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THE TUMOR PROMOTING ROLES OF LYMPHOCYTES DURING RAS INITIATED INFLAMMATION AND SKIN CARCINOGENESIS

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by

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Abstract

The pathological progression of neoplastic tissues requires the concerted activities of normal stromal cells to orchestrate a series of growth promoting effects similar to that found in healing wounds but without resolution. Bone marrow derived leukocytes are instrumental in initiating and mediating many of these processes through various mechanisms employed during periods of inflammation. Recently, studies using chemical and genetic tumor models have demonstrated novel tumor promoting roles for immune cells of the lymphoid lineage, a seemingly counterintuitive discovery.

To model and study the contributions of lymphocytes to oncogene induced pre-malignant inflammation and tumor development, we targeted a Harvey-RAS^{G12V} transgene to basal (K14) and suprabasal (Inv) layers of squamous epithelia under inducible control of a tetracycline transactivator. Reduced doses of doxycycline (dox) induced expression of RAS transgene in InvtTA/tetORas (InvRas) mice leading to outgrowth of benign papillomatous lesions. RAS expression on a lymphocyte deficient *Rag1-/-* genetic background greatly blunted tumor onset and total burdens indicating lymphocyte involvement in the promotion of RAS induced skin carcinogenesis. Antibody depletion of $CD8^+$ T cells in *Rag1+/+* mice partially but significantly reduced the early onset of papillomas while *Rag1-/-* reconstitution of purified $CD8^+$ T cells briefly stimulated lesional growth similar to WT groups that could not be sustained over the course of the study. Examination of the acute inflammatory response to RAS expression revealed diverse myelocytic infiltration into cutaneous tissues predominated by intraepidermal infiltration of cytotoxic $Ly6G^+$ neutrophils. Conversely, when acute RAS expression was observed on *Rag1 -/-* mice, epidermal proliferation and skin inflammation was greatly attenuated. Neutrophilia, microabcesses, and keratinocyte proliferation levels similar to

Rag1+/+ mice could be rescued by reconstitution of transgenic *Rag1-/-* mice with total CD3^{+} T or CD8^{+} T cells. Depleting CD8^{+} but not CD4^{+} T cells diminished or ablated all of these pathologies as well as reduced Th17 and γδ-17 cell activation, demonstrating the requirement for CD8^{+} T cells in driving global RAS inflammatory responses. Neutralization of IFN-γ blocked neutrophilic inflammation and keratinocyte proliferation caused by CD8^{+} repletion of *Rag1-/-* mice thereby implicating this cytokine as a mechanism of action.

RAS expression driven by an epithelial basal layer promoter (K14Ras) also activated severe systemic and skin inflammation but was characterized more predominantly by the expansion of Ly6C^{hi} inflammatory monocytes capable of suppressing T cell proliferation *ex vivo*. This immunosuppressive behavior could be ameliorated by titration of RAS transgene quantities that correlated linearly with reduced GM-CSF expression in keratinocytes. Strikingly, the acquisition of immunosuppressive functionality in these myeloid subsets could also be abrogated by crossing RAS transgenes onto the *Rag1-/-* background. Depletion of CD4^{+} and/or CD8^{+} T cells was ineffective at reproducing this phenomenon. However, B cell reconstitution into K14Ras/*Rag1*-mice completely recovered Ly6C^{hi} mediated blockade of T cell proliferation suggesting B cells were the lymphocyte mediating these activities. B cell depletion, in contrast, did not reverse these effects because of an anti-CD20 resistant Breg population that likely stimulates MDSC phenotypes through the paracrine activities of IL-10.

Finally, we show that the unique inflammatory responses observed in basal and suprabasal RAS expressing mice may be caused by inherently different signaling properties in differentiated and proliferating keratinocytes. RAS activation of ERK1/2 was greatly inhibited in differentiated keratinocytes *in vitro* and *in vivo,* whereby MKK4 activation was increased. As AP-1 blockade during RAS activation revealed a repressive role for AP-1 in cytokine

transcription, we hypothesize that increased AP-1 activation due to enhanced JNK signaling in post-mitotic keratinocytes may dampen RAS mediated immunosuppressive pathways in mutated non-dividing epidermal layers. In turn, this disparity in the ability to provoke regulatory myeloid cells that inhibit anti-tumor immunity may provide a reasonable explanation for the increased malignant potential in basal/stem cell driven epithelial cancers.

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Abbreviations

CD – cluster of differentiation antigen
Gr-1 – granulocyte differentiation antigen 1
Ly6G – extracellular marker expressed by neutrophils
Ly6C – extracellular marker expressed by monocytes, neutrophils, and some T cells
NK – natural killer
DETC – dendritic epidermal T cell
PMN - polymorphonuclear
RAS – gene encoding human rat sarcoma viral proto-oncogene GTPase
RAS^{G12V} – oncogenic form of RAS with a valine substitution at amino acid position 12
H-Ras – v-Ha-ras Harvey rat sarcoma viral oncogene homolog
K-Ras – v-Ki-ras Kirsten rat sarcoma viral oncogene homolog
N-Ras – neuroblastoma RAS viral oncogene homolog
v-ras – viral RAS gene
MMTV-PyMT – mouse mammary tumor virus polyoma middle T antigen transgene
HPV16 – human papillomavirus type 16 E6/E7 transgene
GTP – guanine tri-phosphate
GDP – guanine diphosphate
BCC – basal cell carcinoma
SCC – squamous cell carcinoma
HNSCC – head and neck squamous cell carcinoma
UV –ultraviolet light
EMT – epithelial to mesenchymal transition
BcL – B cell lymphoma gene
PKCα – protein kinase C alpha
PLC – phospholipase C
ERK1/2 – extracellular regulated kinase type 1 and 2
MEK1/2 – mitogen extracellular kinase type 1 and 2
MKK4 – dual specificity mitogen activated protein kinase kinase 4
MKK3/6- dual specificity mitogen activated protein kinase kinase 3 and 6
JNK – jun amino-terminal kinase
p38 – MAPK protein 38
PI3K - Phosphoinositide 3-kinase
IRS-1 – insulin receptor substrate 1
AKT – protein kinase B
NFκb – nuclear factor kappa B
AP-1 – adaptor protein 1
IκBα – inhibitor of NFκb alpha
IKK – IκB kinase
p50 – NFκb subunit protein 50
p65/RelA – NFκb subunit protein 65
EGFR – epidermal growth factor receptor
ERB2 - erythroblastic leukemia viral oncogene homolog 2
Raf – murine leukemia viral oncogene homolog 1
GAP – GTPase activating protein

