of systemic IFN-γ was approximately equal to that observed in the asymptomatic rIL-12 group reported by Osaki et al. (163) and was far less than the pathologic 17 ng/ml levels of serum IFN-γ noted for toxic rIL-12 + rIL-18 administration (163). Given this modest level of systemic IFN-γ production and our inability to discern any treatment-associated modulation in animal behavior or physical appearance, we believe that intralesional AdIL-12/IL-18DC therapy is not only very effective, but safe. Our findings support intralesional delivery of DC-based, IL-12/IL-18 gene therapies as therapeutic regimens for cancer.

2.6. Acknowledgements

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3. Preface Chapter 3

In this chapter, we further evaluated mechanism(s) of DC-mediated tumoricidal function against cancer cells and whether cancer cells could be sensitized to this activity, thereby increasing the efficacy of DC-based therapies. The initial set of experiments showed that DCs could mediate apoptosis in lymphoma cell lines via both Fas-FasL-dependent and -independent pathways, with the former playing a more dominant role. To modify the sensitivity of tumor cells to apoptosis, we chose to pre-treat them with a pharmacological agent, an NO donor compound PAPA-NO. This agent is a protein phosphatase inhibitor that alters protein phosphorylation status, and thus promotes the proteasomal degradation of certain cellular proteins. Here we observed the preferential turnover of the anti-apoptotic protein survivin in A20 lymphoma cells treated with NO donors through a proteasome-dependent pathway. In association with the down-regulation of survivin, we noted enhanced sensitivity of tumor cells to DC-induced apoptosis. This activity involved the concerted action of multiple TNF ligand/receptor pathways, including FasL/Fas, TRAIL/TRAIL-R, and possibly TNF/TNF-R as well. More importantly, NO treatment facilitated DC uptake of tumor apoptotic bodies and the subsequent cross-presentation of tumor derived-antigens to specific T cells.

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3.1. Abstract

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) associated with efficient antigen processing and presentation to T cells. However, recent evidence also suggests that DCs may mediate direct tumoricidal functions. In this study, we investigated the mechanism by which murine DCs mediate the apoptotic death of murine lymphoma cell lines, and whether DC effector function could be enhanced by pre-conditioning tumor cells with the protein phosphatase (PP) inhibitor nitric oxide (NO) by altering the balance of pro-/anti-apoptotic proteins in the treated cells. We observed that NO donor compound sensitized lymphomas to DC-mediated cytotoxicity *in vitro*. Both immature and spontaneously matured bone marrow-derived DCs (SM-DC) were capable of inducing tumor cell apoptosis, with SM-DCs serving as comparatively better killers. Fas ligand (FasL)-Fas engagement proved important in this activity, since elevated expression of membrane-bound FasL was detected on SM-DCs, and DCs derived from FasL-deficient mice were less capable of killing NO-sensitized tumor cells than wild-type DCs. As FasL-deficient DCs were still capable of mediating a residual degree of tumor killing, this suggests that FasL-independent mechanisms of apoptosis are also involved in DC-mediated tumor killing. Since NO-treated tumor cells displayed a preferential loss of survivin protein expression via a proteasome-dependent pathway, enhanced tumor sensitivity to DC-mediated killing may be associated with the accelerated turnover of this important anti-apoptotic gene product. Importantly, NO-treated tumor cells were also engulfed more readily than control tumor cells and this resulted in enhanced cross-presentation of tumor-associated antigens to specific T cells *in vitro*.

3.2. Introduction

Dendritic cells (DCs) are the most efficient antigen presenting cells (APCs) and appear largely responsible for the induction of primary immune responses (19). In a tumor-bearing host, DCs are believed to cross-present tumor peptide epitopes in the context of major histocompatibility complex (MHC) class I and class II molecules in order to stimulate specific (CD8+ and CD4+) CTL responses, respectively. Interestingly, apoptotic tumor cells have been reported to provide DCs with a comprehensive source of tumor antigens used to cross-prime effector T cells (179, 180). The origin of apoptotic tumor bodies *in situ* has been generally presumed to be the result of spontaneous tumor cell death or to be mediated by cytotoxic effector NK or T cells. However, tumor infiltration by these two killer cell populations may not be a common event early in tumor progression and cross-priming would occur more efficiently if DCs themselves were able to directly induce tumor cell apoptosis as an innate function, thereby generating an antigenic substrate for consequent T cell cross-priming *in situ*.

It has been previously demonstrated that human DCs, particularly after treatment with pro-inflammatory cytokines, are capable of mediating the *in vitro* apoptosis of tumor cells via a mechanism involving membrane-bound Fas ligand (FasL) or TNF-related apoptosis inducing ligand (TRAIL) (43-46). Recently we reported that immature human DCs are preferentially able to directly induce the apoptotic death of cancer cell lines and fresh tumor cells *in vitro*. This cytotoxicity was antagonized by inclusion of blocking Abs or receptor-Fc constructs specific to TNF family ligands, demonstrating that human immature DCs mediate tumoricidal activity by simultaneous engagement of multiple transmembrane TNF family ligands, TNF, FasL, LTα1/β2 and TRAIL (49, 50). In the current report, we have determined that spontaneously matured

murine bone marrow-derived DCs (SM-DC) are superior to immature DCs in mediating the apoptotic death of B and T lymphoma cells *in vitro*.

Since our current observations and previous reports (44-46) suggest that DC-mediated killing of tumor cells *in vitro* was most evident at high DC-to-tumor cell ratios, which may not accurately reflect the physiological situation where low frequencies of tumor infiltrating DCs are observed (181), we sought means by which tumor cells could be further sensitized to this lytic pathway in order to define a potentially translatable therapy for established cancers. In particular, we chose to sensitize tumor cells to DC-mediated apoptosis by pharmacologically altering the balance of the anti-/pro-apoptotic protein expression in tumor cells using a nitric oxide (NO) donor compound PAPA-NO. This agent is capable of oxidizing and ablating the enzymatic activity of protein phosphatases (PP) that regulate the proteasome-dependent turnover of many cellular proteins (182-185), including the pro-/anti-apoptotic proteins in the Bcl-2 and the inhibitor of apoptosis (IAP) families, as well as the inhibitor nuclear factor- κB (IκB) (186-189). In the present study, we report that NO induces the enhanced proteasome-dependent degradation of survivin in tumor cells, in association with the increased sensitivity of treated tumor cells to DC-mediated apoptosis and uptake, leading to enhanced cross-presentation of tumor cell-expressed antigens to specific T cells *in vitro*.

3.3. Materials and methods

3.3.1. Mice

Female 6-8-week old BALB/c mice and FasL-deficient (gld) mice on the BALB/c background were purchased from the Jackson laboratory (Bar Harbor, ME) and maintained in microisolator cages. Animals were handled under aseptic conditions per an institutional Animal Care and Use Committee-approved protocol and in accordance with recommendations for the proper care and use of laboratory animals.

3.3.2. Cell lines and culture

The A20 cell line is an H-2^d B lymphoma cell line (American Tissue Culture Collection, Manassas, VA). The EL4 cell line is an H-2^b T lymphoma cell line and EG7 is a derivative of EL4 cells that contains the chicken ovalbumin (OVA) transgene (190) both cell lines were the kind gifts of Dr. Louis Falo III, University of Pittsburgh). All cell lines were maintained in complete media (CM; RPMI-1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 10mM L-glutamine; all reagents from Invitrogen, Carlsbad, CA) and 50μM 2-mercaptoethanol (2-ME; Sigma Chemical Co., St. Louis, MO) at 5% CO_2 and 37ºC in a humidified incubator. The mock- and mouse FasL-transfected L5178Y T lymphoma cell lines were obtained from Dr. Hideho Okada. The cells were cultured in CM supplemented with 1mM sodium pyruvate and 0.1mM non-essential amino acid (both reagents from Invitrogen). G418 sulphate (Geneticin, from Invitrogen) was used at 1 mg/ml for maintenance of the transfected line. The DO11.10 cell line is a T cell hybridoma that recognizes I-A^d MHC class II/OVA $peptide_{323-339}$ complex (a generous gift from Dr. Louis Falo III). This CD4+ T cell line was

maintained in DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 10mM L-glutamine.

3.3.3. Peptides

The $OVA_{323-339}$ and control C. Falciparum ($MCS_{326-345}$) I-A^d binding peptides were synthesized using 9-fluorenylmethoxycarboxyl (fMOC) chemistry by the University of Pittsburgh Cancer Institute's (UPCI) Peptide Synthesis Faculty. The peptides were >95% pure based on high-performance liquid chromatography, with identities validated by mass spectrometric (MS/MS) analyses performed by the UPCI Protein Sequencing Facility.

3.3.4. Generation of DCs *in vitro* from bone marrow (BM)

DCs were generated as previously described (167). Briefly, BALB/c BM cells were cultured in CM supplemented with 1000 U/ml recombinant murine granulocyte/macrophage colony-stimulating factor (mGM-CSF; Schering-Plough, Kenilworth, NJ) and 500 U/ml recombinant mIL-4 (Peprotech Inc., Rocky Hill, NJ) at 37ºC in a humidified, 5% CO_2 incubator for 7 (immature) or 9 (mature) days. DCs were then purified using CD11c magnetic beads (MACS^{TM}; Miltenyi Biotec., Auburn, CA) and subjected to the phenotypic and functional analyses described below. In some experiments, an additional metrizamide gradient centrifugation (167) was performed prior to CD11c MACS^{TM} isolation, resulting in purities exceeding 99% (Supplement figure 1).

3.3.5. Experimental design of nitric oxide sensitization of tumor cells

Tumor cells were pretreated with the NO donor compound PAPA-NO (a generous gift from Dr. Lawrence Keefer, National Cancer Institute, Bethesda, MD). After 1h incubation, the media were removed and cells maintained in fresh media for additional 18h at 37ºC. The cells were then harvested and used as targets in cytotoxicity assays or lysed for Western blot analyses.

3.3.6. Flow cytometry

For phenotypic analyses of DC, PE- or FITC- labeled monoclonal antibodies (mAbs) against mouse cell surface molecules I-A^d, CD80, CD86 and appropriate isotype controls (all purchased from BD-Pharmingen, San Diego, CA) were used. Flow cytometric analyses were performed using a Coulter Epics XL (Beckman Coulter, Fullerton, CA) flow cytometer. To assess cell surface expression of Fas or FasL, a previously described three-step flow cytometric technique (82) was used. Briefly, the DC were labeled with unconjugated anti-mouse FasL mAbs or appropriate isotype-matched controls (MBL, Medical & Biological Laboratories, Nagoya, Japan), washed twice and incubated with biotin-conjugated secondary Abs (Vector Laboratories, Burlingame, CA), washed twice, then labeled with PE-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA). Results are presented as overlays of single-color histograms, with mean fluorescence intensity (MFI) units reported on an arbitrary scale.

To evaluate the cytotoxicity of DC against A20 tumor cells, DC were co-cultured with tumor cells for 4h at an effector/target (E:T) ratio of 5:1 at 37ºC. The cells were then harvested and stained with PE-conjugated anti-mouse B220 mAb (BD-Pharmingen) and FITC-conjugated pan-caspase inhibitor z-VAD-FMK (Promega, Madison, WI) on ice for 30min. The results are reported based on the percentage of cytotoxicity calculated as the number of apoptotic tumor cells (B220+VAD-FMK+) divided by total number of tumor cells (B220+). To block the

interaction between the TNF family ligands and their receptors, DC were preincubated for 1h with antagonist Abs against TRAIL (e-Bioscience, San Diego, CA) and/or TNFα (Endogen, Woburn, MA) or appropriate isotype control Abs at a final concentration of 20 μg/ml (49, 191). Effector DC and targets were then mixed at a 5:1 (DC/tumor) ratio and cytotoxicity assays performed, as described above. To test the cytotoxicity of DC against normal B cells, bulk splenocytes were cultured at 37ºC in the presence of 10μg/ml LPS. Three days later, cells were harvested and treated with or without PAPA-NO at a final concentration of 25 μg/ml, with cytotoxicity assays performed, as described above.

To evaluate antigen uptake, untreated or PAPA-NO treated tumor cells were stained with Hoechst 33342 (Molecular Probe, Carlsbad, CA) for 30m prior to co-culture with DCs for 18h at a DC/tumor ratio of 5 to 1. DC uptake of Hoechst-positive tumor cells by DCs was then analyzed using a MoFlo cytometer (Cytomation, Fort Collins, CO).

3.3.7. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays

To assess the apoptotic sensitivity of A20 cells to agonist anti-mouse Fas mAb (Jo2; BD-PharMingen), A20 cells were cultured in the presence or absence of the Ab at the indicated concentrations for 24h. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays (MTT) assays were then performed, as described previously (49). Briefly, the Experimental wells (Ex) contained medium and both A20 tumor cells and Ab. Wells with medium alone were used as background control (BG) and wells for total viability/spontaneous death of untreated cells (TS) contained only medium and A20 cells. The percentage of cytotoxicity was calculated using the following formula: %cytotoxicity = [(TS-BG) – (Ex-BG)]/(TS-BG)×100%.

3.3.8. *In vitro* antigen cross-presentation assays

EG7 and EL4 cells were pretreated with PAPA-NO prior to coculture with DCs for 24h, 36h or 48h at a DC/tumor ratio of 5 to 1. DO11.10 T hybridoma cells were then added to wells at a T/DC ratio of 1 to 1. After 18h, the supernatants were harvested and IL-2 production was quantitated by specific ELISA (Endogen, Woburn, MA). IL-2 secretion by hybridoma cells stimulated with DCs pulsed with 400 ng/ml of the $OVA_{323-339}$ synthetic peptide served as a positive control in these experiments.

3.3.9. Western blot analyses

A20 cells were treated with PAPA-NO as described above. The cells were next washed in PBS and lysed in buffer (1% Triton X-100, 0.5% NP-40, 3.7 mg SOV, 150 mM NaCl, 1 mM EDTA, 10 mM Tris; all reagents from Sigma) containing protease inhibitors (Complete, Roche Diagnostic, Mannheim, Germany). Total cellular lysate from each sample was separated by 12% SDS-PAGE electrophoresis, prior to transfer to a PVDF membrane (Millipore, Bedford, MA), and incubation of the blots with anti-survivin, anti-Bcl-2, anti-Bcl-X_L and anti-Bax Abs (all purchased from Santa Cruz Biotechnology, Santa Cruz, CA) in 2% non-fat dry milk in 0.5% Tween 20 in PBS. After 3 washes, the membrane was incubated with appropriate HRP-conjugate mouse or rabbit secondary Abs (Biorad, Hercules, CA). Bound Abs were detected using an ECL chemiluminescence detection kit (PerkinElmer, Boston, MA). Mouse anti-β-actin Ab (Abcam, Cambridge, MA) was used as a loading control. Densitometric quantitations were performed using a White/UV Transilluminator (UVP Products, Upland, CA) and analyses performed using Labworks software (UVP).

For the functional analyses of proteasome involvement, A20 cells were pretreated with PAPA-NO. One hour later, media were removed and cells maintained in fresh culture for additional 4h in the presence or absence of proteasome inhibitor lactacystin (concentration, 0.2 μM or 1 μM; Sigma). Cells were lysed and immunoblots were performed as described above.

3.3.10. Statistical analyses

Statistical differences between groups were evaluated using a two-tailed Student's t test, with statistical significance defined as a p value ≤ 0.05.

3.4. Results

3.4.1. Spontaneously matured bone marrow-derived murine DCs (SM-DC) mediate superior killing of tumor cell lines

Based on our previous report that cultured human monocyte-derived DCs were capable of mediating the apoptotic death of tumor cell lines *in vitro* (49), we analyzed whether BM-derived murine DCs generated in GM-CSF + IL-4 supported cultures were similarly capable of killing murine tumor cells. Approximately 30×10^6 CD11c+ (MACS-isolated; 90-95% pure) DCs per BALB/c donor mouse were obtained reproducibly on day 7 (immature; i.e. iDC) and day 9 (spontaneously matured; i.e. SM-DC) of culture. SM-DCs expressed markedly higher levels of MHC class II molecules ($I\text{-}A^d$) and costimulatory molecules (CD80 and CD86) than their immature day 7 counterparts (Figure 13A).

Since our recent studies demonstrated a role for TNF family ligands expressed on human iDCs in the direct anti-cancer effector function mediated by these cells (50), we assessed the potential role of the murine homologues in DC-mediated killing of the A20 B lymphoma cell line. As it has been reported that activated B cells express abundant membrane-bound Fas and are sensitive to Fas-mediated apoptosis (192), we initially targeted an analysis of the FasL-Fas pathway. Compared with expression on the FasL-transfected L5178Y (L5178.FasL; positive control) cell line, FasL expression on DCs was clearly evident, albeit at a lower level (Figure 13B). Notably, SM-DCs expressed higher levels of FasL than iDCs, indicating that SM-DCs might be more effective at inducing the apoptotic death of Fas+ tumor cells. To test this hypothesis directly, we incubated A20 tumor cells with either iDC or SM-DCs for 4h at 37^oC at an DC/T ratio of 2.5 to 1 or 5 to 1. Cells were then stained with antibodies against the B cell

marker B220 to distinguish tumor cells from DCs, in addition to the fluorescent caspase substrate z-VAD-FMK to detect apoptotic cells. As shown in Figure 14, in the absence of DCs, only 5.4% of A20 cells were determined to be undergoing spontaneous apoptosis. However, co-cultures of DCs and A20 cells contained significantly greater frequencies of apoptotic A20 cells. Of interest, killing was DC dose-dependent and regardless of the E/T ratio evaluated, SM-DCs were more cytotoxic against A20 cells than iDCs. Since effector cell function appeared to correlate with the relative expression level of membrane-bound FasL, these data suggest an important functional role for FasL in DC-mediated tumor cell apoptosis.

To provide further support for the role of the Fas-FasL pathway in DC-mediated tumoricidal activity, we analyzed A20 tumor cells for their level of expression of membrane-bound Fas by flow cytometry. We detected uniform, high levels of Fas expression on the surface of A20 cells (Figure 15A). However, since expression of Fas is necessary, but not sufficient for cells to undergo Fas-mediated apoptosis (192), we also determined whether ligation of Fas could trigger A20 cells to become apoptotic (in 24h-MTT assays) after addition of various concentrations of agonist anti-Fas Ab (Jo2) to cultures. As shown in Figure 15B, the anti-Fas Ab induced A20 cell death in a dose-dependent manner, while the isotype-matched control Ab had little impact on A20 viability. Similarly, nearly 30% of A20 cells became apoptotic after a 4h co-incubation with L5178Y.FasL cells but not control L5178Y cells (Figure 15C). These data demonstrate that A20 tumor cells were sensitive to the apoptosis mediated through FasL-Fas pathway induced by Ab agonist, FasL+ transfected cell lines or FasL+ DCs.

As a further demonstration of the relevance of FasL in DC-mediated tumor killing, and to determine whether additional Fas-independent pathways were also involved in this effector mechanism, we assessed the tumoricidal activity of BM-derived DCs generated from FasL-

deficient mice. While DCs generated from wild-type animals efficiently induced the apoptosis of A20 cells, DCs derived from FasL-deficient mice had approximately 3-fold less tumor cell killing capacity (Figure 16). These data indicate that FasL plays a dominant role in DC-mediated tumor apoptosis and that Fas-independent pathway(s) may also partially contribute to this killing activity.