GEF – guanine nucleotide exchange factor
ETS – erythroblastosis virus E26 oncogene homolog
RSK – ribosomal protein S6 kinase
mTOR – mammalian target of rapamycin
PTEN – phosphatase and tensin homolog
RalGDS – Ral guanine nucleotide dissociation stimulator
NF1 – neurofibromatosis
PDE5 – phosphodiesterase 5
MAPK – mitogen activated protein kinase
STAT3 – signal transducer of activated T cells type 3
JAK – Janus kinase
BRDU – 5-bromo-2-deoxyuridine
H&E – hematoxylin and eosin
MPO – myeloperoxidase
NBF – neutral buffered formalin
DAB – diaminobenzadine
MLR – mixed leukocyte reaction
LPS - lipopolysaccharide
PDGF – platelet derived growth factor
TCR – T cell receptor
CTL/Tc – cytotoxic $CD8^+$ T lymphocyte
Tc17 – CTL expressing IL-17A/F
Th – helper $CD4^+$ T lymphocyte
Th1 – Th cells expressing IFN-γ and T-bet
Th2 – Th cells expressing IL-4 and GATA-3
Th17 – Th cells expressing IL-17A/F and RORγT
Treg – Th cells expressing TGF-β and FoxP3
γδT cell – T lymphocyte expressing γδ chains of the T-cell receptor
FLHC – fetal liver hematopoietic cells
Ig - immunoglobulin
FcγR – high affinity receptor for IgG
NKG2D – natural killer group 2 member D
MICA/MICB - major histocompatibility complex class I chain-related A and B
RAE1 – retinoic acid earl inducible gene 1
MHC – major histocompatibility complex
H60 – minor histocompatibility antigen
FoxP3 – forkhead box P3
RORγT – RAR-related orphan receptor gamma isoform T
TNF-α – tumor necrosis factor alpha
TGFβ- tumor growth factor beta
IFN-γ – interferon gamma
IL – interleukin
IL-4Rα – interleukin 4 receptor alpha
CSF – colony stimulating factor
GM-CSF – granulocyte/monocyte colony stimulating factor
G-CSF – granulocyte colony stimulating factor

CCL – chemokines with consensus consecutive cysteine-cysteine sequence
CXCL – chemokines with consensus cysteine-X-cysteine sequence
MMP – matrix metalloprotease
VEGF – vascular endothelial growth factor
Rag1 – recombination activating gene 1
CLL – chronic lymphocytic leukemia
MDSC – myeloid derived suppressor cell
TAM – tumor associated macrophage
M1 – classically activated macrophage
M2 – alternatively activated macrophage
CC – chemical carcinogenesis
K14 – cytokeratin type 14
K5 – cytokeratin type 5
K1 – cytokeratin 1
K10 – cytokeratin type 10
Inv – Involucrin
rTA – reverse tetracycline transactivator
tTA – tetracycline transactivator
tetO – multimeric tetracycline operon regulatory sequence
InvRas – InvtTA/tetORAS^{G12V} mice
K14Ras – K14-rTA/tetORAS^{G12V} mice
TPA - 12-*O*-tetradecanoylphorbol-13-acetate
DMBA - 7, 12-dimethylbenzathracene
CC – 2-stage skin chemical carcinogenesis
HNSCC – head and neck squamous cell carcinoma
FACS – fluorescently activated cell sorting
H5N1 – influenza virus expressing hemagglutinin 5 and neuraminidase 1
HBV – hepatitis B virus
HCV – hepatitis C virus
EBV – Epstein Barr virus
HCC - Hepatocellular carcinoma\
CFSE – carboxyfluorescein succinimydyl ester
PCR – polymerase chain reaction
QRT-PCR – quantitative reverse transcribed polymerase chain reaction
ROS – reactive oxygen species
NO – nitric oxide
Arg1 – arginase 1
iNOS – inducible nitric oxide synthase
IRS-1 – insulin receptor substrate 1
AID – activation induced cytidine deaminase
HOCl – hypochlorous acid
ADCC – antibody dependent cell mediated cytotoxicity
DNA – deoxyribonucleic acid
RNA – ribonucleic acid

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Chapter 1: Introduction

1.1 Inflammation: A cancer perspective

Virtually all living organisms inherently contain at least the primitive ability to defend themselves from infectious attack as well as repair damaged cells and tissues (1). One mechanism by which Animalia distinguishes itself from other organismal kingdoms is by the complexity in their ability to carry out these functions (2). In order for many animals to maintain homeostasis and survive potentially injurious environments, an inflammatory process must be initiated and resolved. Inflammation is an integral biological response to numerous pathological insults to vascularized tissues including but not limited to: physical injury, microbial invasion, and neoplastic development with the goal of restoring homeostatic balance (3). Parturition is even initiated by an inflammatory event (4) and age associated diseases are thought to be mediated by low-level inflammation (5). An inflammatory process involves the dynamic interplay between cells of the epithelia, stroma, and a variety of resident and tissue infiltrating leukocytes that seek to reshape tissue, repair wounds and eliminate infections (6). Understanding the mechanistic details and properties of inflammation will not only provide clinicians with the therapeutic tools to treat inflammatory related diseases but also educate numerous other scientific disciplines into the biology of eukaryotic cells. Indeed, the involvement of multiple systems during the inflammatory process demonstrates a unique opportunity to study biology holistically at a biochemical, molecular, cellular, and organismal level.

1.1.1 Acute Inflammation

When clinically classifying inflammation, one can divide these events based on the relative duration that the cells or soluble mediators reside and persist within the tissue itself. The

fate and end result of this process and the reasons for these biological checkpoints will be discussed later, but despite the disparity in the outcome of inflammation and the evolution of the soluble and cellular components that make up its consistency, almost all inflammatory processes begin in the same manner: the cellular identification and recognition of something foreign or potentially injurious and the genetic decision to respond accordingly (7). Cells have the innate ability to respond to stressful environments, and inflammation is typically the biochemical and cellular chain reaction of these cells responding to stress (8). Pathological stress commonly causes cell death and these dead cells need to be removed in order for the injured tissue to heal properly and certain leukocytes are specifically equipped to carry out their removal (9). Hematopoietic cells with the ability to engulf foreign material and remove dead/dying cellular debris are known as professional phagocytes for their ability to consume and process macromolecules and microbes. These immunocytes of myeloid origin include neutrophils, monocytes, macrophages, and dendritic cells. Danger signals activate tissue resident macrophages that in turn stimulate chemotaxis and extravasation of neutrophils and monocytes, the first responders during acute inflammation, whose job is to non-specifically seek to destroy potentially harmful bacterium that have invaded tissue compartments (10). Following tissue infiltration and respiratory burst, neutrophils release a myriad of stored intracellular mediators such as leukotrienes (11), anti-microbial polypeptides and proteases (12), and reactive oxygen intermediates that non-specifically destroy bacterial pathogens but also can wield extensive collateral damage if not regulated properly (13). Monocytes differentiate within tissues into macrophages whose function is to help shape and tune more specialized adaptive immune reactions as well as to resolve acute inflammation by phagocytosing apoptotic neutrophils, effectively blunting granulopoesis and neutrophil infiltration (14). The nature and intensity of

this response is usually determined by the nature and intensity of the pathological insult as well as the specific organ and/or tissue that it occurs in. During infections that which occurs in the skin or gastrointestinal tract may be more severe and widespread than that affiliated within immune privileged sites like the brain (15). Clinically diagnosing stages and grades of cancer as well as prediction of disease course based on the severity and type of inflammation is now becoming a common alternative to grading tumors (16;17). A viral hepatitis infection can induce a relatively intense inflammatory response in the liver when compared to alcoholism, a pathology that targets the same organ and can take years to produce similar liver inflammation (18). Furthermore, obesity, which can produce a small, underlying, and clinically undetectable amount of inflammation, is now thought to increase the risk of cancer in certain tissues despite the obvious presence of tissue pathology (19;20). Another aspect affecting the severity of inflammation is the quantitative dose of the infectious or toxicological agent encountered by the organism whereas, for instance, a higher microbial load will require stronger immune activation resulting in greater inflammation in order to eliminate the entirety of the infection. Finally, higher classes of animals such as mammals, have adaptive immune systems providing an organism the capability of generating an immune response that is tailored to more efficiently attack a particular microorganism based on lymphocyte receptor antigen specificity (21). A hallmark of adaptive immunity is immunological memory, a process that retains a proportion of immune cells with a specific killing capacity for the newly encountered microorganism after its primary activation. For infectious microbes that have evolved to escape immune recognition and share very little biochemical homology to other microbes, the first inflammatory process can be relatively intense so as to mount a specific adaptive response as efficiently and quickly as possible (22). Viruses typically seek to multiply efficiently and overwhelm their host but