3.4.2. Pre-treatment of A20 tumor cells with a nitric oxide donor increases their sensitivity to DC-mediated, Fas-dependent apoptosis

Since A20 cells were moderately sensitive to Fas-dependent killing mediated by DCs, we next sought to determine whether tumor cells could become conditionally sensitized to such killing using pharmacologic agents. In particular, we chose to attempt to alter the balance of anti-/pro-apoptotic protein expression in tumor cells by either up-regulating the expression of pro-apoptotic proteins or down-regulating anti-apoptotic proteins, or both, in order to theoretically make the cells more susceptible to DC-mediated apoptosis. We attempted to modulate this balance by affecting the functional activity of protein phosphatases (PP) that regulate the levels of pro-/anti-apoptotic protein expression based on their state of phosphorylation, making them targets for E3 ligases and consequently, the proteasome (193). Since NO is known to oxidize critical cysteine residues within PP catalytic sites, we pretreated A20 cells with the NO donor compound PAPA-NO for 1h and then maintained the cells in fresh media for an additional 18h. The cells were then stained with FITC-conjugated pan-caspase inhibitor z-VAD-FMK to determine the level of cellular apoptosis. As shown in Figure 17, as a single agent, PAPA-NO induced apoptosis in A20 cells in a dose-dependent manner. More importantly, at low concentration (i.e. < 100 μg/ml), PAPA-NO was non-toxic, and did not

promote significant A20 apoptosis by itself. However, the NO-conditioned tumor cells appeared to become sensitized to apoptosis mediated by anti-Fas agonist Ab (Figure 18A) or L5178Y.FasL cells (Figure 18B).

We next determined whether NO-pretreated A20 cells were also sensitized to DC-mediated apoptosis. Figure 19A provides the cumulative results derived from three independent experiments performed. We observed that although treatment with the NO donor was non-toxic to A20 cells, it greatly enhanced their sensitivity to iDC-mediated apoptosis. Tumor cells pre-treated with 25 μg/ml and 50 μg/ml of PAPA-NO showed significant differences in their apoptotic frequencies versus the non-treated tumor control group ($p \leq 0.05$). Similar results were also obtained using SM-DCs as effector cells (Figure 19B). Given minor concerns that the small contaminant CD11c-neg cell populations might be at least partially responsible for mediating tumor cell apoptosis, we performed an additional density gradient centrifugation step using metrizamide prior to CD11c MACS bead selection. This resulted in CD11c+ DC purities exceeding 99%, with no significant change in tumoricidal function mediated by these effector cells (Supplement figure 1).

Given additional concerns that such pre-conditioning could also affect the sensitivity of normal tissue targets to DC-mediated killing, we analyzed whether SM-DCs could promote the apoptotic death of normal B cells or B cells pre-treated with a source of NO. B cell blasts were generated from BALB/c splenocytes cultured with LPS for 3 days. As shown in Figure 20, SM-DCs did not induce apoptosis in normal B cell blasts and B cell pre-treatment with PAPA-NO did not increase the observed activated caspase staining when compared with controls.

3.4.3. Sensitization of A20 cells to DC-mediated apoptosis by NO involves both Fas-dependent and -independent pathways

Since DC killing of A20 cells appeared to be at least partially mediated through a Fas-dependent pathway, we initially hypothesized that this would also be the case for NO-treated tumor cells. Indeed, the capacity of DCs generated from FasL-deficient mice to kill NO-treated A20 cells was greatly impaired when compared with DCs derived from wild type mice (Figure 21A). These results suggest that the Fas-FasL mediated pathway contributes partially to the observed tumoricidal activity associated with DCs. At the same time, these data also suggest the FasL-independent pathway(s) are also significantly involved in DC-mediated killing of A20 tumor cells. The most likely potential candidates would be other TNF family members, such as TNFα and TRAIL, based on our previous results published for human DCs (50).

To test these possibilities, we performed blocking experiments to interfere with the residual tumor cell killing associated with FasL-deficient DCs using antagonist Abs. DCs generated from FasL-deficient mice were pre-incubated with individual or combinations of Abs specific for either TNFα or TRAIL, prior to addition to NO-pretreated A20 tumor cell cultures. As shown in Figure 21B, disruption of either TNF/TNF-R or TRAIL/TRAIL-R interactions served to further (partially) inhibit DC-mediated killing of NO-treated A20 cells. In this setting, anti-TNFα Ab appeared less effective than anti-TRAIL Abs in blocking the residual killing mediated by FasL-deficient DCs. Indeed, since the simultaneous disruption of all three ligand-receptor (FasL/Fas, TNF/TNF-R, TRAIL/TRAIL-R) pairs appeared to inhibit DC-mediated killing of NO-treated A20 cells to a degree comparable to that noted for DCs whose FasL and TRAIL interactions were simultaneously disrupted, TNFα likely plays a very minor role in this model of apoptosis.

Combined with the fact the NO treatment did not modulate the expression of TNF family receptors (TNF-R, Fas, TRAIL-R) on A20 cells (data not shown), these data suggest that pre-treatment of A20 with NO donors sensitizes these target cells not only to FasL-induced apoptosis, but also to (at least) TRAIL-mediated apoptosis. However, the minor but detectable level of apoptosis observed under conditions in which the Fas, TNF and TRAIL pathways were coordinately blocked may suggest the further, minor participation of additional molecule(s) in DC tumoricidal activity.

3.4.4. Treatment of tumor cells with NO donors preferentially accelerates survivin degradation through a proteasome-dependent pathway in association with enhanced sensitivity to DC.

To further investigate the mechanism(s) by which NO donors sensitize A20 tumor cells to DC-mediated killing, we analyzed the NO-treated tumor cells for alterations in their expression of pro- and anti-apoptotic proteins. One of most well-known protein families that regulate cell survival and apoptosis is the Bcl-2 family, in particular, Bcl-2 and Bcl-X_L (194). The protective function of these proteins is, in great part, due to the formation of inactivating heterodimers with the pro-apoptotic protein Bax. An imbalance among these proteins, in favor of the pro-apoptotic activities, might sensitize tumor cells to apoptosis. However, we did not observe any differences in the normalized (vs. β-actin) expression levels of the Bcl-2, Bcl-X_L or Bax proteins within 18h of NO treatment (Figure 22A). In contrast, we noted that expression of another anti-apoptotic protein, survivin, which belongs to the IAP family, was markedly down-regulated in an NO donor dose-dependent manner (Figure 22A).

Since the degradation of survivin is ubiquitin-proteasome dependent (195), we assessed whether the NO-induced effects on tumor cell survivin expression could be inhibited by the proteasome inhibitor lactacystin. A20 cells that were treated with NO donors alone exhibited 50% reduction in survivin expression by 4h post-treatment, with lactacystin completely blocking this process (Figure 22B). Indeed, even control A20 tumor cells treated with lactacystin displayed a slight elevation in their expression of survivin, indicating that constitutive survivin turnover is blocked by this agent.

3.4.5. Pre-treatment of tumor cells with NO donor results in increased DC uptake and cross-presentation of tumor antigen to specific T cells.

We further hypothesized that increased ability of DC to mediate the apoptotic death of NO-treated tumor cells might lead to a more efficient protocol for apoptotic body (ApB) uptake and consequent cross-presentation of tumor antigens *in vitro*. To properly evaluate uptake of ApB by DCs using a flow cytometry-based analysis, we chose to pre-label tumor cells for 30m with the cell-permeable, UV-fluorescent nucleic acid dye Hoechst 33342. After staining with Hoechst 33342, tumor cells were washed and cultured in media for an additional 3h to allow for any unbound dye to diffuse from the cells. To validate that the staining procedure did not promote tumor cell apoptosis directly, labeled tumor cells were cultured overnight, then stained with PI to evaluate viability. At a final concentration of 4 μg/ml Hoechst 33342, 100% of tumor cells were stained and this signal was maintained for at least 24h, without any alteration in cell viability. Moreover, labeling was stable, since the supernatant harvested from labeled cells was not able to consequently label fresh tumor cells (data not shown).

To evaluate ApB uptake by DCs, we treated tumor cells with PAPA-NO, stained them with Hoechst 33342 before addition of DCs, and 24h later, harvested these cultures for flow cytometric analyses. Cells were counterstained with anti-mouse B220 mAb (to distinguish A20 cells from DC) and PI. Viable DCs (PI^-B220^-) were gated and analyzed for uptake of Hoechst 33342+ apoptotic bodies. As shown in Figure 23A, DCs co-cultured with NO-treated A20 cells exhibited much higher MFI than those cultured with untreated tumor cells. This suggests that more apoptotic tumor cells were taken up by DCs per unit time if the tumor cells were pretreated with the NO donor compound.

We next asked whether this NO-dependent uptake of tumor cells would result in the improved cross-presentation of tumor-associated antigens to specific T cells. To test this, we used an OVA model antigen system; H-2^b OVA-expressing EL4 (EG7) tumor cells and the DO11.10 T cell hybridoma that recognizes the $OVA_{323-339}$ peptide epitope presented in the context of I-A^d. As was the case for A20 B lymphoma cells, both EL4 and its derivative T lymphoma cell line EG7 were sensitized to DC-mediated killing by PAPA-NO pre-treatment (Figure 23B and supplement figure 2), providing support for the generality of this mechanism. Also similar to A20 cells, uptake of apoptotic EG7 cells by DCs was also increased if these tumor cells were pretreated with NO-PAPA (Figure 23C).

To access cross-presentation of tumor antigens to T cells, H-2^b EG7 (OVA+) or EL4 (OVA-) tumor cells were pretreated with PAPA-NO, prior to addition of H-2^d DCs to culture. To allow for optimal antigen uptake, processing and presentation, we co-incubated the DC-tumor mix for 24-48h, prior to addition of I-A^d-restricted DO11.10 T hybridoma cells. IL-2 production by the hybridoma cells was used as an indicator for T cell activation. As shown in Figure 24, T cells stimulated with DCs loaded with synthetic $OVA_{323-339}$ peptide produced high levels of IL-2,

while no response was detected for DCs pulsed with the control MCS peptide. Similarly, minimal IL-2 was produced when these T cells were cultured with DCs and EL4 (OVA-) tumor cells. On the contrary, when the EG7 (OVA+) tumor cells were pretreated with 10 or 25 μg/ml of PAPA-NO, IL-2 production from responder T cells was elevated to a level comparable to that of the DC + $OVA_{323-339}$ peptide cohort and this level of production was significantly greater than that observed for T cells + DC + untreated EG7 cells (with maximal effects observed after 36-48h processing periods). To further exclude the possibility of DC uptake of soluble OVA protein elaborated from EG7 cells, we also established trans-well cultures in parallel in which untreated of treated EG7 were added to upper wells while DCs and T cells were loaded into the bottom wells. Under these conditions, IL-2 was not detected in these cultures at any time point evaluated (data not shown), indicating that the activation of T cells in Figure 24 was primarily due to the direct cross-presentation of OVA peptide by H-2^d DC uptake of ApB, which is accentuated by tumor cell pre-treatment with the NO donor compound.

3.5. Discussion

In the current study, our central hypothesis was that apoptotic signals mediated via TNF family ligand/receptor complexes allow for DC-mediated tumor cell apoptosis, thereby defining a simplified cellular pathway capable of supporting the cross-priming of specific anti-tumor T cells. Our data provide novel information that multiple TNF family ligands participate in murine DC-mediated tumoricidal activity against B lymphoma cells in vitro; and, that this innate DC function can be further enhanced by pre-treating tumor cells with the phosphatase inhibitor NO to preferentially extinguish tumor cell expression of the anti-apoptotic protein survivin.

Previous reports have shown that FasL may be used by murine DCs to kill susceptible target cells, such as T cells (43, 88). Here, we report that multiple TNF family ligands, FasL and TRAIL (and possibly TNFα), participate in DC tumoricidal activity. Among the molecules that are involved, FasL clearly played a dominant role, since FasL-deficient DCs were reduced over 65% in their killing capacity. Approximately 85% of the DC-mediated cytotoxicity was ablated when all three ligand/receptor (i.e. FasL, TRAIL, TNFα) pairs were simultaneouly disrupted. However, a low but detectable level of residual tumor cell apoptosis still remained under such conditions, suggesting the minor involvement of an as yet undefined molecule(s). While we were unable to assess its importance in the current study, one obvious candidate in this regard is lymphotoxin-α/β, which belongs to the same TNF ligand superfamily and has been reported to be utilized by human DCs to kill tumor cell lines in vitro (50).

Fas-induced apoptosis of B cells has been extensively evaluated in the past (196). Naïve murine B cells express low levels of Fas, but upon activation, expression of Fas is markedly up-regulated (192). Notably, this is not sufficient to enhance B cell sensitivity to Fas-induced apoptosis. Engagement of B cell receptor or expression of anti-apoptotic proteins may induce

resistance to Fas-mediated B cell death (196). Expression of p53 may also be required for B cells to undergo apoptosis. Crosslinking CD40 induces normal B cells to proliferate and differentiate but causes many tumor cell lines to undergo apoptosis. Murine B lymphoma lines that contain mutated p53, such as A20 cells, are induced to undergo apoptosis upon CD40 crosslinking, while other lines that express wild-type p53 are comparatively resistant (197). In this context, it is perhaps not surprising that we observed that murine BM-derived DCs were selectively cytotoxic against A20 B lymphoma cells and were non-toxic to normal naïve B cells or LPS-activated B cells (either not treated or pre-treated with NO donor compounds). This provides translational support that adoptively transferred DCs may selectively kill B lymphoma cells without damaging their normal counterparts in vivo.

Several groups have also shown that treatment of tumor cells with ionizing radiation, chemotherapeutic or other pharmacological agents can sensitize them to TNF family ligand-induced apoptosis (135, 136, 198, 199). Our findings extend this work by suggesting that the pre-treatment of tumor cells with a source of NO markedly increases their apoptotic sensitivity to DCs that express (at least) three TNF family ligands including FasL, TRAIL and TNFα. Since these molecules are coordinately utilized by CTLs (200, 201) and NK cells (82) to kill tumor cells, the in vivo treatment of tumor lesions with locoregional NO would be anticipated to sensitize tumor cells to not only DC-, but also to specific CTL- and NK-mediated apoptosis, particularly under pro-inflammatory, delayed-type hypersensitivity (DTH)-type conditions.

The mechanism by which NO sensitizes tumor cells to DC-mediated killing was also investigated. Among the anti-/pro-apoptotic proteins analyzed, survivin was found to be uniquely down-regulated after NO treatment. Survivin is a recently identified anti-apoptotic protein that is aberrantly expressed in cancer cells, but is not expressed by normal, differentiated adult tissues

(202). Consistent with the homeostatic turnover/degradation of survivin being regulated by the ubiquitin-proteasome pathway (195), we observed that the proteasome inhibitor lactacystin blocked survivin degradation in NO-treated A20 cells. Based on our current data and previous reports (203, 204), we hypothesize that the mechanism of NO-induced survivin degradation and tumor sensitization involves the following pathway: 1) treatment of tumor cells with an NO donor compound rapidly inhibits the function of PP; 2) leading to the sustained activation of protein kinases, such as p34(cdc2)-cyclin B1, that are capable of phosphorylating survivin (203); 3) enhanced phosphorylation of survivin facilitates its ubiquitination; and 4) its consequent proteasome-dependent degradation. As a result of altering the balance between anti- and pro-apoptotic proteins towards a more pro-apoptotic phenotype, the death signals delivered by DCs, under such sensitized conditions, are enhanced. From an immunotherapeutic point of view, NO-facilitated survivin degradation also provides an experimental basis for the design of new combinational therapy strategies in which a "sensitizing agent" and autologous DCs could be sequentially injected intratumorally. This would promote tumor cell death in an immunologically-important manner yielding enhanced DC-mediated cross-priming of specific T cells in vivo as supported in principle by the results of in vitro experiments reported in Figure 24.

Previous reports suggested the NO has a dichotomous nature. Instead of enhancing apoptosis, some groups showed that NO could inhibit apoptosis induced by different stimuli (205-208). These differences in behavior may be explained by the chemistry of NO. After the treatment of NO donor, released NO is thought to either react directly with radicals, or form intermediates that can facilitate nitrosylation reaction (209). Since various NO donors may induce either direct or indirect effects, this could yield different end effects on treated cells. For instance, it has been reported that pre-treatment of lung fibroblast cells with PAPA-NO and

DEA-NO enhanced tumor sensitivity to cisplatin-mediated death, whereas other NO donors failed to do so (210). Hence, these distinct effects may be ultimately useful in defining clinical strategies for the use of NO donor compounds in cancer patients.

Since survivin is a shared tumor-associated antigen that is differentially expressed in a variety of malignancies and can be recognized by specific CTLs (211), it is also possible that proteasome-derived survivin peptides could be expressed at higher stochastic levels on NO-treated tumor cells, making them better targets for specific T cell clearance. We are currently evaluating the ability of survivin peptide-specific CTLs to differentially recognize control vs. NO-treated tumor cells in vitro.

Somewhat in contrast to our previous studies of human DCs (49, 50), phenotypically mature murine DCs (i.e. SM-DCs), rather than immature murine DCs mediated superior levels of tumor cell killing. Since the lytic capacity of SM-DCs appeared comparable to that of LPS-matured DCs (data not shown), the manner in which DCs become "mature" may not profoundly influence this functional aspect of these APCs.

Of translational importance, SM-DCs and iDCs appeared equally capable of engulfing apoptotic tumor bodies after first mediating their demise (data not shown). Given that DCs may also acquire antigens from viable cells (212), we could not formally exclude the participation of this pathway in the observed DC antigen uptake. Furthermore, our data has shown that sensitization of tumor cells to DC-mediated killing by NO donor treatment also leads to enhanced ApB uptake and cross-presentation of tumor-associated antigens to tumor-specific T cells. This provides confidence that the adoptive transfer of SM-DCs +/- NO intratumorally may lead to improved cross-presentation of tumor-derived epitopes to T cells in vivo. The co-application of NO *in situ* may also impact DC function however, as suggested by an equivocal

literature. Hence, although one group has showed that glioma induces apoptosis in DCs via inducible NO synthase (213), others have presented contradictory data that NO-treated DCs not only reduced the growth of the highly tumorigenic and poorly immunogenic B16 melanoma, but also caused tumor regression and improved animal survival (214). Clearly the direct testing of such combinational therapies in murine tumor models warrants further evaluation.

In summary, our study demonstrates that murine DCs mediate tumor cell apoptosis, and that such lysis may be increased by tumor cell pre-treatment with NO-elaborating agents in association with the depletion of tumor cell survivin protein levels. These findings provide a better understanding of important connections between the innate and adaptive immune effector functions of DCs and provide support for the development and pre-clinical testing of combinational immunotherapy approaches integrating tumor cell apoptosis-sensitization regimen(s) and intratumoral DC administration.

3.6. Acknowledgements

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4. SUMMARY AND CONCLUSIONS

This thesis has considered the working hypothesis that DCs can directly mediate tumor apoptosis and then uptake, process and cross-present tumor Ags, allowing for the definition of an efficient system of cross-priming for poly-specific, anti-tumor T cells *in situ*. Ultimately, I believe that by further studying the mechanism(s) of DC tumoricidal activity and the means to enhance this effect through tumor cell sensitizing modalities, we will gain a better understanding of the important connection between innate and adaptive immune functions associated with DCs in the tumor setting. Furthermore, we may then have a rationale for the formulation of novel DC-based therapies for cancer.

With regard to enhancing the efficiency of DC-mediated tumor apoptosis, I believe there are 2 prime approaches that should be considered. One is through DC modification. Gene-engineered DCs have been shown to promote DC survival, antigen presentation, as well as T cell stimulation, depending on the gene(s) inserted. In our case, I chose two intriguing Type-1 cytokines, murine IL-12p70 and IL-18. Transduction of one/both of these genes was shown to significantly improve DC cytotoxicity against CMS4 tumor cells *in vitro*. Genetic alteration was also associated with enhanced DC survival and antigen uptake, as well as notable therapeutic benefit *in vivo*. An alternative approach is via the sensitization of tumor cells to non-transfected, cultured DC-mediated apoptosis. A lack of balance in anti-/pro-apoptotic protein expression in tumor calls has been shown to alter their relative level of resistance to apoptosis. Accordingly, over-expression of pro-apoptotic proteins or down-regulation of anti-apoptotic proteins would be expected to sensitize tumor cells to apoptotic stimuli. In this study, we treated tumor cells with a pharmacologic NO donor compound, PAPA-NO, in order to accelerate the turnover of an anti-apoptotic protein survivin. I was able to demonstrate that pretreatment of lymphoma cells with PAPA-NO significantly increased their sensitivity to DC-mediated apoptotic death. This

consequently resulted in an enhanced antigen uptake and cross-presentation of tumor antigen-derived epitopes to specific T cells.

Previous reports have shown that human DCs expressing TNF family ligands are capable of inducing apoptotic death in tumor cell targets. Hence, I decided to examine whether the induced expression of TNF family ligands on murine DCs would modulate their ability to kill tumor cells. Since pro-inflammatory cytokines have been reported to increase cellular expression of TNF family ligands, I initially sought to determine whether the molecular engineering of DCs to secrete the pro-inflammatory cytokines IL-12p70 and/or IL-18 would prompt the expression of TNF family ligands on DCs, and whether these engineered DCs would mediate an enhanced capacity to kill tumor cells. Importantly, it had been previously shown that delivery of DCs expressing IL-12 (133) or IL-18 (165) into tumor lesions could induce anti-tumor immune responses, although the mechanism(s) of their enhanced therapeutic activity had not been elaborated. The fact that these cytokines also act in a synergistic manner to enhance IFN-γ production from Th1/Tc1-type T cells provides a strong basis for the use of both factors in a combined immunotherapy. Combining the available aggregate data, we hypothesized that infection of DCs with Ad.IL-12p70 and/or Ad.IL-18 would enhance their constitutive tumoricidal activity and that treatment of tumor bearing mice with cytokine-engineered DCs would induce both a stronger and also broader Type-1 anti-tumor immune response *in situ*, culminating in tumor regression.

Indeed, my initial study was to generate mIL-12 and/or mIL-18 secreting DCs. Following the previous protocol (165), I was able to successfully transduce mIL-12 and/or mIL-18 cDNAs into DCs, resulting in abundant production of rmIL12/rmIL-18, as confirmed by specific ELISA assays. Flow cytometric analyses indicated that these gene-modified DCs exhibited a more

mature phenotype than control DCs, as they expressed higher levels of MHC class I/II, as well as costimulatory molecules, when compared to controls.

To assess whether these DCs had become better killer cells, we next evaluated the cell surface expression of three TNF family ligands, TNF-α, FasL and TRAIL. These molecules are known for their ability to deliver death signal to target cells, and have typically been linked to the lytic capacity of other well-known killer cells, such as NK cells and cytotoxic T cells. Although non-infected, control DCs expressed low-to-moderate (i.e. baseline) levels of TNF family ligands, DCs infected with Ad encoding cytokines expressed significantly enhanced levels of all three molecules. This suggested that the cytokine gene-transduced DCs might represent more potent in their capacity to kill target cells that express the corresponding death receptors; i.e. TNF-R, Fas, or TRAIL-R.

To directly test this possibility, we performed MTT assays in order to determine the comparative tumoricidal activities of the various DC populations. The MTT assay is based on the cleavage of the yellow tetrazolium salt, MTT, into purple formazan by metabolically-active cells, and thus, was initially designed to provide a quantitative index of cellular proliferation and viability. Here, this assay was modified to evaluate the cytotoxicity of effector cells (49). I was very pleased to find that, although viral infection itself did not modulate DC killing activity, AdIL12DCs and AdIL12/IL18DCs became significantly better killers of CMS4 target cells *in vitro*. Inhibition of their killing activity using anti-TNF, anti-FasL, and/or anti-TRAIL blocking Abs demonstrated that both TNF-α and TRAIL played significant roles in this DC effector function. These data support the hypothesis that DC-mediated tumoricidal activity can be significantly enhanced by inserting the genes encoding IL-12 and/or IL-18 into DC, resulting in elevated expression of TNF family ligands by these APCs.

These results also encouraged me to further investigate the functionality and therapeutic efficacy of cytokine engineered-DCs *in situ*. To observe the direct impact of these DCs in the tumor microenvironment, DCs were injected intratumorally into established subcutaneous tumors. One day post treatment, tumor apoptosis was evaluated using the TUNEL assay. In order to distinguish the injected DCs from host DCs, I specifically chose DCs generated from the bone marrow of BALB/c.EGFP Tg mice, where the cells are constitutively flourescent. The results turned out to be striking. While very few DCs or apoptotic tumor cells were found in tumors that had been injected with PBS, or with uninfected or Adψ5DCs, we were able to detect markedly increased frequencies of tumor apoptosis in lesions that were injected with IL-12 and/or IL-18 transduced DCs. This was strongly correlated with a remarkable number of viable DCs localized in or proximal to regions of increased tumor apoptosis. These data suggested that the engineering of DCs to secrete IL-12 and/or IL-18 might not only enhance the stability of DC (i.e. their capacity to resist tumor-induced apoptosis/necrosis), but also improve the killing activity of these APCs (on a per-cell basis) against tumor target cells.

Another interesting finding in these experiments was the observation that some of the injected cytokine engineered-DCs could be imaged with engulfed apoptotic tumor bodies. This was in accordance with my hypothesis that an enhancement in the tumoricidal activity of DCs would directly translate into increased amounts of apoptotic bodies/debris, that could be taken up by the injected DCs *in situ*. Given that my experiment focused on a single time point post-DC injection, a more comprehensive dynamic experiment should be performed in the future where both tumor and draining LN are harvested at 4, 8, 12, and 24 hours post-DC injection and tissues evaluated for both tumor apoptosis and DC uptake of tumor debris in tissue sections. Such

information will be useful in determining the actual kinetics of ApB generation and uptake by injected DC.

These intriguing observations prompted me to further examine the therapeutic potential of DCs after *ex vivo* IL-12 and/or IL-18 gene transfer. As expected, intratumoral delivery of AdIL12/IL18DC, AdIL12DC, or AdIL18DC significantly inhibited tumor growth, when compared with the control uninfected DC treated cohort of animals, with AdIL12/IL18DCs proving statistically superior to either the AdIL12DC or AdIL18DC treatment cohorts. An evaluation of therapy-induced CD8+ T cell reactivity against natural CMS4-derived peptide epitopes revealed a markedly amplified and expanded Tc1-type repertoire in mice treated with gene-modified DCs, particularly for the AdIL12/IL18DC treated cohort. Given my *in vitro* results, I believe that this enhanced cross-priming of poly-specific T cells *in vivo* is likely due to the improved killing ability and stability of the injected DCs. Compared with previous reports that have focused on eliciting therapeutic T cell responses against a (single) specific tumor-associated antigen (106-109), my DC-based vaccine successfully induced much broader anti-tumor T cell responses against multiple, tumor-associated antigens/epitopes. Therefore, it was not surprising to find that not only the treated tumors, but also contralateral untreated tumors were significantly inhibited in their progression, given the expectation that not only locoregional but systemic therapeutic immune responses were promoted by our novel DC-based therapies.

In addition to gene modification to directly improve killing activity of DCs, the next phase of my studies was to consider increasing tumor cell sensitivity to DC-mediated apoptosis to ultimately amplify the ability of intratumorally-delivered DCs to kill treated tumor cells, then cross-prime anti-tumor T cells *in vivo*. I initially analyzed the constitutive tumoricidal activity of wild-type, cultured DCs. For this purpose, I chose a B lymphoma cell line, A20, as a primary

tumor target, since our pilot study showed that this cell line was at the high end of sensitivity to DC-mediated apoptosis among all the cell lines that I tested. In addition, in order to characterize whether there was any correlation between the level of DC maturation and their killing ability, I analyzed both immature (d7) and spontaneously-matured (d9) cultured DCs as effector cells. I observed that mature DCs were better killers of A20 cells, with this enhanced capacity associated with higher levels of FasL expression, when compared to immature d7 DCs. These data contrast somewhat with human DC reports (49) and suggest that spontaneous maturation of murine DC does not negatively impact DC-mediated tumoricidal activity. In follow-up experiments, I determined that FasL plays an important role in DC-mediated A20 tumor killing. Not only did A20 express uniformly high levels of Fas, but these cells were also sensitive to death signals delivered through the FasL-Fas pathway. This is perhaps best illustrated using DC generated from *gld* mice in which the APCs are deficient in expression of FasL. *Gld* DC were largely deficient in their killing ability to kill A20 cells, suggesting a dominant role of FasL in this DC effector function.

Next, I wished to explore a way to conditionally increase the sensitivity of tumor cells to DC-mediated apoptosis. Since the sensitivity of tumor cells to apoptotic stimuli was closely related to the balance of anti-/pro-apoptotic protein expression, we theorized that alteration of this balance using pharmacological agents could sensitize tumor cells to DC-mediated apoptosis. This idea was inspired by a series of preliminary experiments in our lab showing that treatment of human renal cell carcinoma cell lines with protein (tyrosine) phosphatase (PTP/PP) inhibitors could conditionally induce the degradation of a number of cellular proteins via the proteasome pathway, and others had shown that a subset of the sensitive proteins were involved in the apoptosis pathway of cell death.

NO is reported to effectively and reversely inhibit PTP/PPs by oxidizing critical cysteine residues in the catalytic site. Despite its dichotomous nature, our data showed NO induced A20 cell apoptosis in a dose-dependent manner. Of interest, at low concentrations, this drug was nontoxic and did not promote significant A20 apoptosis by itself. While non-toxic, I hypothesized that this low dose of drug might have affected the balance of pro-/anti-apoptotic proteins in A20 cells, in a manner that might sensitize these targets to DC-mediated killing. Among the four proteins tested, treatment of A20 cells with PAPA-NO was shown to preferentially down-regulate cellular expression of the IAP family protein survivin, while little/no changes were detected in the expression of the anti-apoptotic proteins Bcl-2 and Bcl-xL, or the pro-apoptotic protein Bax. Addition of the proteasome inhibitor lactacystin during PAPA-NO treatment of tumor cells completely blocked accelerated survivin turnover, demonstrating the proteasome dependency of this process.

The fact that PAPA-NO treatment selectively down-regulated survivin greatly encouraged me to further investigate whether there was any modulation in the sensitivity of treated tumor cells to apoptosis. When anti-Fas agonist Ab or FasL were used as apoptosis stimuli, we observed a markedly increased sensitivity of A20 cells to apoptosis. Given that the Fas-FasL pathway is essential to the effectiveness of DC-mediated A20 cell death, these data indirectly supported the hypothesis that such treatment would also sensitize A20 cells to DC-induced apoptosis. This was confirmed in the case of both immature and mature DCs. Of critical importance, this pre-conditioning regimen did not affect the sensitivity of normal B cells or B cell blasts to apoptotic stimuli, including DCs. These findings are important, since selective induction of tumor cell death without damaging normal tissue would be a major consideration for the ultimate translation of these findings into cancer immunotherapies.

Similar to the wild-type (control) DCs, my data also suggest the involvement of FasL in the NO-induced tumor sensitization to DC-mediated killing. To confirm this, and at the same time demonstrate the role of any Fas-FasL independent pathway(s) in DC-mediated tumoricidal functions, we utilized DCs generated from FasL-deficient mice. As expected, the lack of FasL expression on DCs made them less efficient killers of A20 tumor cells, regardless of whether the tumor cells were pre-treated with PAPA-NO. However, unlike wild-type DCs, where FasL played a dominant role in their killing activity, our data indicated that FasL was only partially involved in the ability of DCs to mediate cytotoxicity against NO-treated A20 cells. A more detailed study illustrated that TRAIL, and perhaps TNF-α, also actively participated in this process. In addition, these data indicated that NO pre-conditioning could sensitize A20 cells to the apoptosis induced by each of the FasL-Fas, TRAIL-TRAIL-R and TNF-α-TNFR pathways. Considering that these molecules play critical roles in the ability of both NK cells and CTLs to kill appropriate target cells, locoregional treatment of NO *in vivo* would be envisioned to sensitize tumor cells to not only DC, but NK- and CTL-mediated apoptosis as well. Therefore this strategy would not only enhance antigen uptake and cross-presentation, resulting in improved effector cell priming/activation, but also directly improve the killing efficiency of effector NK cells and CTLs within the tumor microenvironment. Such expanded cytolytic activity could then promote additional rounds of cross-presentation of ApB-derived tumor antigens and enforce therapeutic anti-tumor immunity *in situ*. Overall, this would translate into more diversified and profound anti-tumor immune responses via the classical mechanism of "epitope spreading". Moreover, due to their strong apoptosis-inducing capacity, the death receptor ligands FasL, TNF and TRAIL have been widely viewed as potential cancer therapy

targets. If combined with a sensitization regimen, therapeutics targeting TNF family ligands might also result in improved treatment efficacy.

Despite my intriguing preliminary findings, I have not formally provided the evidence supporting the direct correlation between NO-induced survivin down-regulation and tumor sensitization to apoptosis. The best way to do this is to examine whether tumor apoptosis could be augmented after survivin expression is specifically blocked in tumor cells. In recent years many RNA-based silencing mechanisms have been reported. One of the most interesting discoveries was RNA interference (RNAi), a post-transcriptional sequence-specific gene-silencing mechanism initiated by the introduction of double-stranded RNA (dsRNA) that are homologous in sequence to the silenced gene (215). Using small interference RNA (siRNA) specific to survivin, we were able to reduce the specific protein expression by approximately 50% (Supplement figure 3A). A20 tumor cells that have been targeted with survivin siRNA exhibited increased apoptosis frequencies (Supplement figure 3B). Although we could not exclude the possibility that other molecules might also be involved in NO-induced A20 sensitization to apoptosis, I believe that survivin is a crucial contributing factor to this process.

Even though I have now demonstrated a novel and potentially clinically-important effector function of DCs, their major role in adaptive immunity, undoubtedly, remains that of antigen-presenting cells. Based on my results generated during the course of this thesis research, a reasonable question to ask is whether the enhanced tumor sensitization to DC-mediated killing directly impacts ApB-derived antigen cross-presentation to specific T cells. I approached this issue by choosing an OVA-expressing lymphoma cell line (EG7) as a primary tumor model. As a byproduct of this experiment, I was able to generalize my initial conclusions that not only A20, but also other lymphoma cell lines (i.e. EL4, EG7) could be sensitized to DC-mediated apoptosis

upon treatment with PAPA-NO, thereby expanding this type of intervention to multiple lymphomas at least.

The usual way to test DC uptake of cellular antigens is to pre-label target cells with DiD (1,1'-dioctadecyl-3,3,3',3'-tetramethylindodi-carbocyanine perchlorate) or CFSE (carboxyfluorescein diacetate, succinimidyl ester or CFDA-SE) before co-incubating them with DCs. DC engulfment of antigen can then be examined by flow cytometry or via confocal fluorescence imaging. In our study evaluating the uptake of ApB by DC, these traditional dyes were inappropriate since DCs could also acquire membrane and intracellular protein antigens from live cells (212). The best surrogate index of apoptotic antigen uptake would be reflected in the acquisition of tumor cell nucleic acids, which would be released only upon tumor cell death. I selected the cell-permeant nucleic acid dye Hoechst 33342 to pre-label tumor cells due to its specificity and stability (i.e. lack of bleeding from pre-labeled cells). Using this dye, I demonstrated that DCs more readily acquired NO-sensitized vs. control, untreated tumor ApB *in vitro*. This could be clearly due to the increased tumor debris available to DCs after enhanced killing of tumor cells. While perhaps a subtlety, I remain unable to resolve whether the DC that mediates tumor apoptosis is the same cell that ultimately captures the ApB (i.e. antigen). Nonetheless, my interpretation of the available data is that DCs that kill tumor cells may themselves uptake some apoptotic antigens, and meanwhile, the amount of excess debris may recruit additional DCs as a "clean up" measure.

One interesting finding of my studies is the impact of PTP inhibitors on tumor cell resistance to apoptosis. In my study, the accelerated turnover of anti-apoptotic protein survivin enhanced the sensitivity of tumor cells to DC-mediated apoptosis. Since NO-induced, proteasome-dependent degradation of alternative cellular proteins, including tumor-associated

antigens, is expected, this treatment modality could also theoretically improve the processing and presentation of tumor epitopes to specific CD8+ T cells. In addition to my findings implicating survivin as a conditional target in such a model, recent results from the Storkus lab also suggest that this may be the case for multiple tumor cells overexpressed receptor tyrosine kinases, such as EphA2 and EGFR. Hence, a careful selection of conditioning agents might positively and coordinately impact virtually all of the major events crucial to effective Type-1 anti-tumor immunity.

As most immunotherapies would ultimately be translated through *in vivo* animal models, our laboratory now plans to apply this DC and sensitization regimen-combined therapy to tumor-bearing mice. In this proposed study, in order to understand whether the cross-priming of T cells can be promoted, we will establish subcutaneous EG7 (H-2^b) tumors in H-2^b × H-2^d F_1 hybrid mice. Instead of using chemical agents such as NO donor compounds, we will be highly selective in injecting AdiNOS, an adenovirus encoding inversible NO synthesase gene, directly into tumor lesions. Cells infected by this virus will produce sustained levels of NO *in situ*, which will be expected to sensitize tumor cells to subsequently i.t. injected DCs. The efficacy of the sensitization regimen will be assessed by evaluating survivin expression in the treated tumor biopsies. Assuming survivin protein is reduced after AdiNOS injection, one day after virus administration, SM-DCs (H-2^d) will be delivered intratumorally and CFSE labeled CD4+ T cells isolated from DO11.10/Rag2 KO (H-2^d) mice will be adoptively-transferred into these recipient animals 48 hours later. CFSE is a cell-permeant fluorescein-related vital dye. Because of its long-lived staining of intracellular proteins, flow cytometric analysis of lymphocyte proliferation can be achieved by determining serial halving of the fluorescence intensity of CFSE-labeled cells over time as daughter cells are generated (216). Since the tumor cells, transferred DCs and T

cells are MHC mis-matched, labeled T cells could only be activated by H-2^d DCs, which had acquired tumor ApB generated by injected H-2^d DCs. Therefore, the activation of T cells will likely be the result of efficient cross-priming *in situ*. In addition, we will also monitor tumor growth every 2-3 days in order to determine whether there is any therapeutic benefit as a consequence of this therapy. If the therapy is successful, these data would provide confidence that intratumoral delivery of sensitizing agents and autologous DC in combinational therapies represent a viable treatment option for patients with cancer. Studying the advance of such applications, however, has numerous considerations: i.e. what is the best and safest sensitization regimen to apply to humans? What is the proper dose and timing for such treatments? Which kind of tumors is most amenable to such treatments? When should DCs be injected after application of the sensitizing agent? Should IL-12 and/or IL-18 cDNA engineered DCs be considered for injection in combined therapies given their enhanced efficacy noted in pre-clinical models? Hence, while this thesis has generated at least as many questions as it has answered, there is great enthusiasm that effective combinational therapies can be designed based on optimizing DC-mediated killing and cross-presentation within the tumor microenvironment.

Table 1. Cytokine Production from Control and Gene Engineered DCs.

	Level of Cytokine Secreted*:	
DCs Evaluated	mIL-12	mIL-18
Control DC (no Ad)	< .06	< .03
Adψ5DC	< .06	< .03
AdIL12DC	85.6 ± 1.1	< .03
AdIL18DC	0.86 ± 0.03	6.3 ± 0.2
AdIL12/IL18DC	85.3 ± 3.2	9.6 ± 0.8

* ng/5 x 10^6 DCs/48h. Results are mean ± SD of triplicate determinations and are representative of 3 independent experiments performed.

Table 2. Phenotypic Characteristics of Control and Gene Engineered DCs.

	Mean Fluorescence Intensity (MFI)[a]				
DCs Evaluated[b]	MHC I	MHC II	CD40	CD80	CD86
Control DC (no Ad)	221	242	59	108	652
Adψ5DC	214	244	64	103	644
AdIL12.DC	317[c]	339[c]	51	106	685
AdIL18DC	351[c]	349[c]	65	168[c]	1020[c]
AdIL12/IL18DC	359[c]	414[c]	66	93	918[c]

[a]Values represent the mean of MFI values obtained in 3 independent experiments.