without being detected by the host immune system so as to avoid the dramatic effects of intense, acute inflammation (23). For instance, H5N1 influenza and African trypanosomes are constantly evolving as a result of the influential selective pressures administered by their host's immune system (24;25). This culminates in a never ending evolutionary battle between pathogens and the immune systems they seek to escape from. From an evolutionary perspective, it generally does not benefit the microorganism to kill its host unless it has the ability to infect another viable host; thus the most infectious and Darwinian fit pathogens are those with the ability to propagate within a host and escape its host's defense system (26). In all of these cases, acute inflammation is generally beneficial to the diseased organism where innate immunity precedes and conditions the subsequent adaptive immune response as necessary. The damaged tissue is repaired and/or the foreign invader is eliminated efficiently.

1.1.2 Chronic inflammation

While acute and self-limiting inflammation is ultimately advantageous to the host organism, it is now well established that when left unchecked, inflammatory components can endure and maintain a chronic environment that actually increases tissue pathology (27). Indeed, in the context of carcinogenesis, chronic inflammation is often associated with tumor progression and malignancy and is now widely considered a risk factor for cancer development (28). One caveat of note is the chronic inflammatory environment established during psoriasis in the skin which is, conversely, an inhibitory factor for cancer development of cutaneous origin (29). Although quantitatively defining the hallmark traits of physiological versus pathological inflammation can be an ambiguous process, there are certain cellular and molecular features of chronic inflammation that distinguish it from that of the acute nature. For instance, the immuno-cellular consistency of chronic inflammation tends to be dominated by lymphocytes and

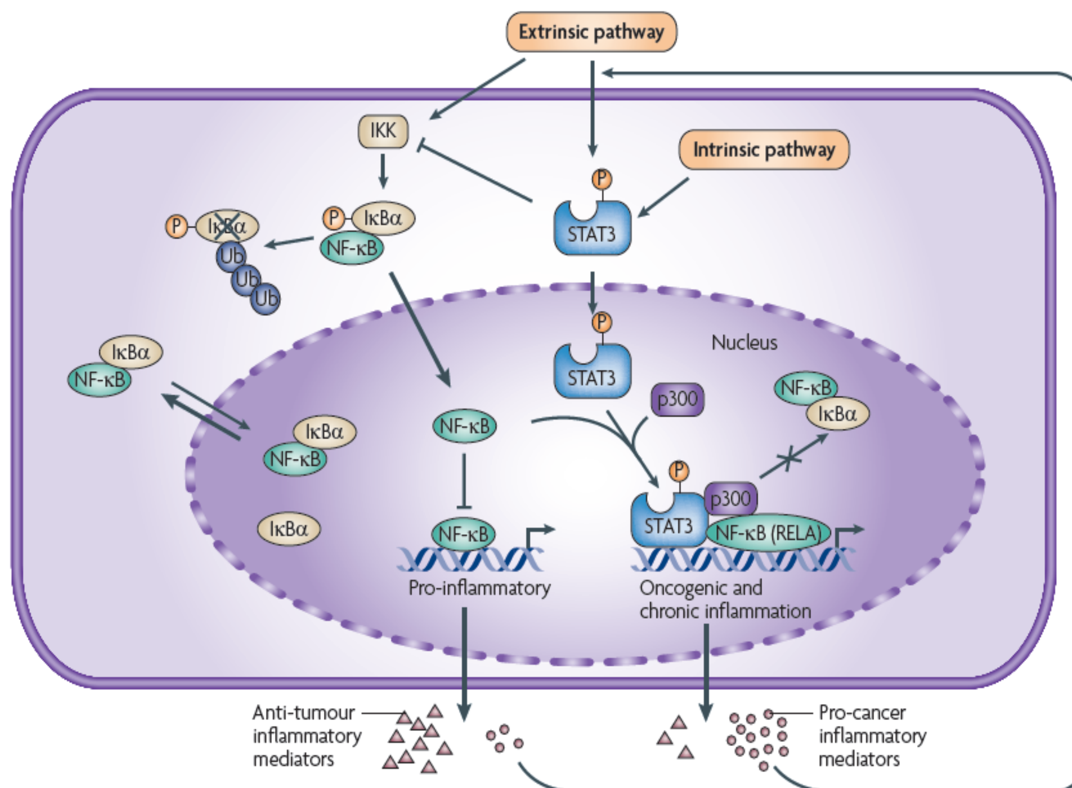
macrophages whereas acute inflammation tends to favor chemotaxis and tissue residency of myeloid cells such as neutrophils and monocytes (30;31). Typically, myeloid cells respond more rapidly due to their innate nature and ability to mobilize from the bone marrow upon chemotactic and cytokine storms, hence these cells have an expanded role during acute inflammation (32). In contrast, lymphocytes undergo a self/non-self antigen-education process where T and B cells are genetically selected to respond only to a particular foreign antigen in the thymus and bone marrow respectively (22). This adaptation to non-self antigens also allows for greater functional diversity as compared to myeloid cells which typically are programmed to perform a more limited number of biological processes as well as a more restricted capacity for phenotypic and morphological variability. At odds with this generality is that macrophages are almost always the most abundant immune cell type within solid tumor masses and can have extremely polarized and variable phenotypes (33). This immune cell and its specialized functions contributions to tumor development will be discussed at great lengths in later sections as its role is becoming increasingly more appreciated for its pro-tumorigenic properties. Finally, chronic inflammation linked to cancer can be further classified based upon the order and manner in which it develops. That which arises as a consequence to oncogene activation is regarded as cell intrinsic inflammation and that which is established preceding the initiating genetic alteration is referred to as extrinsic inflammation as is the case with infection or autoimmune conditions.