[b]DCs were harvested at day 7 of culture and either not infected or infected with the indicated adenoviral vectors. Forty-eight hours later, DCs were recovered from culture and phenotyped for expression of the indicated markers.

[c] $p < 0.05$ compared with mock-infected DCs.

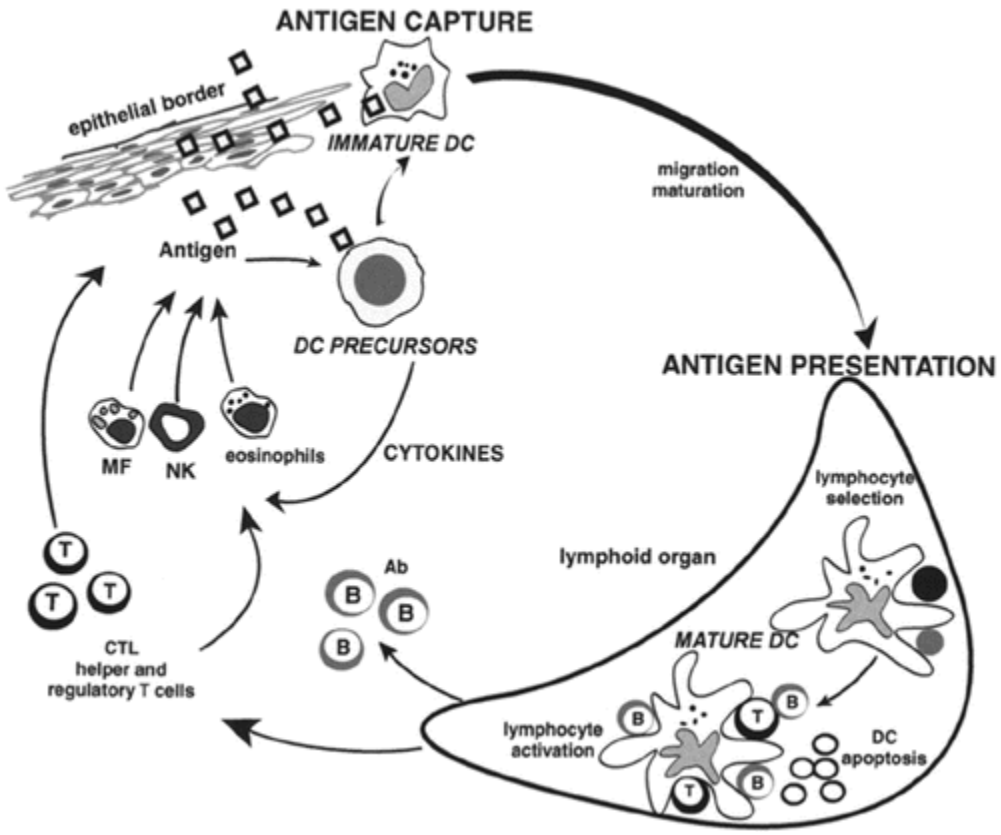

Figure 1 The life cycle of dendritic cells (DC).

Circulating precursor DCs enter tissues as immature DCs. They can also directly encounter pathogens (e.g. viruses) that induce secretion of cytokines (e.g. IFNα), which in turn can activate eosinophils, macrophages (MF), and natural killer (NK) cells. After antigen capture, immature DCs migrate to lymphoid organs where, after maturation, they display peptide-major histocompatibility complexes, which allow selection of rare circulating antigen-specific lymphocytes. These activated T cells help DCs in terminal maturation, which allows lymphocyte expansion and differentiation. Activated T lymphocytes migrate and can reach the injured tissue, because they can traverse inflamed epithelia. Helper T cells secrete cytokines, which permit activation of macrophages, NK cells, and eosinophils. Cytotoxic T cells eventually lyse the infected cells. B cells become activated after contact with T cells and DCs and then migrate into various areas where they mature into plasma cells, which produce antibodies that neutralize the initial pathogen. (Figure cited from Annu Rev Immunol. 2000;18:767-811)

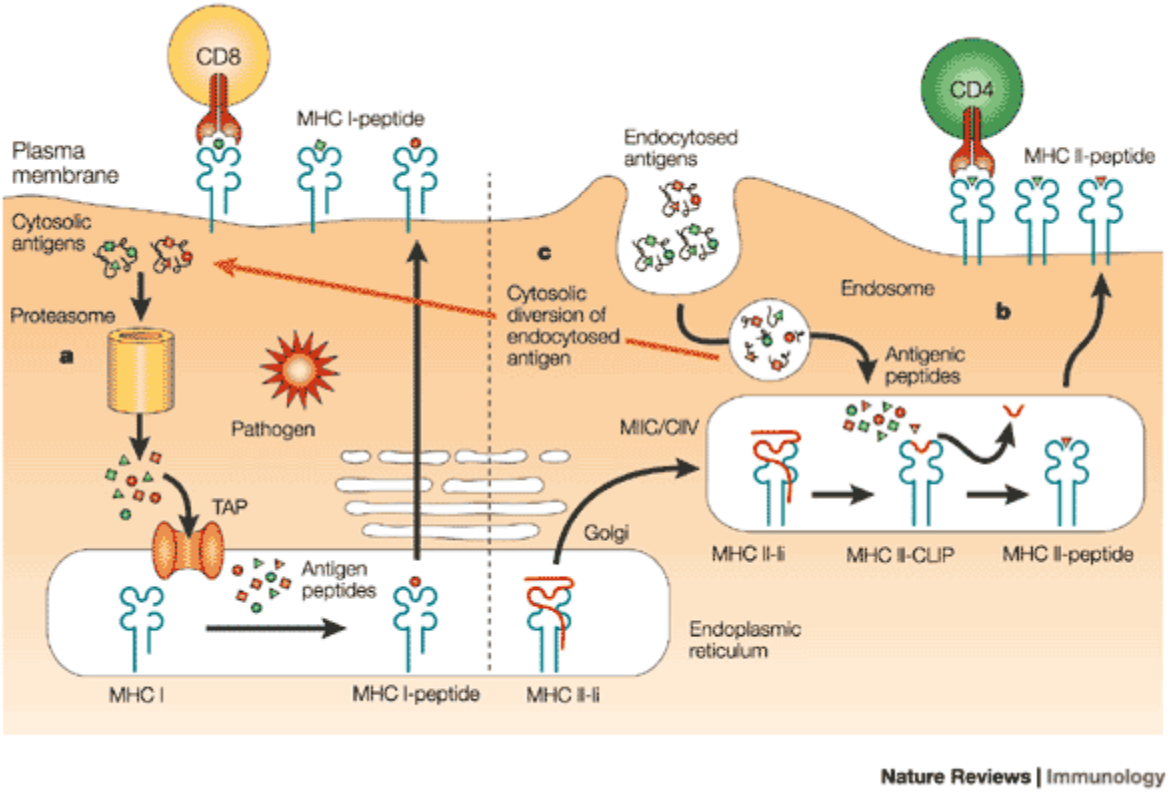

Figure 2 Different antigen-processing pathways for the MHC class I and class II molecules.

a | MHC class I molecules present peptides that are primarily derived from endogenously synthesized proteins of either self or pathogen origin. These proteins are degraded into peptides by the proteasome and then transported through the transporters of antigen-processing (TAP) molecules into the endoplasmic reticulum for loading on MHC class I molecules. **b** | By contrast, MHC class II molecules present proteins that enter the cell through the endocytic route. During maturation of MHC class II molecules, they are prevented from binding to endogenous antigens in the endoplasmic reticulum by association with the invariant chain (Ii). Invariant chain–MHC class II complexes (MHC II–Ii) move through the Golgi to the MIIC/CIIV compartment where the invariant chain is degraded to CLIP (for class II-associated invariant-chain peptide). CLIP is then removed from the CLIP–MHC class II (MHC–CLIP) complexes and exchanged for antigenic peptide. **c** | Dendritic cells can endocytose antigens from other cells and cross-present them to CD8+ cytotoxic T lymphocytes. The TAP-dependence of such cross-presentation[44, 52], indicates that it involves diversion of the cellular antigens into the conventional MHC class I pathway, although the mechanism(s) for this diversion are as yet undefined. In most cases, these antigens will also be processed into the MHC class II presentation pathway for recognition by CD4+ helper T cells. (MIIC, MHC II loading compartment; CIIV, MHC II vesicles.) (Figure cited from Nat Rev Immunol. 2001;1:126-135)

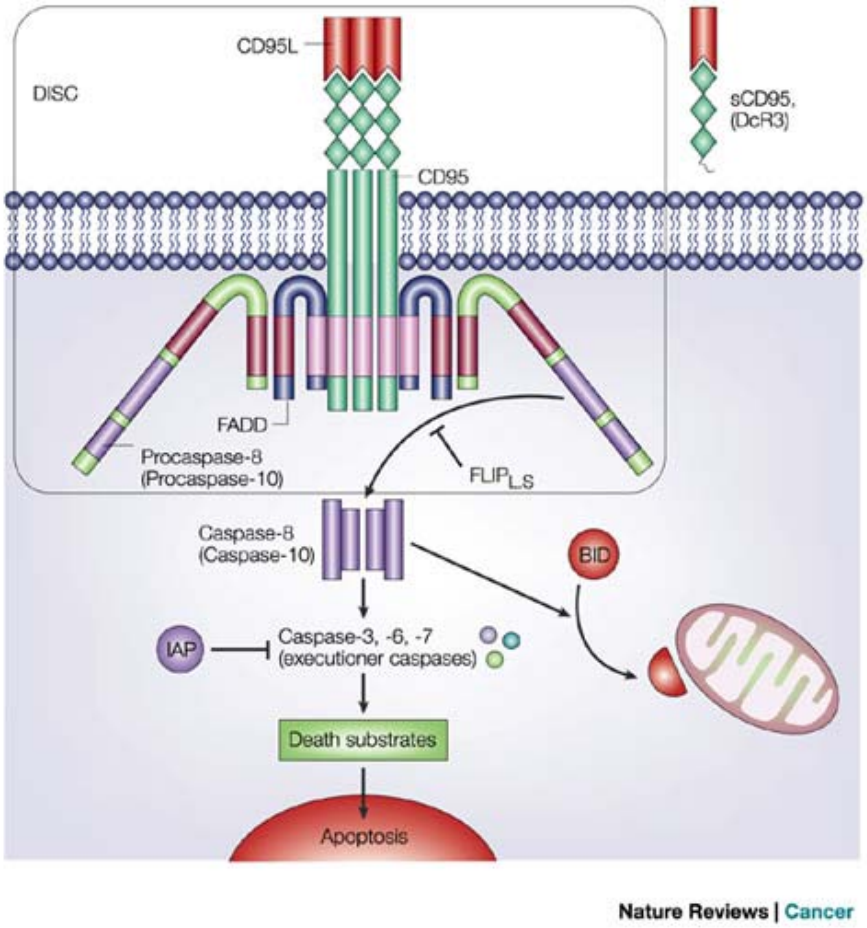

Figure 3 Apoptosis signalling through death receptors.

Binding of death ligands (CD95L is used here as an example) to their receptor leads to the formation of the death-inducing signalling complex (DISC). In the DISC, the initiator procaspase-8 is recruited by FADD (FAS-associated death domain protein) and is activated by autocatalytic cleavage. Death-receptor-mediated apoptosis can be inhibited at several levels by anti-apoptotic proteins: CD95L can be prevented from binding to CD95 by soluble 'decoy' receptors, such as soluble CD95 (sCD95) or DcR3 (decoy receptor 3). FLICE-inhibitory proteins (FLIPs) bind to the DISC and prevent the activation of caspase-8; and inhibitors of apoptosis proteins (IAPs) bind to and inhibit caspases. $FLIP_L$ and $FLIP_S$ refer to long and short forms of FLIP, respectively. (Figure cited from Nat Rev Cancer. 2002;**2**:277-288)

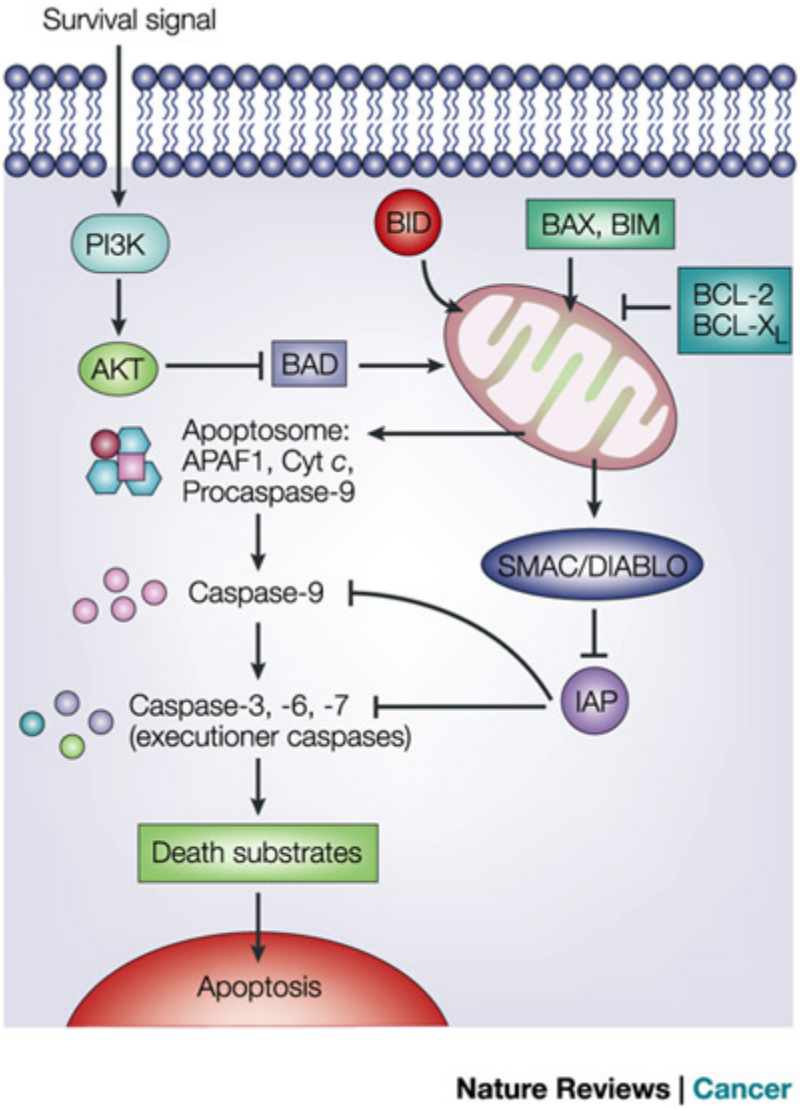

Figure 4 Apoptosis signalling through mitochondria.

Chemotherapy, irradiation and other stimuli can initiate apoptosis through the mitochondrial (intrinsic) pathway. Pro-apoptotic BCL2 family proteins — for example, BAX, BID, BAD and BIM — are important mediators of these signals. Activation of mitochondria leads to the release of cytochrome *c* (Cyt *c*) into the cytosol, where it binds apoptotic protease activating factor 1 (APAF1) to form the apoptosome. At the apoptosome, the initiator caspase-9 is activated. Apoptosis through mitochondria can be inhibited on different levels by anti-apoptotic proteins, including the anti-apoptotic BCL2 family members BCL2 and BCL-X_L and inhibitors of apoptosis proteins (IAPs), which are regulated by SMAC/DIABLO (second mitochondria-derived activator of caspase/direct IAP binding protein with low pI). Another way is through survival signals, such as growth factors and cytokines, that activate the phosphatidylinositol 3-kinase (PI3K) pathway. PI3K activates AKT, which phosphorylates and inactivates the pro-apoptotic BCL2-family member BAD. (Figure cited from Nat Rev Cancer. 2002;**2**:277-288)

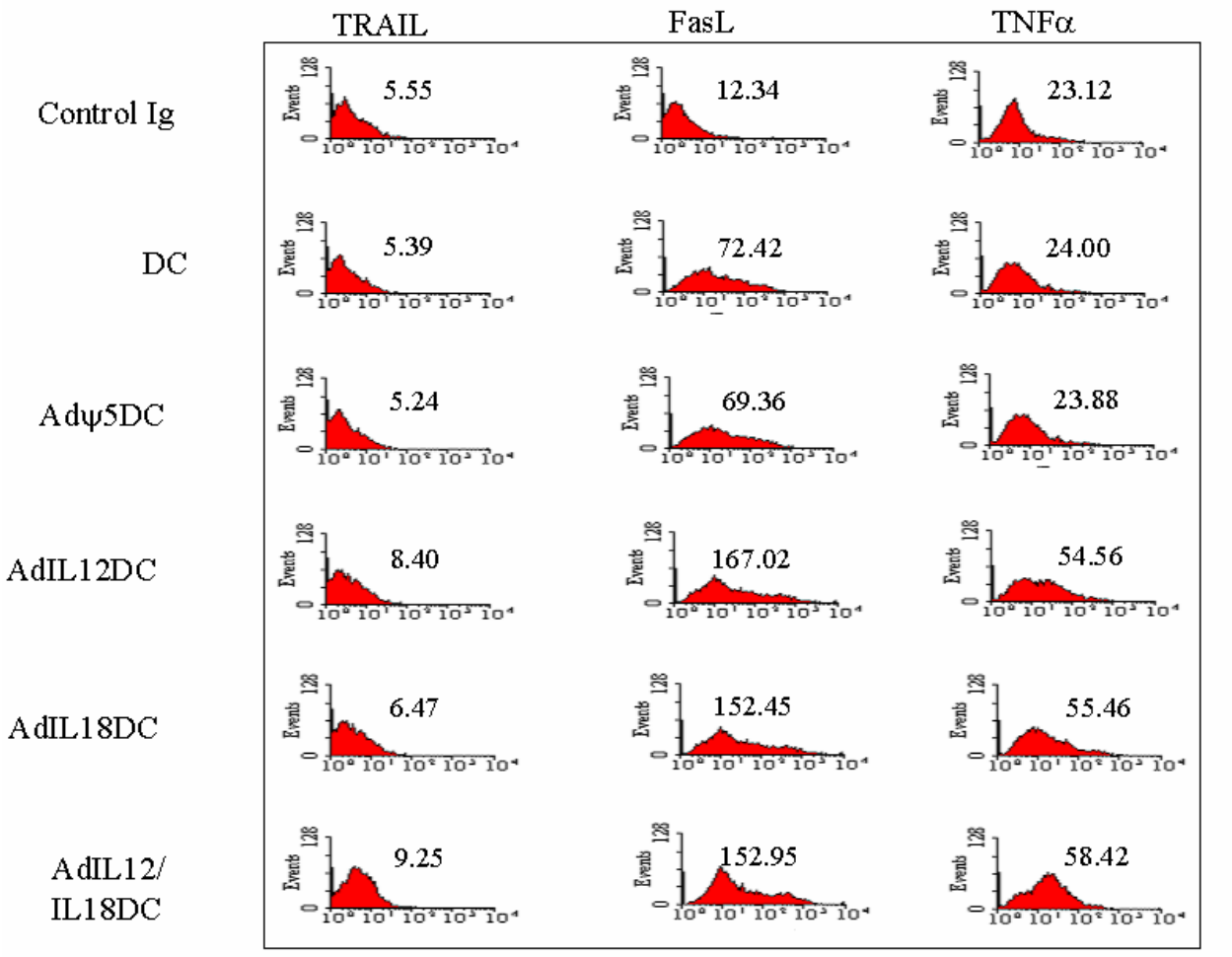

Figure 5 Expression of TNF family ligands on adenoviral trasnfected DCs.

Bone marrow-derived DCs were infected with adenoviral vectors encoding mouse IL-12 (AdIL12DC), mouse IL-18 (AdIL18DC), both AdIL12 and AdIL18 (AdIL12/IL18DC) or mock vector (Adψ5DC). Adenoviral transduced or nontransduced DCs (DC) were stained with TRAIL, FasL or TNF-α specific antibodies, or isotype control antibodies and flow cytometry was performed. Mean fluorescence intensities are indicated in each histogram panel. Similar results were obtained in three independent experiments.

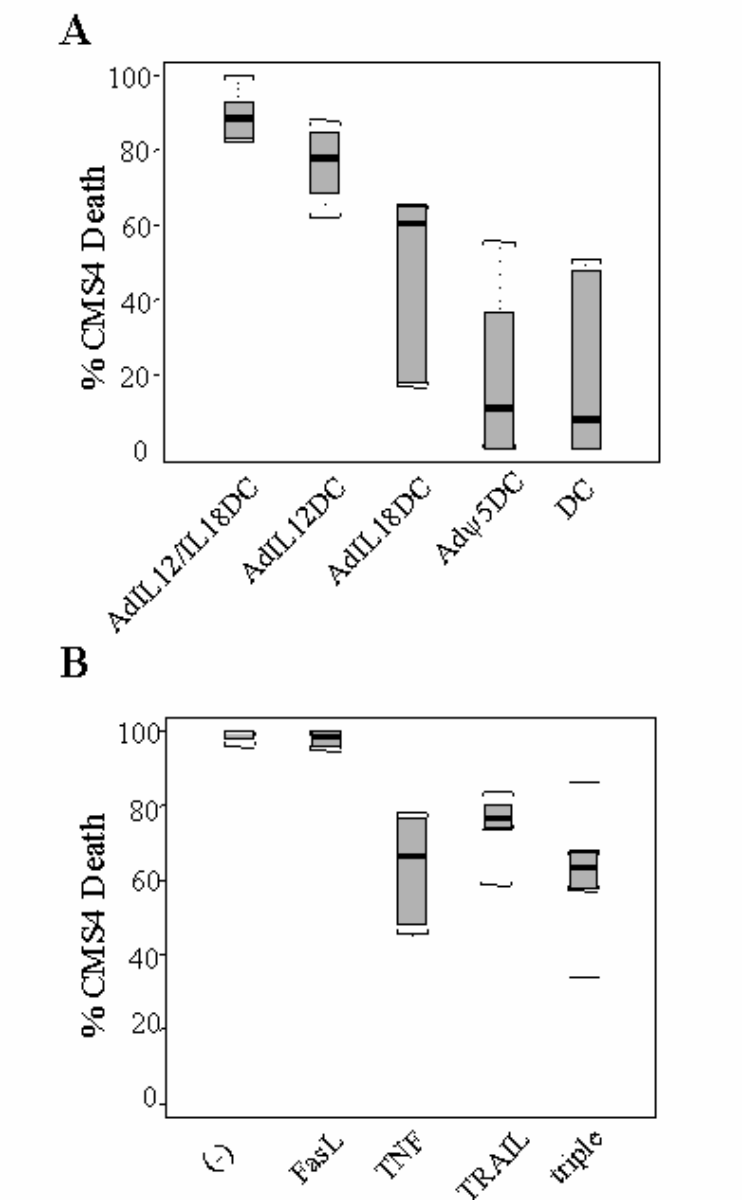

Figure 6 Differential killing of CMS4 cells by adenovirally-infected DCs.

(A) The tumoricidal activity of adenovirally infected DCs against CMS4 cancer cells was evaluated by using 24hr MTT assays. The effector DCs and CMS4 target cells were mixed in a 5:1 (DC:T) ratio and cytotoxicity assays were performed. The percentages of specific killing are presented as box and whisker plots which display the median percent cytotoxicity (black bar) for each groups of adenovirally transfected DCs; the boxes show the interquartile range, the whiskers to 1.5 times the interquartile range. (B) For blocking of the interactions between the TNF family ligands and their receptors, adenovirally infected DCs (AdIL12/IL18DC) were preincubated for 60 minutes without antibody (**(-)**) or with antagonist antibodies against TRAIL (**TRAIL**), Fas ligand (**FasL**), TNF-α (**TNF**) or the combination of all three antibodies (**triple**). Box and whisker plots show the distribution of the percent cytotoxicity for each group.

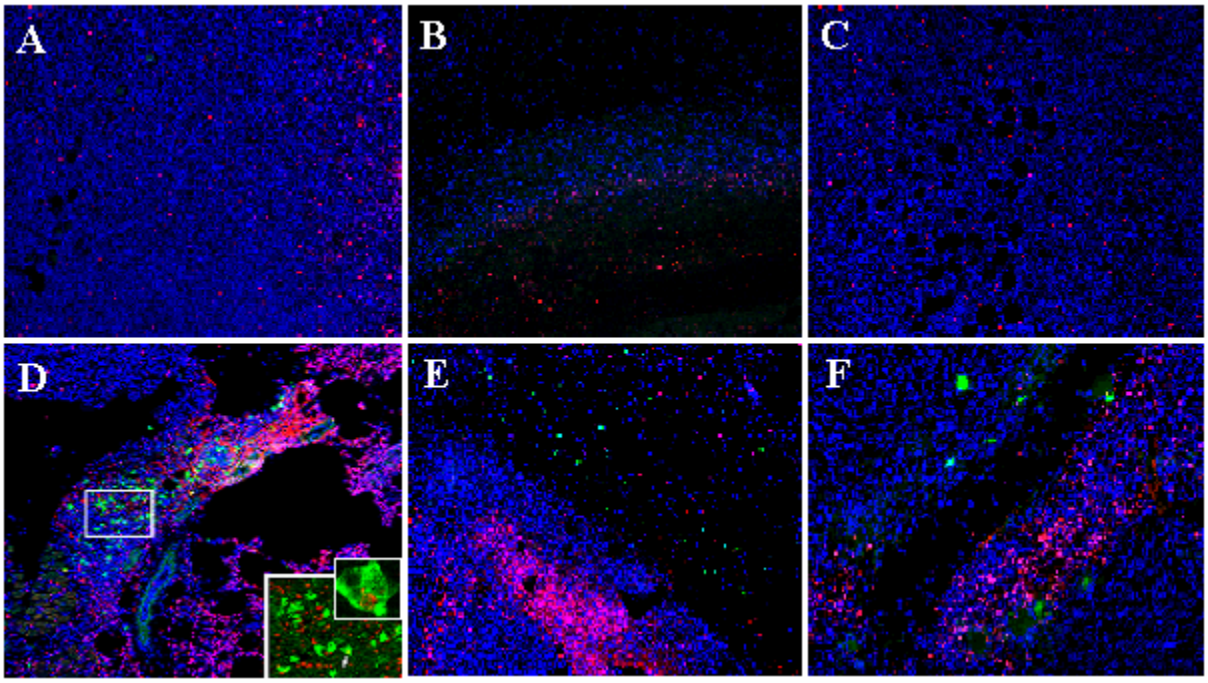

Figure 7 Cytokine gene engineering of DCs promotes DC survival, tumor apoptosis and antigen uptake in situ.

Day 7 BM-derived DCs were generated from BALB/c.EGFP mice and either not infected, or infected with the recombinant adenoviruses for 48h. One million control or cytokine gene engineered DCs (or saline) were then injected into established day 7 CMS4 tumors. After 24h, tumor lesions were resected, fixed, frozen, sectioned and stained for apoptotic cells using TUNEL. Sections were also counterstained using Hoechst to detect nuclei. Panel A = Saline control; Panel B = Uninfected DCs, Panel C = Adψ5DC; Panel D = AdIL12DC; Panel E= AdIL18DC; Panel F = AdIL12/IL18DC. Stained sections were then evaluated by flourescence microscopy at 20X magnification, with Panel C insert providing a 40X regional magnification and a 100X view of an EGFP+ DC containing an apoptotic (tumor) body.

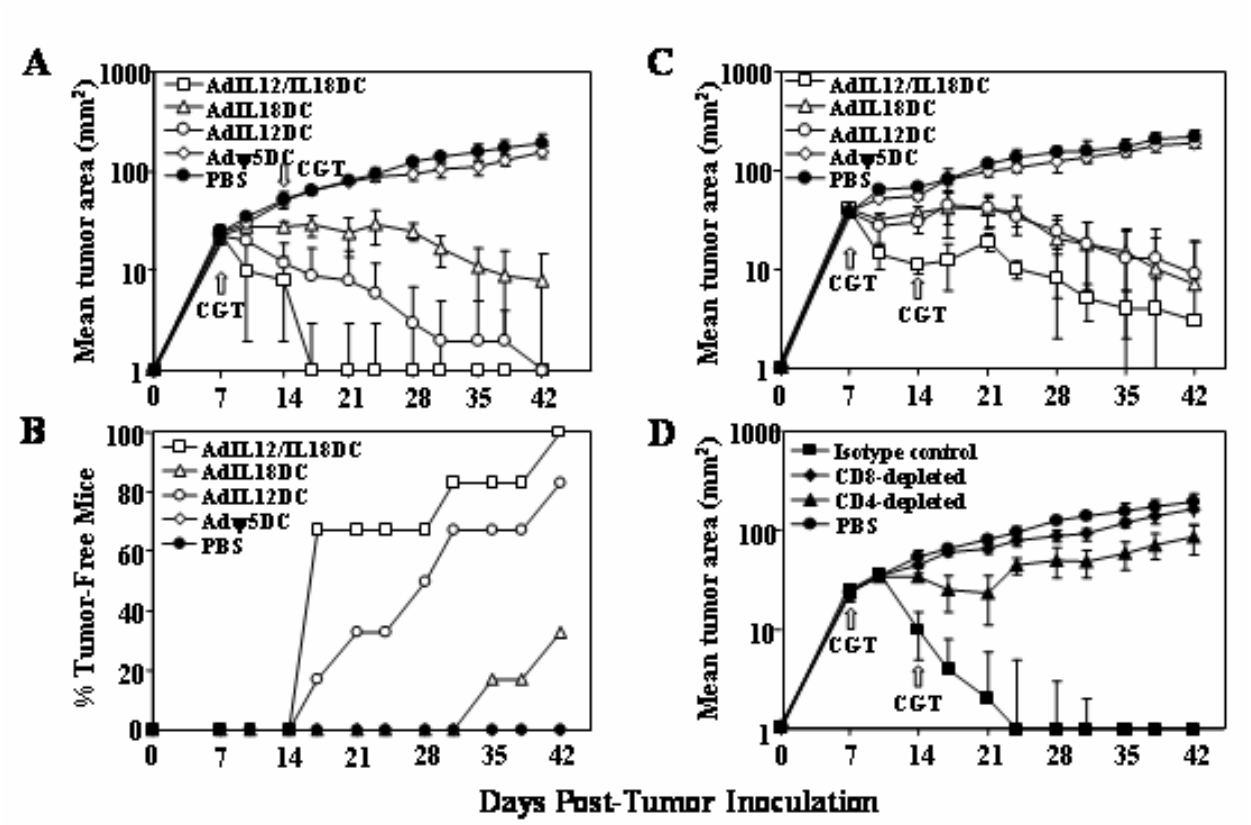

Figure 8 Therapeutic effects of vaccination with IL-12 and/or IL-18 transduced DCs.

Seven days after tumor inoculation, BALB/c mice bearing established CMS4 or MethA tumors were treated with the indicated intratumoral injection therapies (6 mice per treatment groups). (A) A mixed linear model was fit to the data and used to describe change in CMS4 tumor area over time. Each data point represents the least squares mean tumor size derived from the model along with the estimate of standard error. (B) CMS4 tumor rejection rate expressed as percentage for the same mice shown in Figure 4A. (C) Impact of intratumoral DC-based therapies on the growth of MethA tumors. A mixed linear model was fit to the data and used to describe change in MethA tumor area over time. Each data point represents the least squares mean tumor size derived from the model along with the estimate of standard error. (D) Ab-mediated *in situ* depletion of CD4+ or CD8+ T cells (as described in Materials and Methods) markedly reduces the therapeutic efficacy of AdIL12/IL18DC therapy. Each data point represents the least squares mean tumor size derived from the model along with the estimate of standard error.

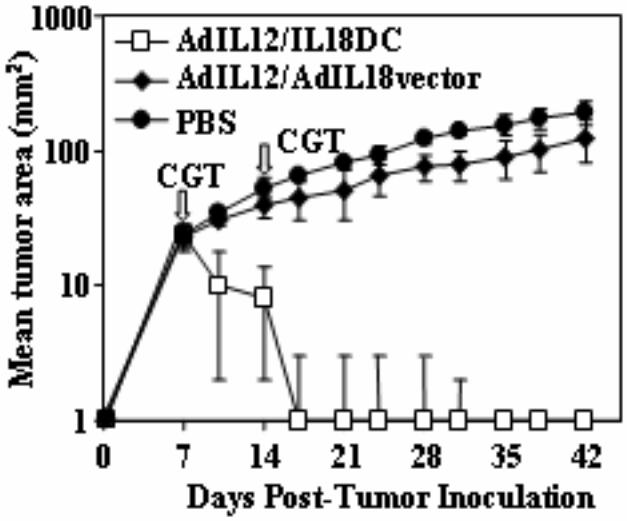

Figure 9 AdIL12/IL18DC-based therapy is superior to AdIL-12 + AdIL-18 intratumoral therapy.

BALB/c mice bearing established day 7 CMS4 tumors were treated with intratumoral injections of either AdIL12/IL18DC or a mixture of the recombinant adenoviruses encoding IL-12 and IL-18 (AdIL-12 and AdIL-18). One week later, therapies were repeated. Tumor size was monitored on the indicated days, with the reported values representing the mean tumor size ± standard error. Similar results were obtained in two independent experiments.

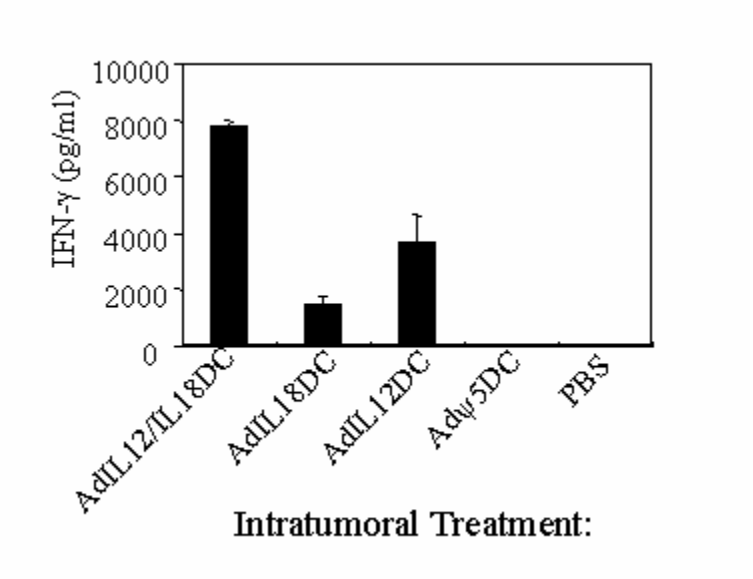

Figure 10 Specific IFNγ production from immune splenocytes.

Mice bearing day 7 CMS4 tumors were treated with intratumoral injection of AdIL-12 and/or AdIL-18-infected DCs. Splenocytes were harvested from mice 14 days after the last intratumoral injections (i.e. 28 days post-tumor inoculation), then cocultured with irradiated CMS4 cells for 5 days, prior to assessment of IFN-γ production from responder splenocytes against CMS4 target cells using specific ELISA assays. Cytokine levels are reported in pg/ml (mean ± SD of triplicate samples). Similar results were obtained in two independent experiments.

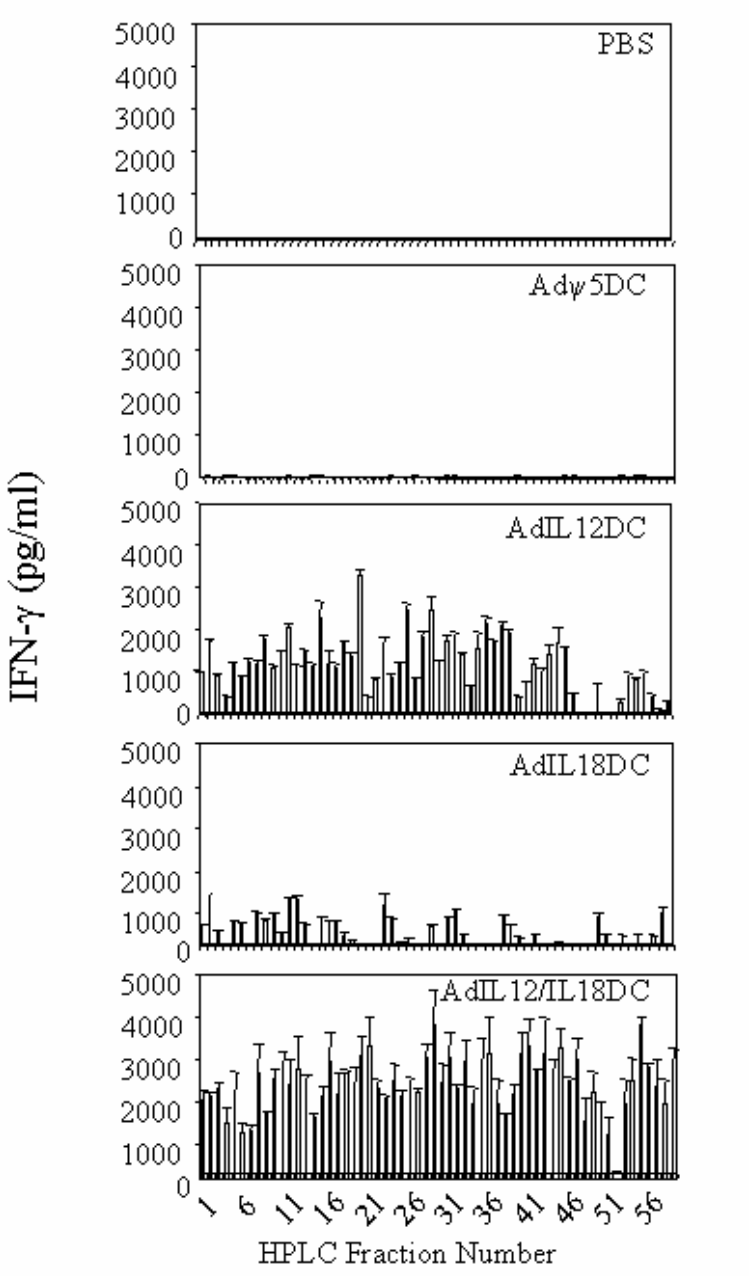

Figure 11 Evaluation of the repertoire of natural CMS4-derived peptide epitopes recognized by CD8+ T cells in responder mice.

Splenic CD8+ T cells were isolated from mice treated with adenovirally transfected DCs (AdIL12/IL18DC:IL-12 and IL-18 transduced DCs, AdIL18DC:IL-18 transduced DCs, AdIL12DC:IL-12 transduced DCs, AdΨ5DC:AdΨ5 transduced DCs, PBS: control). Syngeneic DCs were pulsed with individual HPLC fractions containing resolved CMS4 peptides and used to stimulate CD8+ T cells for 48 hr, prior to harvesting culture supernatants for determination of IFN-γ concentrations measured by ELISA (in pg/ml; mean ± SD of triplicate samples). Similar results were obtained in two independent experiments.