1.1.3 Cooperative crosstalk between mediators of inflammatory signal transduction

Chronic inflammation arising extrinsically is unique in the ability to orchestrate multiple signaling feed forward loops that ultimately potentiate an environment favoring maintenance of the inflamed state. Stimulation of this complex intracellular circuitry is rarely beneficial to the host. One of the more well known feed forward loops is through the synergistic activation of

NFκb and STAT3 (34;35). NFκb and STAT3 are transcription factors expressed in all cell types and their activation leads to expression of pro-inflammatory and cell survival genes in many different cell types (36). Direct protein/protein binding interactions between the transcription factors have been demonstrated in multiple studies (37-39) and adjacent, bi-regulated genomic consensus sites have also been shown for these two transcription factors (40;41) The augmentation of this reinforcing pro-inflammatory activity is fully realized when cytokine expression initiated by NFκb or STAT3 leads to the autocrine and paracrine effects of these secreted cytokines by binding cytokine receptors whose signal transduction culminates in further NFκb and/or STAT3 transcriptional activation. IL-6, for instance, is a known NFκb target gene and ligand engagement of its receptor causes the gp130 subunit to directly activate STAT3 which subsequently can upregulate genes such as IL-17, IL-23, CCL2, and IL-1β that potentiate further NFκb activation in other microenvironment or malignant cells (42;43). A model of colitis induced colon cancer revealed IL-6 was a critical tumor promoter whose effects could be abrogated by genetic STAT3 ablation in intestinal epithelial cells (44). Unabated IL-6 signaling rendered U266 myeloma cells resistant to apoptosis (45) and similarly, constitutive STAT3 activation targeted to the epidermis by means of transgenic manipulation enhanced malignant progression during 2-stage chemical carcinogenesis (46). This feed forward cycle is further propagated by the fact that STAT3 and NFκb cooperate analogously in stromal immune cells where numerous tumor promoting functions are regulated by the nuclear workings of these transcription factors (47-50). Additionally, STAT3 has been shown to prolong the nuclear retention of NFκb through p300 mediated acetylation of p65, thereby inhibiting IκB directed nuclear export and enhanced NFκb transcriptional activity (39). The synergistic relationship between NFκb and STAT3 is just one of many examples supporting the molecular mechanisms

of shared and redundant pro-inflammatory signaling pathways within progressing malignancies. Other signaling proteins mediating similar tumor cell/stromal cell crosstalk include AP-1, JNK, PI3K, and ERK1/2 (51-55). When inflammatory dysregulation is firmly established, oncogene expression in cancer cells leading to these pro-inflammatory feed forward loops can cause the accumulation of additional mutations in cell cycle genes and constitutive activation of various other cytokine and chemokine inducing transcription factors. For these reasons, solid tumors that reach a critical mass for inflammation, proliferation, and vascularization rarely regress without therapeutic or surgical intervention.



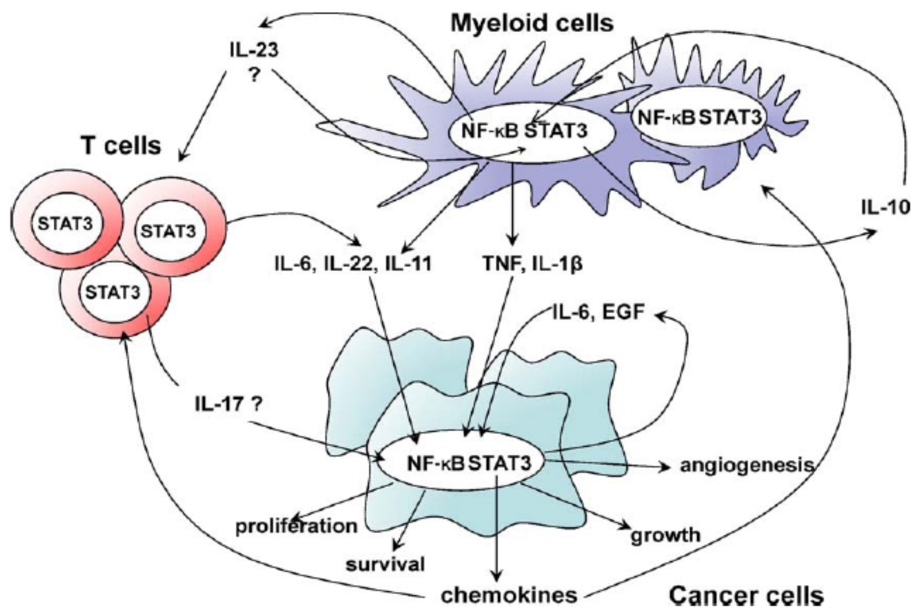


Figure 1-1: Extrinsic and intrinsic NFκb and STAT3 cross-talk between cancer and immune cells leads to chronic reinforcement of pro-inflammatory feedback pathways. *Top*: Activation of either signal transducer can be initiated upstream by cancer cell intrinsic oncogene activity leading to de novo production of autocrine/paracrine factors that subsequently engage cell surface receptors linked to further NFκb and STAT3 signal transduction. Upregulation of a gene mediated by one transcription factor also can lead to activation of the other as is the case of NFκb regulated IL-6 transcription whereby ligand binding to the IL6 receptor directly activates STAT3. Persistent STAT3 signaling may also aid in p65/RelA nuclear retention by inducing p300 acetyltransferase activity. *Bottom*: Alternatively and cooperatively, proximal inflammatory cells can secrete a variety of NFκb and STAT3 activating cytokines that chronically sustain signaling through these pathways in cancer cells as well as other leukocytes leading to recruitment of additional inflammatory cells. These leukocytes harness NFκb and STAT3 crosstalk much in the same manner malignant cells do, providing a permissive and favorable environment for tumor promotion and malignant progression. ***Top****: Reprinted by permission from: Macmillan Publishers Ltd: Nat. Rev. Cancer,Yu et al., Vol. 9,Issue 3, pp. 798-809, © 2009* (top). ***Bottom****: Reprinted from Cytokine and Growth Factors Review, Vol. 21, Grivennikov and Karin, Dangerous Liasons: STAT3 and NF-κB collaboration and crosstalk in cancer pp. 11-19, © 2010 with permission from Elsevier and Lancet.*

1.1.4 Autoimmunity, infection, and cancer risk

The most well known example of chronic inflammation is autoimmunity. Autoimmunity is a disease defined by chronic and persistent immunocyte activation against self-antigens, absent of canonical immunological resolution, and an abnormal persistence of leukocytic tissue residency. Chronic inflammatory diseases can predispose certain tissues to carcinogenesis such as IBD in the colon, hepatitis in the liver, and diabetes in the pancreas (28). Genetic predisposition often precedes establishment of a true autoimmune disease but is not an absolute prerequisite. Indeed, many rheumatologists believe the underlying cause of many (but not all) autoimmune pathologies may arise due to an abnormal immune responses to infection (56). Helicobacter pylori is a bacterium whose etiology is known to cause chronic gastritis and ulcers which lead to the development of stomach cancer (57). HBV and HCV infections are well known to establish persistent autoimmune-like hepatitis that greatly increase the risk for hepatocellular carcinoma development (58). Multiple sclerosis is linked with respiratory pathogens (59), EBV (60), and systemic infections (61). PCR amplified transcripts of Parvovirus B19 were found in 75% of rheumatoid arthritis patients compared with 17% of other chronically inflamed joint sufferers

Table 2. Inflammatory conditions that predispose to cancer

Malignancy	Inflammatory stimulus
Bladder cancer	Schistosomiasis
Gastric cancer	*H. pylori*-induced gastritis
MALT lymphoma	*H. pylori*
Hepatocellular carcinoma	Hepatitis virus (B and C)
Kaposi's sarcoma	HHV8
Bronchial carcinoma	Silica
Mesothelioma	Asbestos
Bronchial carcinoma	Asbestos
Ovarian cancer	Salpingitis/talc/ovulation/endometriosis
Colorectal cancer	Inflammatory bowel disease
Oesophageal cancer	Barrett's metaplasia
Papillary thyroid carcinoma	Thyroiditis
Prostate cancer	Prostatitis

Table 1-1: The links of inflammation to cancer can be well appreciated when examining all of the chronic inflammatory syndromes that expose tissues to malignant growth. *Reprinted from: Cancer Cell, Vol. 7, Balkwill et al., Smoldering and polarized inflammation in the initiation and promotion of malignant disease, pp. 211-217, © 2005, with permission from Elsevier and Lancet Ltd.*

(62). Regardless of the underlying infectious mechanisms, the clinical manifestations of autoimmunity are generally a prognosticator for cancer development within the tissues autoimmunity has arisen in. For instance, specific species of commensal gut flora are known etiological agents for the development of Crohn's disease (63); an autoimmune precursor to colon cancer (64). When examining these two diseases from a purely immunological standpoint, the mechanisms and effector functions in which immune cells become pathological and cause disease can be very similar. As many as 15-20% of all cancer related deaths worldwide are attributed to infection and inflammation (65) and multiple tumor types respond favorably to non-steroidal anti-inflammatory treatments that can alleviate mortality rates (66-68). These similarities beg the question of if many more immuno-therapies applied to autoimmunity can also be efficacious against advanced staged malignancies.