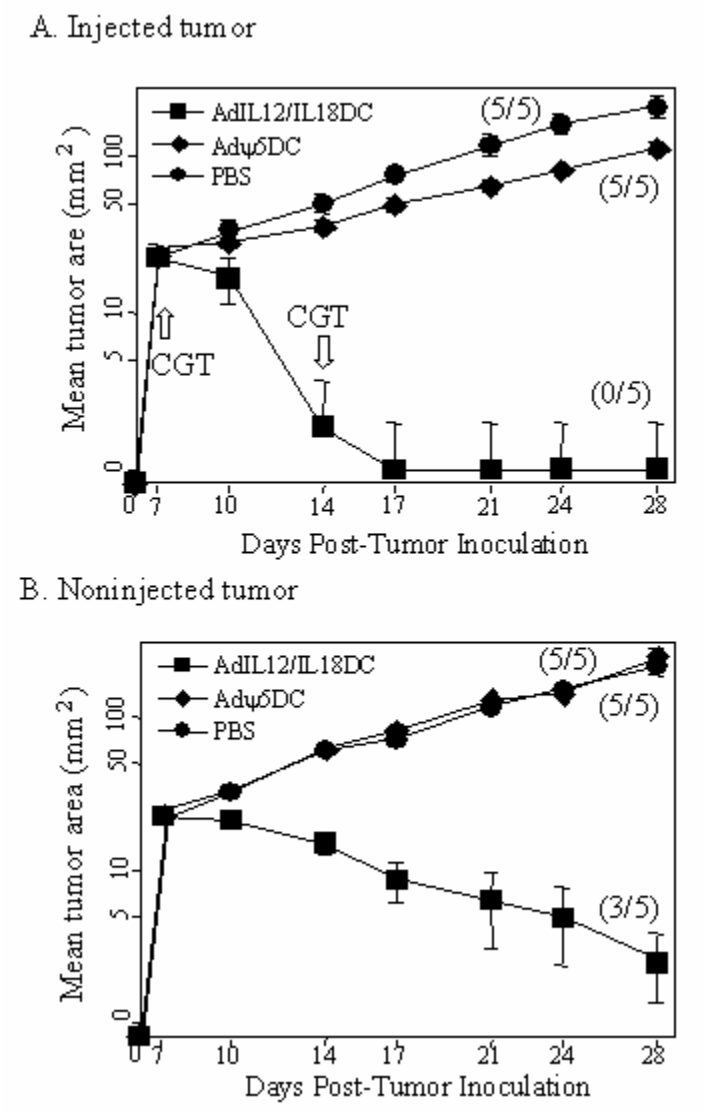

Figure 12 Therapeutic effect of the AdIL12/IL18DC therapy on contralateral nontreated CMS4 tumors.

Mice were first injected with $3x10^5$ CMS4 cells in both flanks on day 0. Seven days after tumor inoculation, BALB/c mice bearing established CMS4 tumors were treated in the right flank with the indicated intratumoral injection therapies as indicated in panel inserts (5 mice per treatment group). Mean tumor area on each flank was determined for all mice bearing tumors (A: treated tumor growth, B: contralateral, non-treated tumor growth). Each data point represents the mean tumor size ± standard error. The fraction of mice bearing a tumor in each treatment group at day 28 is indicated in parentheses. AdIL12/IL18DC-treated tumors were significantly smaller in the injected tumor by day 10 and the non-injected tumor by day 14 (all $p<0.01$).

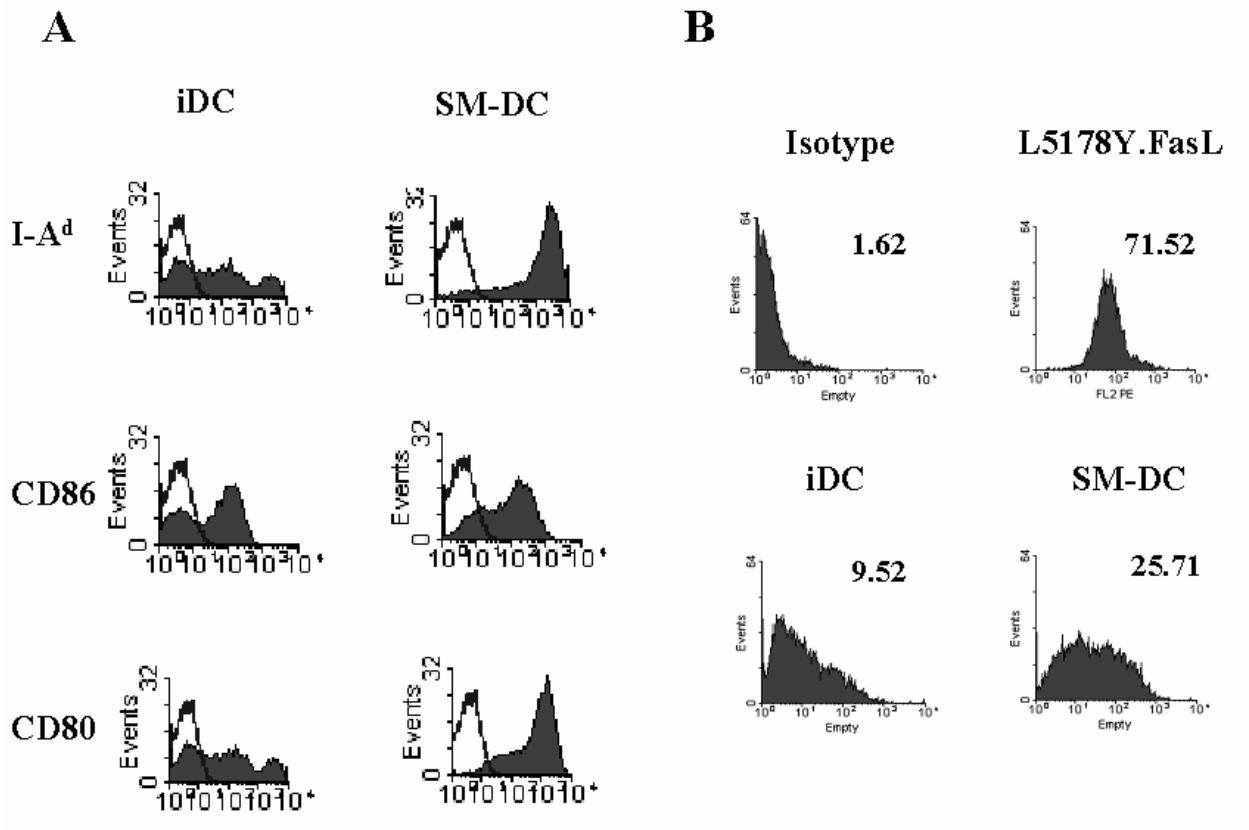

Figure 13 Phenotype of iDC and SM-DC.

BM-derived DC were generated using a bulk-culture method (167). On day 7 and day 9, immature (iDC) and spontaneously-matured DC (SM-DC), respectively, were purified using CD11c MACS beads. DC were stained with specific Abs against (A) MHC class II and costimulatory molecules or (B) FasL, and flow cytometry performed. The results are presented as (A) overlays of single-color histograms obtained with isotype-matched control (open histograms) and specific Abs (filled histograms); and (B) single-color histograms. MFI are indicated in each histogram panel. These data are representative of three independent experiments performed.

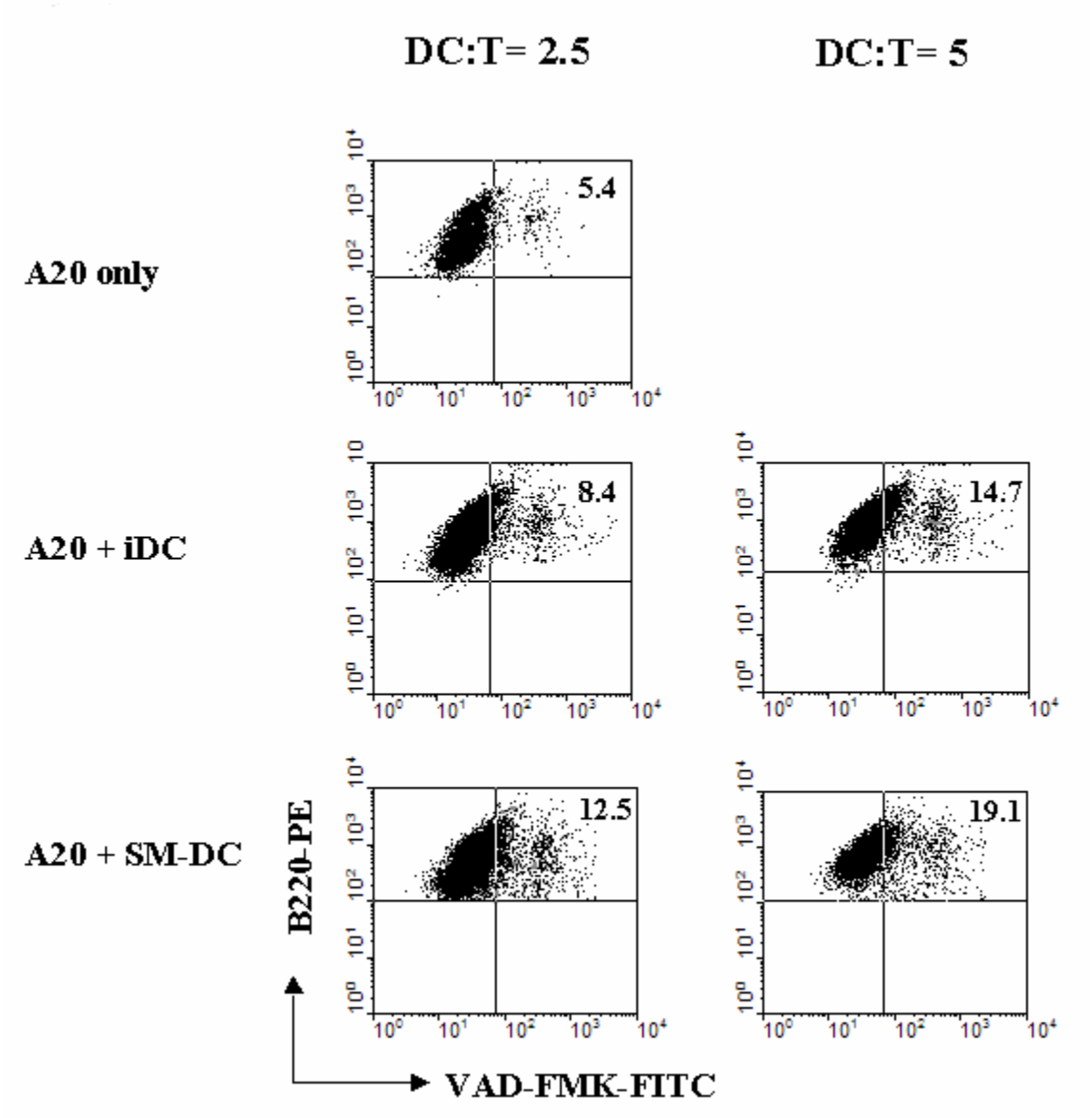

Figure 14 SM-DC induce a greater degree of apoptosis in A20 B lymphoma cells than iDC.

IDC and SM-DC were co-cultured with A20 tumor cells at a DC/T ratio of 2.5:1 or 5:1 for 4h. Cells were stained with PE-conjugated anti-B220 Abs and FITC-conjugated pan-caspase inhibitor z-VAD-FMK and analyzed by FACS. Tumor cell population was gated on B220 positive staining. The percentage of cytotoxicity, indicated in the upper right quadrant, was calculated based on the fraction of apoptotic tumor cells (B220+VAD-FMK+) divided by total number of tumor cells (B220+). These data are representative of two independent experiments performed.

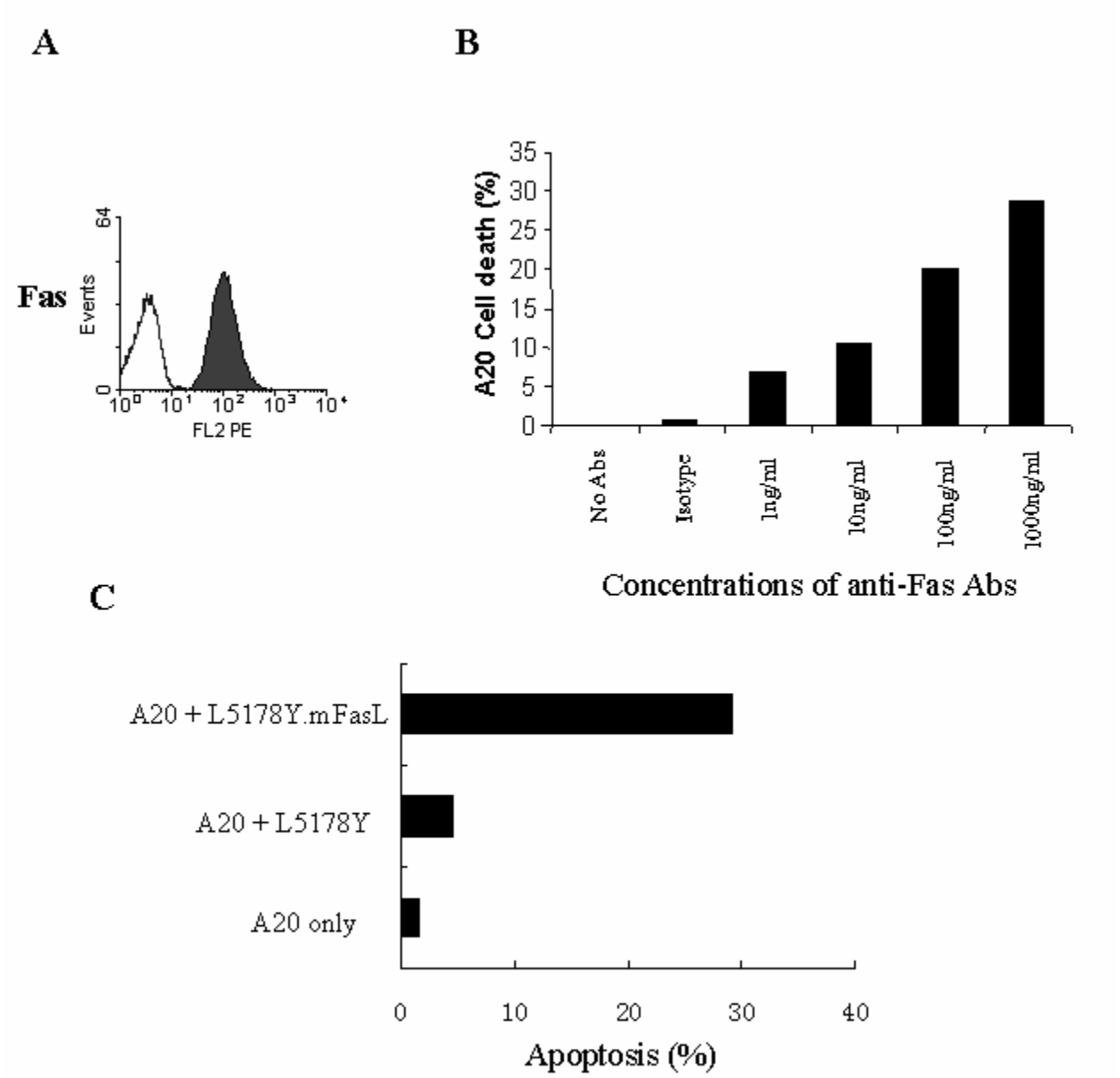

Figure 15 A20 cells are sensitive to Fas-mediated apoptosis.

(A) A20 cells express membrane-bound Fas. Cell surface expression of Fas was assessed using a three-step flow cytometry technique, with the results depicted as overlays of isotype-matched control (open histogram) and Fas Ab (filled histograms) staining profiles. (B) Agonist anti-Fas Ab (Jo2) induces apoptosis in A20 tumor cells. A20 cells were incubated with anti-Fas Ab at the indicated concentrations for 24h at 37°C and cytotoxicity was evaluated by MTT assay. (C) Ligation of FasL by Fas triggers apoptosis in A20 cells. FasL-transfected (L5178Y.mFasL) or mock-transfected L5178Y cells (L5178Y) were co-incubated with A20 tumor cells at an E/T ratio of 5:1 for 4h. Cells were stained with a PE-labeled B220-specific mAb and FITC-labeled caspase activation marker z-VAD-FMK. DC cytotoxicity was calculated as described above. The results presented here are converted from density plots as shown in Figure 14.

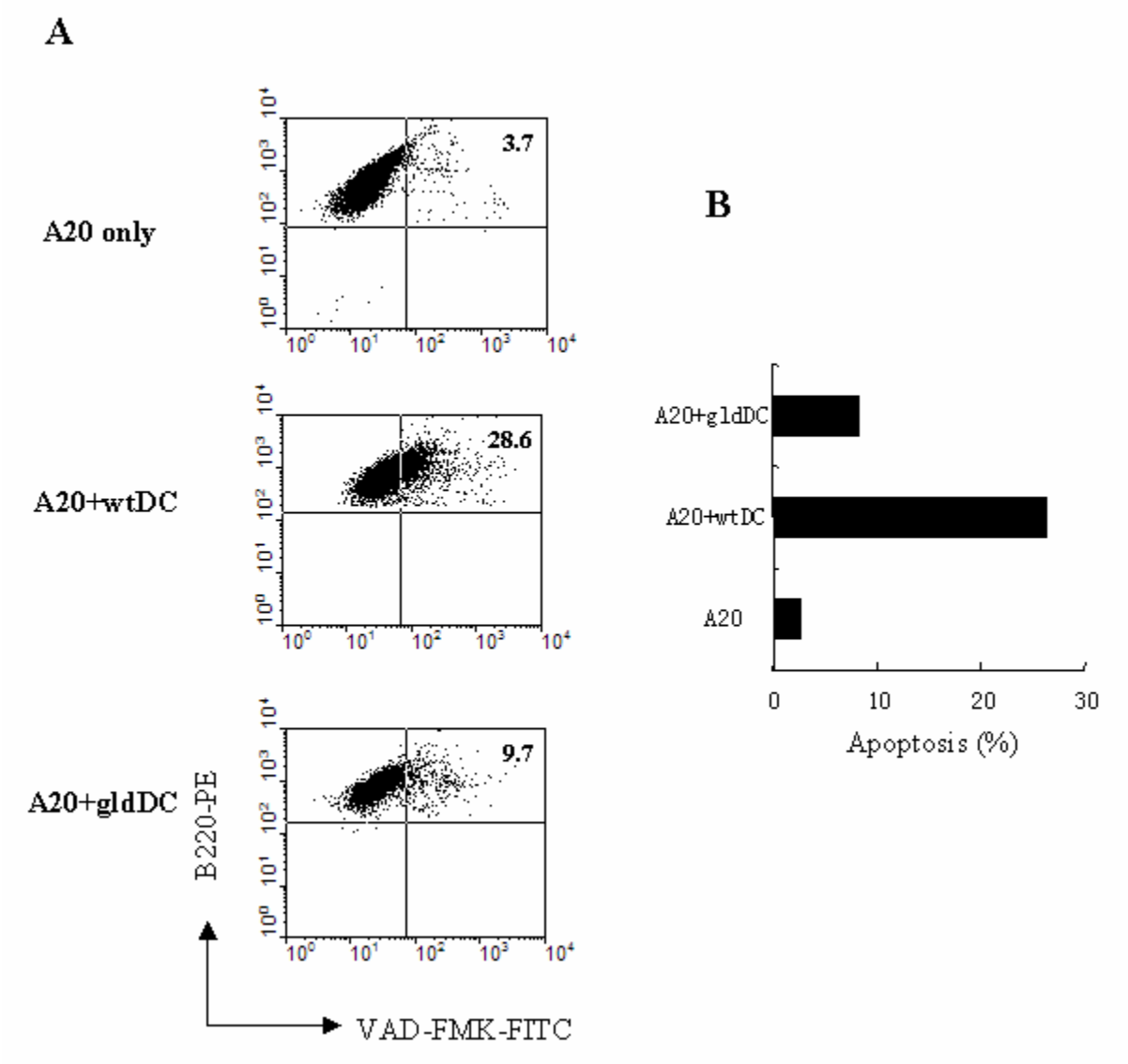

Figure 16 DC-mediated A20 tumor apoptosis is mediated predominantly via the FasL-Fas pathway.