1.1.5 Biological parallels between solid tumors and healing wounds

Scientists have frequently defined solid tumor development as "wounds that do not heal" (69) because many of the biological processes occurring in a healing wound parallel those occurring in a developing and/or established solid tumor. Infiltration of inflammatory cells, clearance of apoptotic cells, hyperproliferation leading to tissue re-epithelialization, collagen deposition and fibrosis, and an increased vasculature are all distinct traits shared by solid tumors and healing wounds (70). Furthermore, many of the same immunological responses, both in cell type and function, mark wounds and tumors similarly (71). While Th1 mediated immunity controls most microbial infections and acute inflammatory processes, Th2 responses dominate the environments of both wounds and tumors as well as parasitic infections (72). Th2 polarized immune activity is defined primarily by the involvement of eosinophils, mast cells, alternatively activated macrophages and $CD4^+$ T lymphocytes that express IL-4 and IL-13 (73). The

cytokines produced during this specialized immune response not only stimulate recruitment and activation of those cell types listed but also can repress hematopoietic lineages and cytokines that are most frequently found in Th1 responses. The major difference between wounds and developing cancers is that wound repair is resolved through self limiting regulation of these inflammatory processes. (74). An example of this is the induction of CCL2, a chemokine leading to monocyte and lymphocyte recruitment, also simultaneously stimulates upregulation of the Th2 cytokine IL-4 in T cells while shutting down IL-12 production (75) thus establishing a classic negative feedback loop. This is important because Th2 mediated immunity also stimulates tissue repair programs and immunosuppressive genes that ultimately blunt the wound responding Th1 driven acute inflammation that can lead to autoimmunity and collateral tissue damage. Meanwhile, many advanced staged tumors demonstrate an abundance of Th2 type leukocytes in their stroma along with cytokine/chemokine profiles that are strongly Th2 polarized and this correlates with poor patient prognosis suggesting that tumors chronically hijack Th2 immunity to stimulate support their expansion and block Th1 driven anti-tumor responses. (76-78). TGFβ, a pleiotropic cytokine with multiple context dependent functions, is highly secreted and active in wounds and aids in fibrosis or scarring (79). TGFβ is also greatly upregulated in numerous tumors and is a potent tumor suppressor (80). Its presence signals to epithelial cells to reshape their morphology towards a more fibroblastic-like phenotype that allows for enhanced mobility, a process referred to as "epithelial to mesenchymal transition" (81;82). This cytoskeleton reorganizing event is required for epithelial cells to acquire greater motility so they may fill in open space within the tissue where the physical damage has occurred but also becomes another deregulated event during metastatic dissemination of cancer cells (83). TGFβ also can have significant impact on angiogenesis and numerous studies have implicated its

requirement for new blood vessel formation (84-87). Angiogenesis is the process by which tissues lay down new blood vessels and increased blood flow is necessary to repairing wounds in order to allow nutrients and inflammatory cells to flow into the wound and waste products and immune cells to be carried away when healing is complete (88). All accelerating solid tumors eventually outgrow their blood supply and initiate seminal blood vessel formation to keep pace with their increased tissue bulk. Many immune cell derived cytokines have been

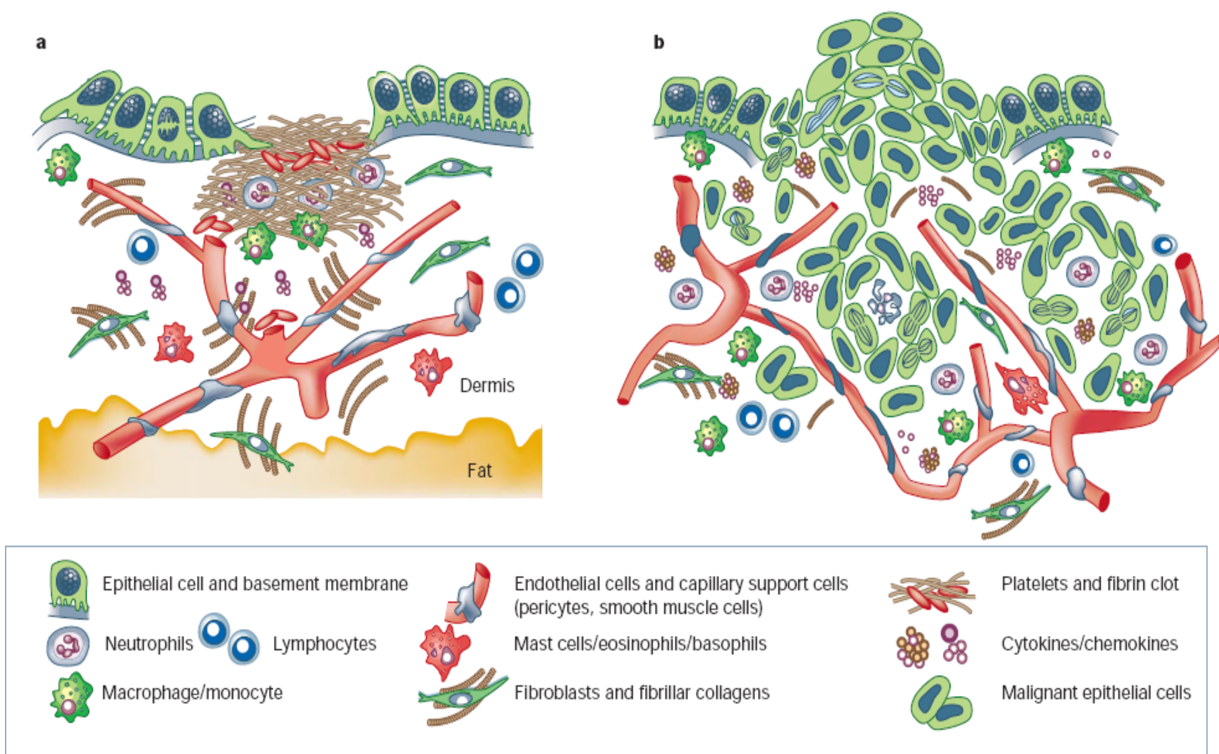


Figure 1-2: Epithelial wound healing (a) vs. deregulated malignant growth (b). While epithelial proliferation, angiogenesis, matrix remodeling, and leukocyte infiltration are biological processes shared in both contexts, only healing wounds maintain self-regulatory networks leading to resolution of those phases. Conversely, with the aid of leukocytes tumors deregulate these self-limiting signals thereby establishing progressive cancer cell proliferation and expansion of the stromal components to support its growth. *Reprinted by permission from Macmillan Publishers Ltd: Nature, Coussens and Werb, Vol. 420, Issue 6917, pp. 860-867, © 2002.*

shown to contribute to this process including IL-8, VEGF, MMP's, and IL-17 (89-92). Not inconsequently, TGFβ also affects locally resident immune cells, shaping their phenotypes towards any number of well studied immune profiles (93;94). Production of TGFβ in copious quantities within tissues generally has anti-inflammatory properties but this is an extreme

overgeneralization because its presence along with other cytokines, such as IL-6 and IL-2, demonstrates far different signaling properties than when cells experience TGFβ alone; an environment that never occurs in the context of wounds and tumors. This cytokine is an absolute requirement in the generation of the specialized $CD4^+$ T lymphocytes, Th17 and Treg cells (95;96), and can induce further cytokine expression from fibroblasts and epithelial cell that ultimately potentiates inflammation (97;98).