(A) BM-derived DCs were generated from H-2^d wild type (wtDC) or FasL-deficient gld mice (gldDC). DC effector cells were then co-incubated with A20 tumor cells at a DC/tumor ratio of 5:1 for 4h. Cells were stained and analyzed as described above. The data are representative of two independent experiments performed. (B) The data shown in (A) have been converted from density plots into histogram.

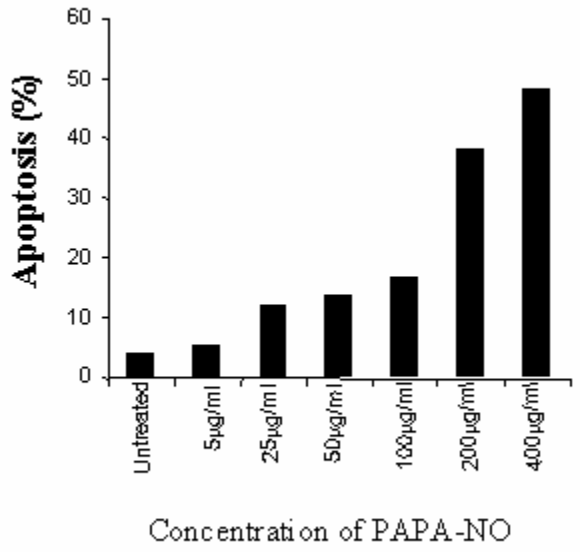

Figure 17 NO donor induced A20 cell apoptosis in a dose-dependent manner.

A20 tumor cells were pre-incubated with PAPA-NO at the indicated concentrations for 1h at 37°C. Control culture media were then replaced and the tumor cells maintained in culture overnight. Cells were then stained with VAD-FMK-FITC to detect apoptosis. These data are representative of two independent experiments performed.

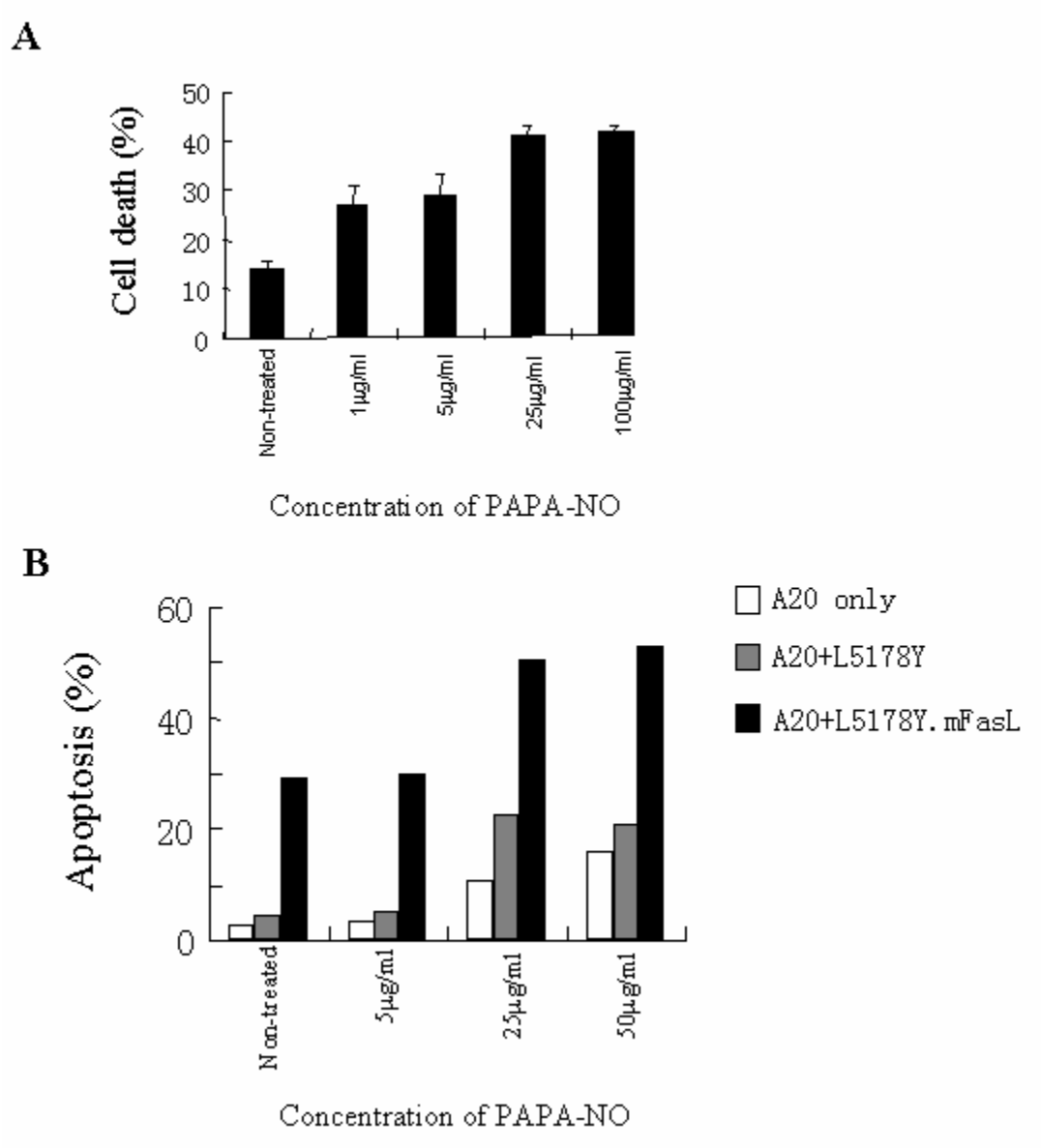

Figure 18 A20 cells treated with low doses of PAPA-NO are sensitized to Fas-mediated apoptotic death.

(A) NO-treated A20 cells were sensitized to anti-Fas Ab-induced apoptosis. Tumor cells were treated with PAPA-NO at the indicated concentrations for 1h at 37°C, and then incubated in fresh media in the presence or absence of anti-Fas Abs overnight. The MTT assay was used to determine cytotoxicity. These data are representative of three independent experiments performed. (B) NO-treated A20 cells were sensitized to FasL-mediated apoptosis. A20 cells were treated with NO donor as described in (A). 18h post treatment, FasL- or mock-transfected L5178Y cells were added to culture for 4h and cytotoxicity was evaluated as described above. These data are representative of two independent experiments performed.

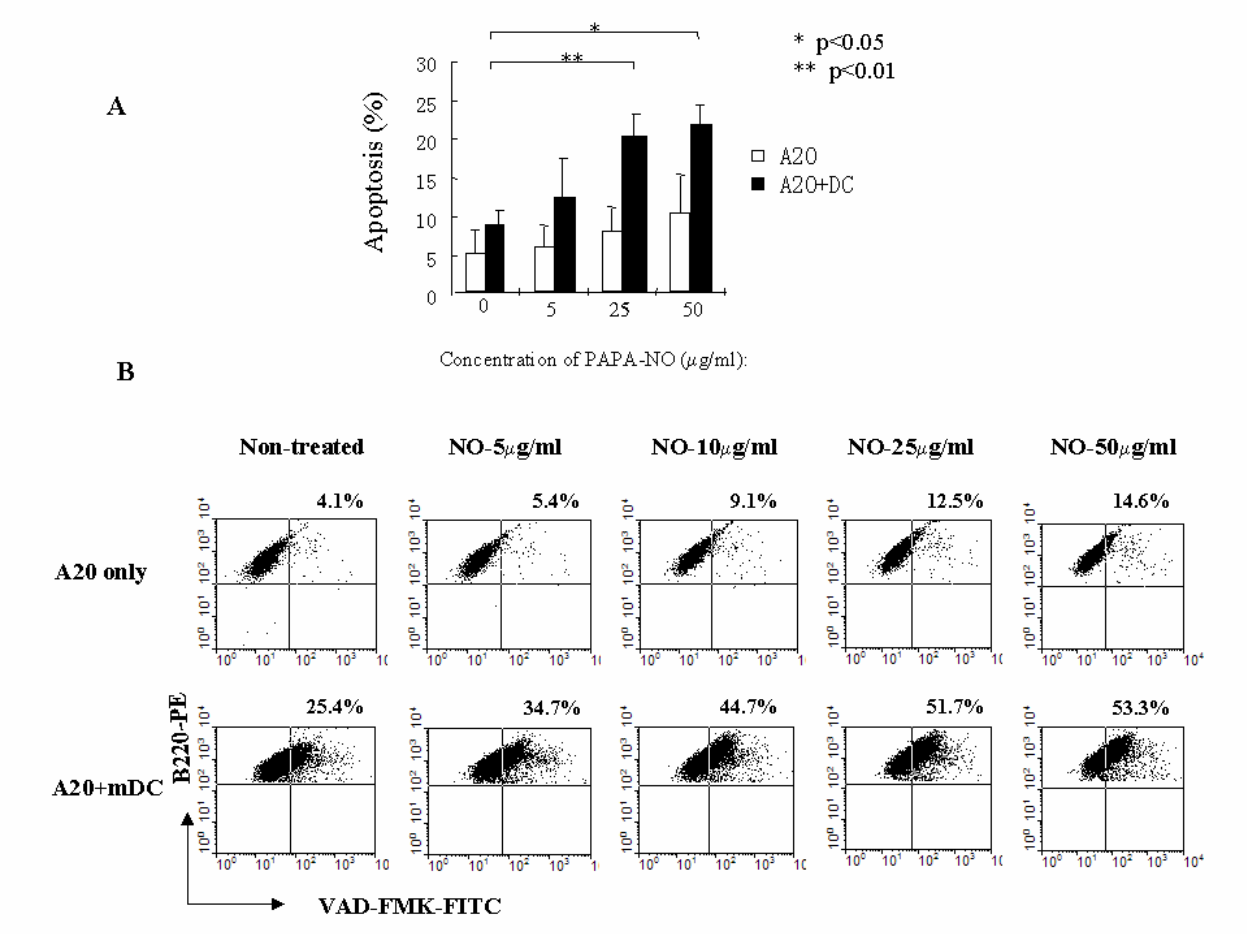

Figure 19 NO pre-treatment significantly sensitizes A20 tumor cells to both iDC- and SM-DC-mediated apoptosis.

A20 cells were pre-treated with PAPA-NO as described in Figure 18 and then co-cultured with iDC (A) or SM-DC (B) for 4h in cytotoxicity assays. The statistical analyses of three independent experiments are provided in panel A. Panel B results are representative of 2 independent experiments performed.

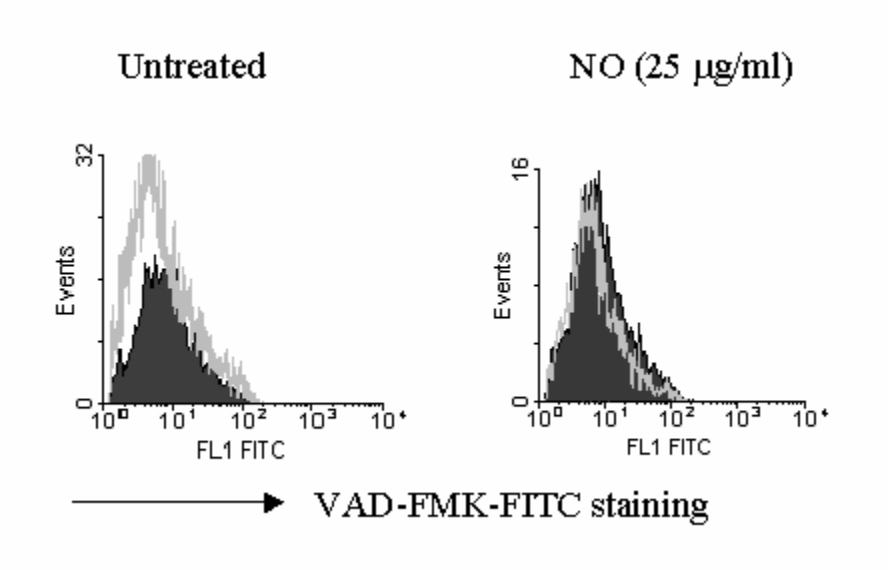

Figure 20 Pretreatment of PAPA-NO do not promote the apoptotic death of normal B cells.

Bulk splenocytes were cultured at 37ºC in the presence of 10μg/ml LPS. Three days later, cells were harvested and treated with or without PAPA-NO at a final concentration of 25 μg/ml, before being co-cultured with SM-DCs as described above. Cells were gated on B220+ events and the data presented as overlays of histograms obtained with B cell only culture (open histograms) and B-DC co-culture (filled histograms).

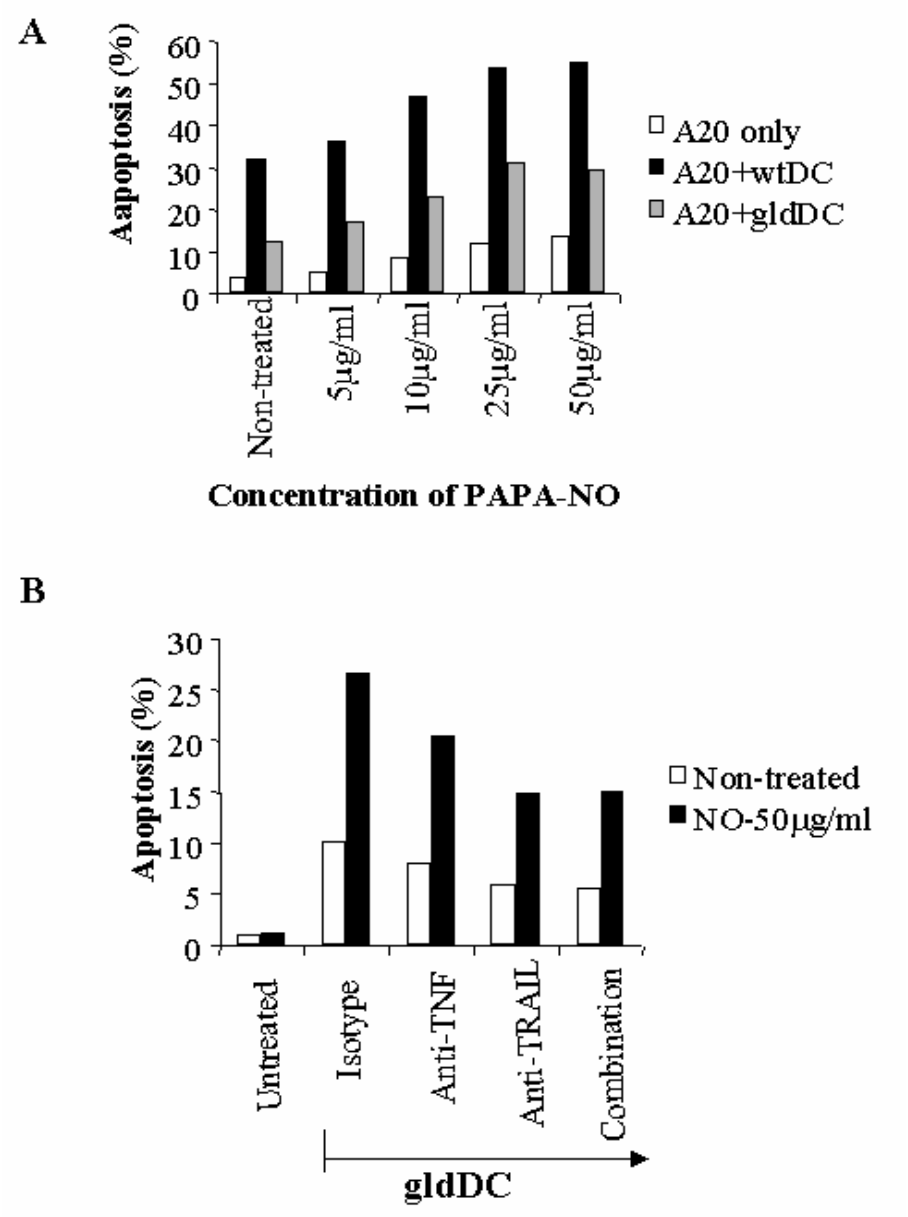

Figure 21 Treatment of A20 tumor cells with PAPA-NO enhances their sensitivity to DC-induced apoptosis through both Fas-dependent and -independent pathways.

(A) A20 tumor cells were pretreated with PAPA-NO at the indicated concentrations before being co-incubated with DCs that were generated from either wild type mice (wtDC) or FasL-deficient mice (gldDC). DC cytotoxicity against tumor cells was analyzed as described in the Figrue 14 legend. (B) DCs generated from the BM of FasL-deficient mice were cultured for 1h in the presence or absence of 20 μg/ml of anti-TNF Ab and/or anti-TRAIL Ab, or isotype control Ab. The DCs were then added to A20 cell cultures that has been pre-treated with 50 μg/ml of PAPA-NO and analyzed for DC-mediated cytotoxicity as shown in (A). Data of density plots have been converted into histograms.

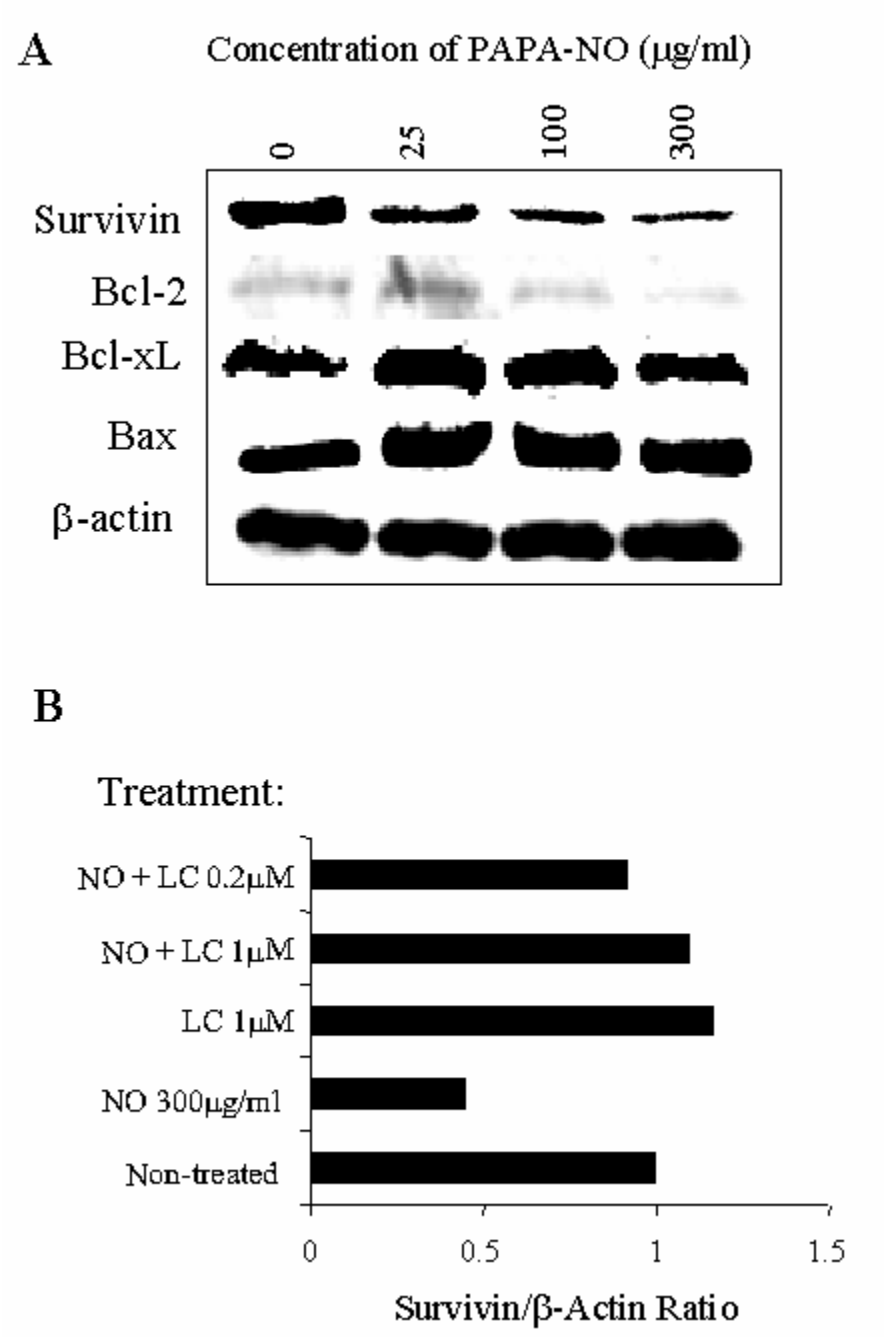

Figure 22 NO donor pre-treatment preferentially accelerates survivin protein turnover in A20 cells.