1.1.6 Questions to consider

Despite these studies, questions remain about how uniform inflammatory responses are in these different pathological contexts as well as what are the key mechanisms for avoiding resolution of them in causing disease. Furthermore, are there immune responses unique to certain tissues and what types of cells or genes govern the specificity of that tissue? For instance, do epithelial cells regardless of tissue localization (i.e. skin, lung, gut, and breast) have an equal capacity to regulate and influence similar immune activities? Is there a qualitative difference in the inflammation responding to danger (DAMP) signals derived from epithelial or mesenchymal cells? What is the role of symbiotic tissue microbiota in preventing or tuning chronic inflammation? Finally, can the quantity or intensity of inflammation determine the nature of the evolving immune response?

1.2 Inflammatory contributions to specific stages of carcinogenesis

1.2.1 Initiation

Cancer biologists have compartmentalized experimental tumor development into 3 well-characterized molecular and/or histopathological stages: initiation, promotion and malignant conversion (99). These events are analogous to the acquisition and progression of human tumors

as well. Although these classifications represent a fluid continuum of neoplastic progression, distinct differences can be identified and are implicit in determining treatment courses and the level of clinical intervention. Initiation encompasses the seminal and irreversible mutational activation of oncogenes or deactivation of tumor suppressors in somatic cells and is an event rarely, if ever detected in human cancers because biopsies of palpable tumor masses contain numerous mutations that could be the initial cancer causing event. The detection, correction and elimination of mutated genomic DNA are normal homeostatic processes of somatic cells. Considering mutational events are actually quite common, arising spontaneously 10^{-6} to 10^{-11} in each round of DNA replication, genomic editing is a standard and necessary mechanism of ensuring balanced cell cycling (100). When the editing machinery fails to correct these genetic errors or remove cells with mutations, these cells have a selective advantage over neighboring cells for division and growth. It is postulated that the chronic presence of inflammatory cells within tissues contributes to increased DNA damage. In support of this, one study found mutation rates nearly 4 times higher in inflamed non-cancerous tissue than normal adjacent (101). One mechanism inflammatory cells may achieve this is through the induction of oxidative stress by reactive oxygen species (ROS) and nitric oxide (NO), which produce perioxynitrates and hypochlorous acid, thereby greatly increasing rates of mutation and transformation (102). Both neutrophils and macrophages are rich sources of these reactive oxidative products during inflammatory activation normally intended for anti-microbial defenses. NO is also a potent inhibitor of p53 function (103;104) and the amount of NO is directly proportional to the number of p53 mutations in inflamed tissue due to its genotoxic effects (105;106). Not surprisingly, genes with dominant functions in regulating cell cycle entry, proliferation, growth arrest, apoptosis and senescence are the most frequently mutated in cancer cells (107). Even non-

cancerous cells residing within chronic inflammatory conditions such as arthritis and IBD contain p53 mutations at rates similar to those found in solid tumors of the same tissue (108). Production of cytokines also augments genomic instability. IL-6 signaling creates hypermethylated regions in the p53 promoter (109) and TNF-α, IL-1β, IL-4, IL-13, and TGFβ all induce upregulation of activation induced cytidine deaminase (AID) (110-112) which promotes double stranded DNA breaks in p53, c-MYC (113;114), and BCL-6 (115). Known and putative chemical and infectious carcinogens often target many of these same genes. Depending on the functions of these proto-oncogenes, these mutations may cause constitutive activation or inactivation. A single mutation is rarely adequate or sufficient to induce unregulated, cancerous cell growth since activation and upregulation of tumor suppressing genes such as p53 will occur as a negative regulatory consequence to the mutational event (107;116). Therefore, the simultaneous chronic presence of pro-proliferative and DNA damaging inflammatory agents would be a favorable cancer conditioning environment. To that end, sequences of cancer genomes show mutations in multiple genes with overlapping functions and often a pro-mitotic activating mutation will occur contemporaneously with an inactivating mutation in a pro-apoptotic or growth arrest protein (117).

1.2.2. Promotion to Malignant conversion

Following initiation, pre-malignant outgrowth must occur through expansion of cancer cells and cancerous tissue so as to establish a palpable benign tumor mass. Evasion of growth arrest and apoptosis leading to sustained proliferative signaling and the capacity for unlimited self-renewal must occur in order for cells with initiating mutations to lead to palpable tumor masses. At this stage proliferation is promoted in mutated cells through any number of inflammatory mediated mechanisms which can encourage these cells to acquire additional trailer

mutations that augment its ability to form neoplastic lesions. Cytokines can perpetuate proliferative signaling in cancer cells through direct and indirect mechanisms. IL-1α upregulates IL-6 production in Kupffner cells leading to compensatory cell survival in hepatocytes (118). The risk of HCC progression correlates well to the relative circulating IL-6 levels in tumor bearing patients (119). TNF-α can stimulate epithelial to mesenchymal transition (120) and regulates chemokine expression in ovarian cancer cells (121). High expression of TNF-α in human tumors is also associated with poor prognosis, loss of hormone responsiveness and cachexia (122-124). TNF-α is also a potent mitogen for hepatocytes (125) making it currently an attractive target for cancer therapy (126). IL-23, a potentiator of IL-17 mediated inflammation, is secreted by macrophages and dendritic cells and has been linked to the Warburg effect, a metabolic process in cancer cells favoring glycolysis to oxidative phosphorylation (127). As stated previously, constitutive cytokine signaling can propagate permanent cellular changes. For instance, increased expression of BCL-2 and BCL-X1, two anti-apoptotic proteins, is mediated by the increased activities of NFκb, STAT3, and/or AP-1 (128). STAT3 inhibits p53 synthesis (129) and positively regulates cyclin D1, D2, B, and c-MYC, thereby fostering proliferative cell cycle entry (130). However, solid human tumors are never strict masses of genetically monoclonal cancer cells, contrary to tumor models utilizing syngeneic and/or orthotopic transplantation of homogenous, transformed, and highly malignant cell lines. Until much later stages, benign solid tumors maintain many of the morphological tissue organization found in normal tissue where proliferation and apoptosis are increased concomitantly suggesting most cells within the tumor mass still maintain homeostatic cell cycle regulation. Promotion of these tumors leads to progressive deterioration of this organization where the most undifferentiated phenotypes lead to more malignantly aggressive forms (131-133). This process of tumor

promotion is most evidently carried out indirectly by immune cells that provide pro-angiogenic and tissue remodeling soluble factors as well as chemokines that augment immunocellular

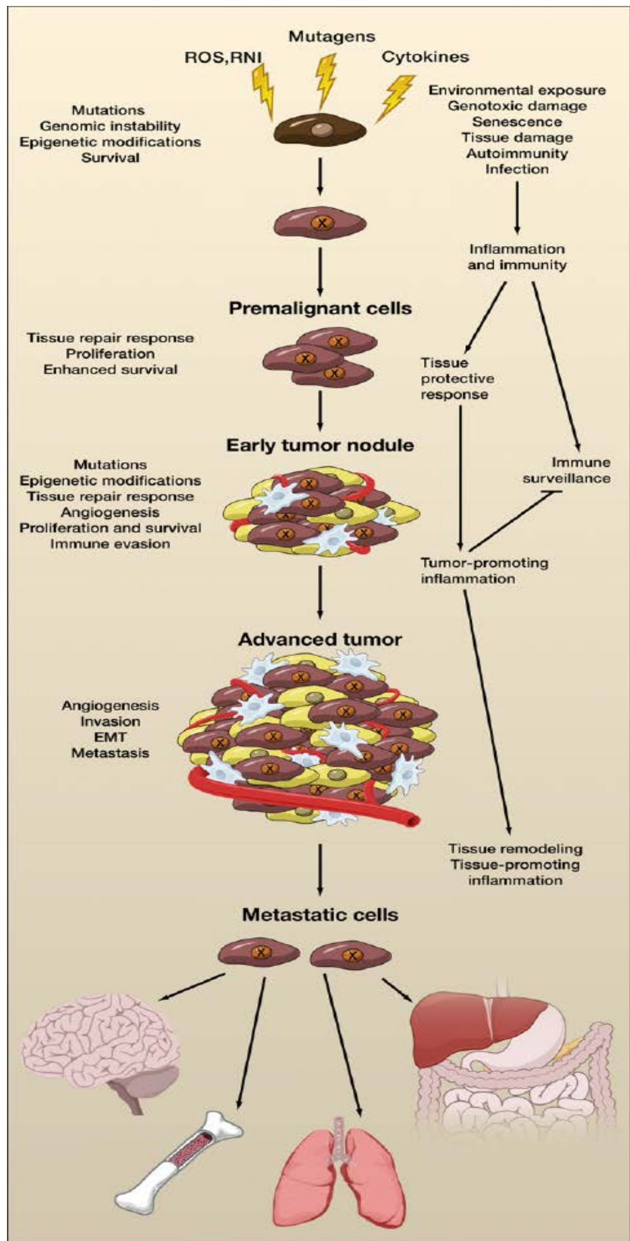


Figure 1-3: **Stage specific contributions of leukocytes to carcinogenesis**. Immune cells can affect every stage of carcinogenesis. Phagocytes produce genotoxic oxidative products which increase mutation rates. Myeloid cells provide matrix remodeling, pro-angiogenic, and immunosuppressive factors that increase pr e-malignant progression and avoid anti-tumor immunity. Chemokines direct metastatic cells to distal tissue sites where specific cytokines promote cancer cell survival. *Reprinted from: Cell, Vol. 140, Grivennikov et al, Immunity, Inflammation, and Cancer, pp. 883-899, © 2010, with permission from Elsevier and Lancet.*

recruitment into the tumor. VEGF is necessary to stimulate endothelial cell proliferation and migration and immature Gr-1^{+} myeloid cells and macrophages are a key intratumoral source of VEGF (134;135). Production of the chemokine CXCL12 activates the recruitment of CXCR4^{+} endothelial cells during hypoxic conditions which is thought be the seminal event in stimulating the angiogenic switch (136). Mast cell derived IL-8, TGF-α, PDGF, tryptase and chymase also contribute to vascular development during tumorigenesis (137). Matrix metallo-proteases (MMP) are critically important for remodeling tissues through their abilities to proteolytically degrade extracellular matrix proteins such as collagen and cell to cell adhesion molecules such as

integrins (138). MMPs can contribute to angiogenesis and as is the case with VEGF, leukocytes such as macrophage, neutrophils and mast cells are rich sources of these proteins and release them upon inflammatory activation (139;140). Bone marrow derived MMP9 was required for myelomonocytic-derived vasculogenesis in a mammary carcinoma model (141) as well as in the skin restricted HPV16 driven transgenic squamous tumor mouse (90). These studies have led to numerous clinical trials targeting the inhibition and depletion of MMP activity and signaling, the majority of which have been unsuccessful (142). MMPs can also regulate the bioavailability of growth factors, including cytokines (143). In keratinocytes, IFN-γ and TNF-α induced upregulation of MMPs leads to shedding of membrane bound pro-TGF-α and enhanced ERK1/2 mediated CCL2, CCL5, and CXCL10 mRNA stability. This skews the inflammatory infiltrate from neutrophils to monocytes and T cells (55). Finally, chemokine expression leading to cyclically augmented leukocyte recruitment and ultimately enhanced tumor growth, is upregulated in immune cells, stromal fibroblasts, and cancer cells as the result of autocrine and paracrine cytokine signaling (144-146).

1.2.3 Malignant Conversion to Metastasis

Once pre-malignant tumor masses have successfully been established, some cancer cells acquire malignant properties that can allow for the formation of metastasis, a mobile bolus of cancer cells that travels away from the autochthonous primary tumor and invades distal organs and tissues. Malignant conversion is defined by the histological observation that the cancer cells have breached the basement membrane, a proteinacious barrier between the epithelium and fibroblast stroma that maintains organization and tissue orientation (147). When cancer cells degrade and invade through this barrier, metastasis is made possible through proximal access to the systemic roads of lymphatic channels and blood vessels (148). The dissemination of

malignancy and metastasis is, again, a process that is clearly promoted by contributions by inflammatory cells. Immune cell derived proteases play a large role in mediating these events (149). Abrogation of the type 2 TGFβ receptor in mammary carcinomas led to increased Gr-1/CD11b cell recruitment into the invasive front by upregulation of CXCL5/12 and these myeloid cells reciprocally aided tumor invasion through an MMP-dependent mechanism (150). Ablating the downstream mediator of TGFβR signaling, SMAD4, in the colon, produced similar tumor biology through CCL9/CCR1 chemotaxis of $CD34^+$ immature myeloid cells (iMCs) that employed MMP2/9 to increase carcinomas invasion (151). TNF-α signaling can biochemically stabilize Snail, a key mediator in EMT induction and metastasis (152). CSF-1 and CXCL12 may mediate a physical interaction between TAMs and cancer cells, thus facilitating metastasis (153) and *CSF1* null mice have strikingly attenuated PyMT-driven pulmonary metastasis (154). Additionally, chemokine gradients cooperatively direct the coordinated migration of metastatic seeds through the chemokine receptors CXCR4, CCR4, CCR7, CCR9, and CCR10 (155). Malignant cells exposed to inflammatory mediators upregulate many of these receptors, making them more responsive to the chemotactic factors leukocytes secrete (156). CXCR2 expression on keratinocytes has been shown to be critical for proliferation, migration and tumor volume in the skin two-stage carcinogenesis model (157). Other examples include: CXCR5 and liver metastasis from primary colon tumors (158), CXCR4 and breast carcinoma metastasis (159) and CXCR1, CXCR2 and CXCR3 in malignant melanoma (160). Since it is estimated that 0.01% of cancer cells survive in circulation to produce metastatic colonies at distant sites, the combined actions of TNF-α, IL-6, and epiregulin promote metastatic spread by inhibiting NK cell destruction (161) or by directly promoting cell survival genes (162). The arrival of metastasis into secondary organs is regulated by the concerted actions of integrin-dependent arrest onto

endothelium, and depletion of macrophages reduced the propensity of this process during breast cancer metastasis to the lung (163).