(A) Whole cell lysates of PAPA-NO treated or untreated A20 tumor cells were prepared for Western blot analyses using anti-survivin, anti-Bcl-2, anti-Bcl-xL and anti-Bax Abs. (B) A20 cells were treated with 300 μg/ml of PAPA-NO. Media were then changed and tumor cells maintained in CM in the presence or absence of 0.2 μM or 1 μM lactacystin (LC) for 4h. Whole cell lysates were prepared for immunoblot analyses using anti-survivin Ab, as described in the Materials and Methods. The results shown here have been normalized by expression of β-actin.

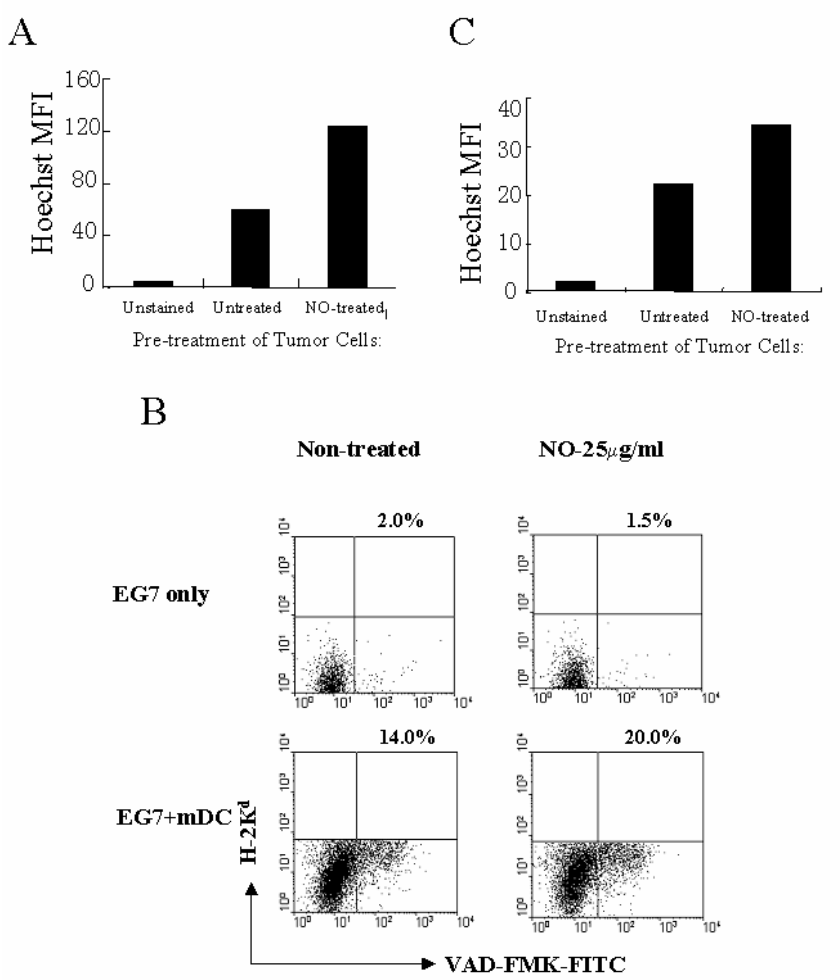

Figure 23 PAPA-NO pre-treatment enhances DC-mediated ApB uptake.

(A) Pre-treatment of A20 tumor cells with PAPA-NO enhanced DC uptake of apoptotic tumor cells. A20 tumor cells were treated with 25 μg/ml of PAPA-NO as described above and stained with 4 μg/ml Hoechst 33342 for 30m. Cells were washed and maintained in culture for additional 3h before washing again and adding DCs at a DC/tumor ratio of 5 to 1. After 24h, the cells were harvested and stained with anti-mouse B220 mAb and PI, prior to flow cytometry analysis. Viable DCs ($B220^{-}PI^{-}$) were gated for analysis and MFI of Hoechst 33342 staining is reported. (B) Pre-treatment of EG7 (OVA+; $H\text{-}2^{b}$) cells with PAPA-NO sensitized them to DC ($H\text{-}2^{d}$)-mediated apoptosis. EG7 tumor cells were treated with 25 μg/ml PAPA-NO and coincubated with DCs, as described above. The cells were then stained with anti-mouse $H\text{-}2K^{d}$ mAb and VAD-FMK-FITC to distinguish EG7 (K^{d}-neg) and DC (K^{d+}), and to determine viability, respectively, in the flow cytometry assays. (C) NO donor pre-treatment of EG7 cells increases uptake of tumor ApB by DCs. EG7 tumor cells were treated with PAPA-NO and stained with 1 μg/ml Hoechst 33342 before addition of DCs, as described in *Material and Methods*. Twenty-four hours later, cells were stained with anti-mouse $H\text{-}2K^{d}$ mAb and PI. The DC populations were gated on $H\text{-}2K^{d}$ and PI negative events and MFI of Hoechst 33342 staining was evaluated.

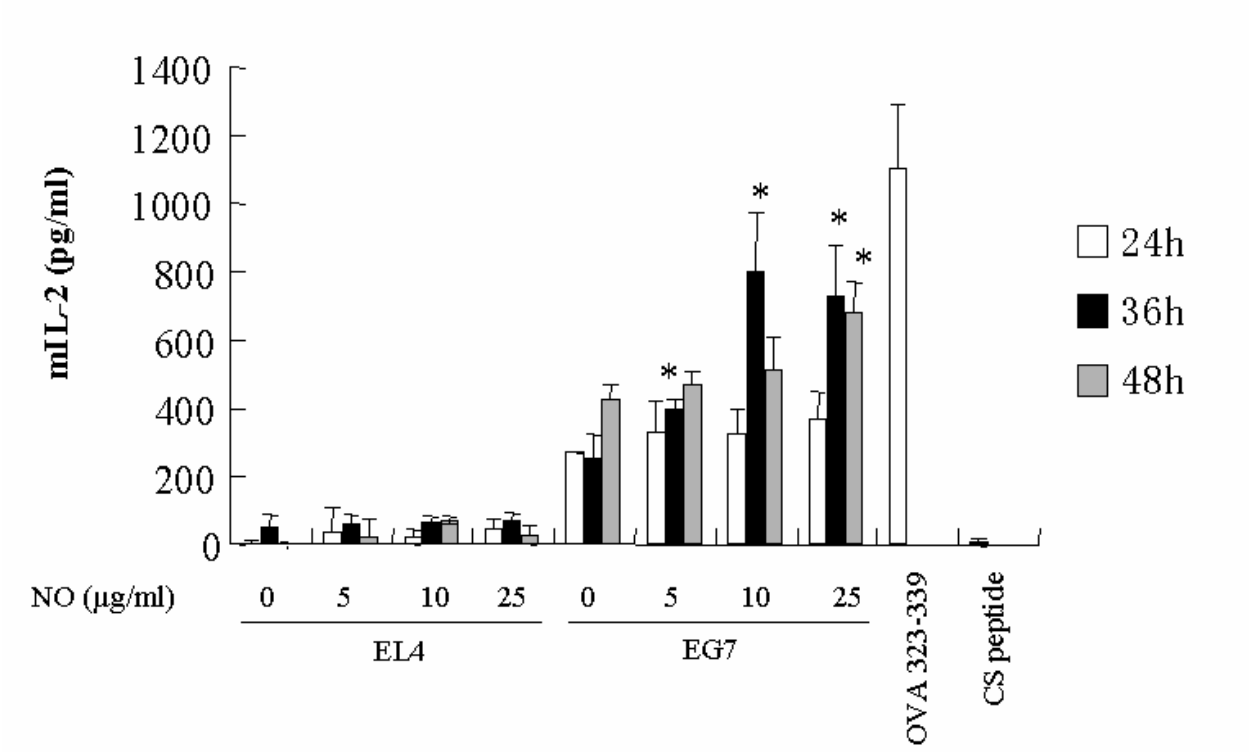

Figure 24 Pre-treatment with PAPA-NO enhances DC-mediated cross-presentation of OVA to tumor specific T cells.

EL4 and EG7 tumor cells were pretreated with PAPA-NO at the indicated concentrations, prior to the addition of DCs. After 24h, 36h or 48h, DO11.10 T cell hybridoma was added to the culture and IL-2 production (per 10^6 DO11.10 cells) within 24h was tested by ELISA. Positive controls included DC pulsed with the $OVA_{323-339}$ peptide, with negative controls including DC pulsed with the MCS peptide and cohorts including the OVA-neg EL4 tumor cell line. Data are representative of two independent experiments performed. *$p < 0.05$ vs. corresponding data from untreated (i.e. NO 0 μg/ml) controls.

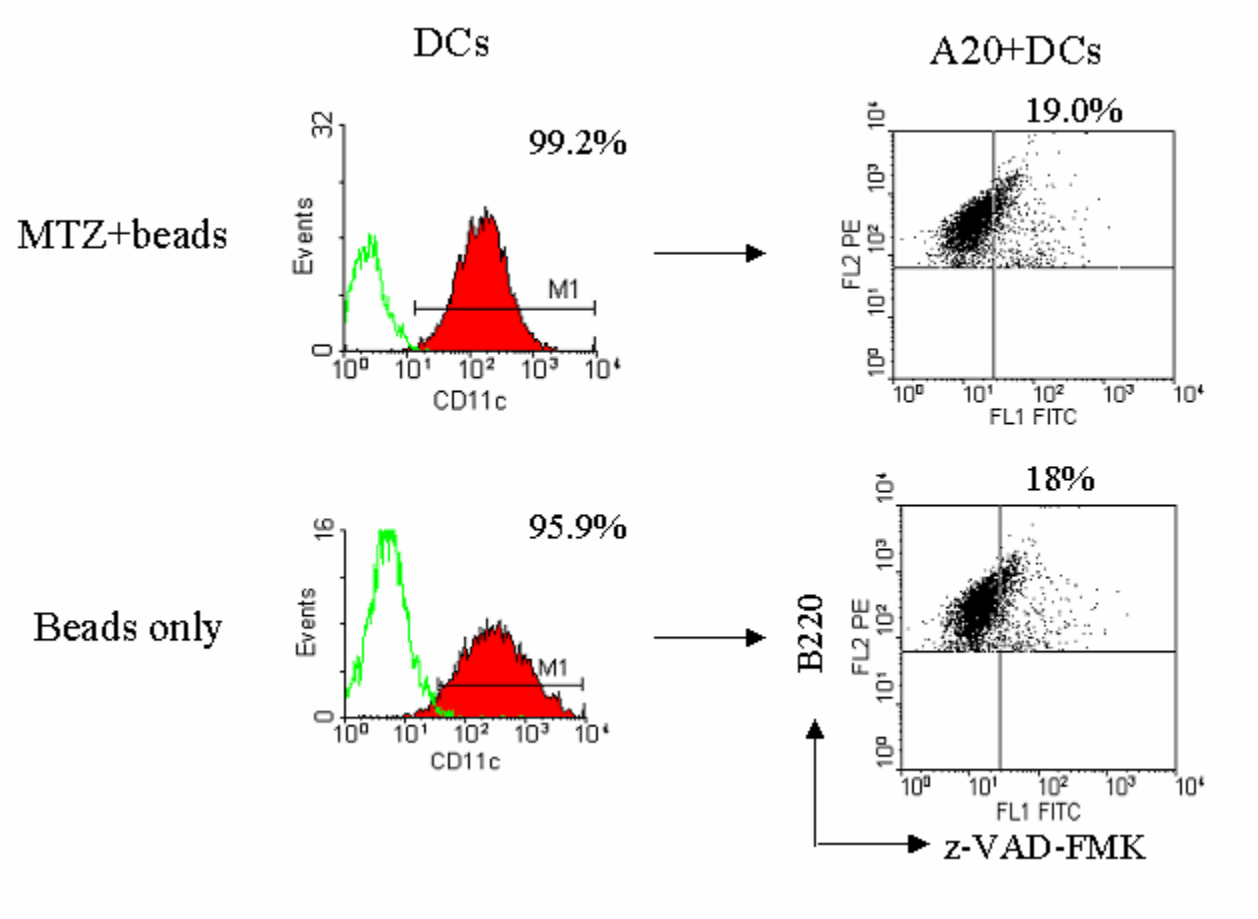

Figure 25. Further enrichment of CD11c+ DC over metrizamide gradients does not affect their capacity to mediate the apoptotic death of lymphoma cells in vitro.

BM cells were cultured as described in our original Materials and Methods for 9 days. The cells were harvested and subjected to centrifugation on 14.5% (w/v) metrizamide gradients prior to CD11c MACS bead isolation. CD11c+ purity of the Metrizamide + (MACS) Beads vs. Beads only preparations are depicted in the left panels of the figure. Each population of DC was then used as effector cells against B220+ A20 cells (at a 5:1 DC-to-tumor cell ratio), with apoptosis monitored by flow cytometry as described in our Materials & Methods. The DC populations mediated comparable levels of apoptotic killing of A20 cells.

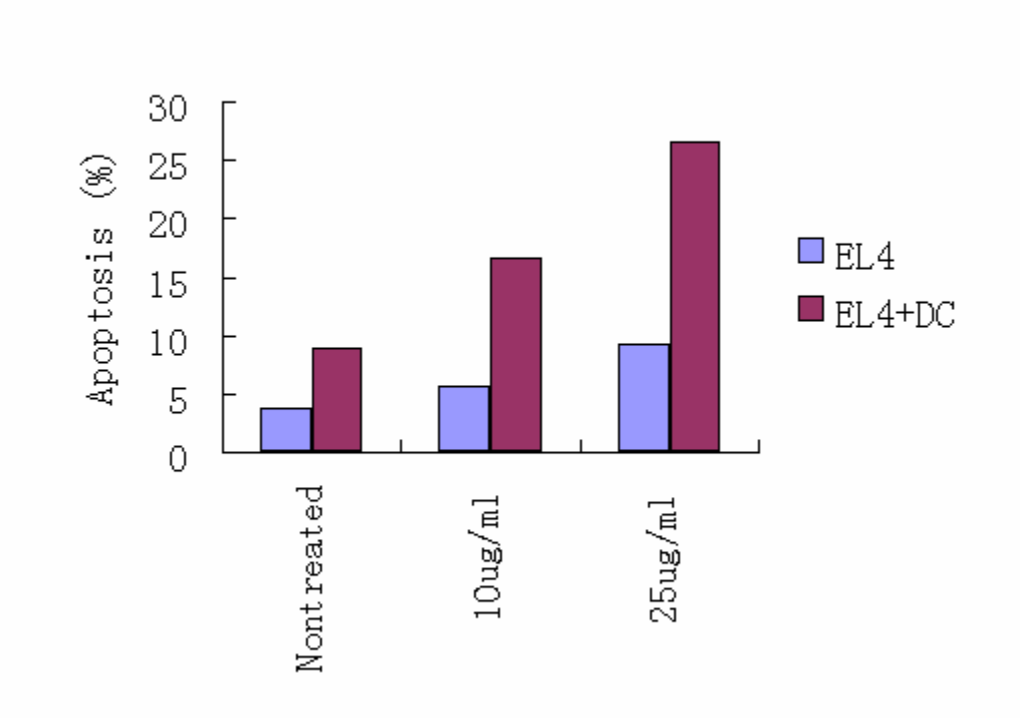

Figure 26. Pretreatment with PAPA-NO enhances the sensitivity of EL4 cells to DC-mediated apoptosis.

EL4 tumor cells were pretreated with PAPA-NO at the indicated concentrations before being co-incubated with SM-DCs. DC cytotoxicity against tumor cells was analyzed as described previously. The histogram shown here has been converted from density plots.

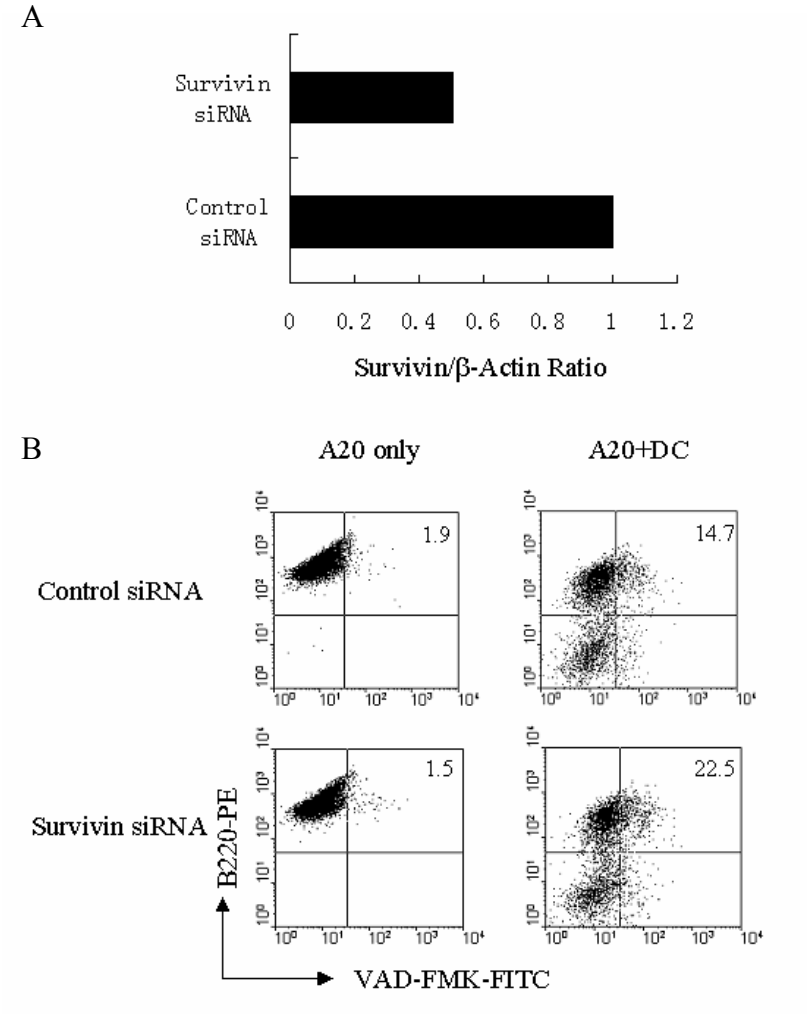

Figure 27. Inhibition of survivin expression in A20 cells using siRNA enhances their sensitivity to DC-mediated apoptosis.

(A) Transfection of siRNA into A20 cells specifically reduced survivin expression by 50%. Negative control siRNA or siRNA (Ambion, Austin, TX) specific to murine survivin was transfected into A20 tumor cells at a final concertration of 100pmol using Lipofectamine^{TM} 2000 following the manufacturer's instruction. Twenty-four hours later, the same transfection procedure was repeated to enhance gene knockdown. After additional 24h culture at 37°C, cells were harvested and lysed for survivin expression analysis using Western blot. (B) Survivin-siRNA transfection sensitized A20 tumor cells to DC-mediated apoptosis. A20 cells were transfected with siRNA as described in (A). Forty-eight hours after the first transfection, the cells were harvested and co-cultured with SM-DCs and 4h cytotoxicity assays were performed as described above.

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