1.3 Skin structure and physiology

The skin represents the outer-most barrier for a vertebrate animal and is constantly exposed to a variety of potentially injurious pathological insults. This integumentary organ is designed to positively regulate water loss, maintain a symbiotic relationship with commensal surface microorganisms, sample and protect against foreign environments, grow hair in mammals, thermo-regulate, absorb harmful UV radiation, provide sensory information for external stimuli and synthesize vitamin D (164). Keratinocytes are the primary cell type of the epidermis arranged in a stratum of progressively differentiating squamous cells beginning with a basal proliferating layer and ending with the apoptotic stratum corneum; a process regulated by calcium and cellular orientation (165;166). This differentiation process is marked biochemically by a specific keratin expression pattern where K5/K14 pairs are expressed in basal layers, and K1/K10 as well as involucrin and filaggrin are induced in suprabasal, spinous and granular layers respectively (167). In mice, the epithelium may only be 1 or 2 cell layers thick but humans homeostatically maintain a stratum of 4-6 cells (164). Directly beneath the epidermis is the dermis, primarily composed of fibroblasts interdigitized within a dense matrix of collagen. Blood vessels, lymphatic channels, nerves and hair follicles also extend and reside within the dermis, structures that provide a complex support system to the entire cutaneous tissue. Lastly, a layered bed of smooth muscle and adipose tissue mark the hypodermis just below the dermis. For the purpose of this review, the focus will be on the relationship between keratinocytes, resident immune cells, and infiltrating leukocytes during inflammation and highlighting their

roles in skin carcinogenesis. Like most tissues, bone marrow derived immune cells are present in significant quantities within the skin and serve an important role in regulating homeostasis. Langerhan cells are a specialized type of dendritic cell and make up the largest proportion of $CD45^+$ immunocytes while inhabiting the epidermis. These professional antigen presenting cells populate the epidermis in a TGFβ dependent manner during development and are the key mediators of skin immunity and tolerance (168). Furthermore, in murine and bovine, but not human epidermis, a morphologically similar hematopoetically derived cell to the Langerhan cell is the aptly termed dendritic epidermal T cell (DETC) (169). These cells are γδTCR/CD3 double positive and require TCR gene rearrangement and selection in the thymus similarly to other TCR^+ subclassed lymphocytes. However, they function in a more innate-like fashion where less biochemically stringent polyclonal antigens are seemingly able to activate these cells (170). DETCs are also thought to function in wound healing (171). Indeed, multiple groups have demonstrated that DETC (and human counterparts) activate effector functions through upregulated stress-induced self antigens such as RAE-1, H60, and MULT; all of which are NKG2D ligands (172-174). Analogously, humans maintain significant populations of $\gamma\delta TCR^+$ T cells within other epithelium, most evidently the lining of intestines. Human γδT T cells are also activated by NKG2D ligands, MICA and MICB suggesting their involvement in maintaining tolerance dealing with stress responses (175). In addition, quiescent mast cells are present in large numbers within the dermis and when activated are responsible for many pro-inflammatory reactions including releasing histamine during an allergic response (176). Beyond these cells types, numerous dendritic cell subsets inhabit the dermis, with different phenotypes than that of the Langerhan cell but perform similar antigen sampling and immunoregulatory functions specific to dermal immunity (177). Macrophages, monocytes, granulocytes, NK cells and

αβTCR^{+} T cells typically reside in small numbers within normal skin tissue and will be discussed later in regards to their roles during carcinogenesis (178).

1.4 Two-stage skin chemical carcinogenesis

The two-stage chemical carcinogenesis (CC) model provides a biologically relevant tool for studying squamous cell carcinoma development *in situ* where observations can be extrapolated to many other epithelial-derived malignancies (179). This protocol faithfully mimics the stages of initiation, promotion and malignant conversion. The initiating mutation, caused by single 7, 12-dimethylbenze[a]anthracene (DMBA) application to the dorsal skin of mice, occurs 90% of the time in the H-*ras* gene of epidermal keratinocytes which produces a constitutively active oncogenic form of the GTPase (180). Most frequently a point mutation, caused by the irreversible DNA damaging activities of diol epoxide metabolites, substitutes a glycine residue at position 61 with a valine and hence inactivation of the GTPase activity (179). The type and position of the initiated keratinocyte in relation to the rest of the epidermis is currently unknown but is thought to likely occur in a basal interfollicular cell or a stem cell residing within the bulge of the hair follicle (181). However, most of these hypothesis are speculative and based on circumstantial evidence that proliferation and cell cycle dysregulation can only be occur in a moderately to highly undifferentiated keratinocytes. Terminal differentiation of cells by definition must signal acquisition of permanent genetic changes that prevent the cell from infinite self-renewal and provoke programmed cell death or arrest without further entry into the cell cycle (182). Once initiation following DMBA exposure is complete, chemical promotion is carried out by repeated application of TPA, a potent phorbol ester and PKC activator. Constant PKC activation provokes upregulation of numerous NFκb regulated

pro-inflammatory cytokines and chemokines within keratinocytes including but not limited to TNF-α, CXCL1/2, S100A8/9, G-CSF, and GM-CSF that provoke continual chemotaxis of inflammatory cells thereby promoting cancer cell proliferation and neoplastic progression (183-186). This protocol is highly effective at inducing consistent, repeatable, papilloma outgrowth. Among these benign lesions there are a small fraction of which contain cells at a high risk for malignant conversion into SCC (132;187). SCC and BCC are the malignant forms of benign skin papillomas and are the most commonly acquired malignancies in the United States (188).

Cytokine/Immunocellular Knock-out Phenotypes during Skin 2-stage Chemical Carcinogenesis

Gene	**Tumor Progression**	**Mouse Strain**	**Reference**
Rag2	Resistant	C57BL/6	(189)
JH (B cell)	Resistant	C57BL/6	(189)
TCRδ	Susceptible	FVB/n	(190)
TCR γV5/δV1 (DETC)	Susceptible	FVB/n	(191)
TCRβ	Resistant	FVB/n (high dose TPA)	(192)
CD4	Susceptible	FVB/n (low dose TPA)	(193;194)
CD8	Resistant	FVB/n (high dose TPA)	(194;195)
CD4	Resistant	C3H/HeN	(196)
CD8	Susceptible	C3H/HeN	(197)
Langerin-DTA	Resistant	FVB/n	(198)
p19 (IL-23)	Resistant	C57BL/6	(199)
p35 (IL-12)	Susceptible	C57BL/6	(199)
p40 (IL-12/IL-23)	Resistant	C57BL/6	(199)
IL-17	Resistant	C57BL/6	(200)
INFγR	Resistant	129/SV/EV	(201)
TNF-α	Resistant	C57/BL/6 and Balb/c	(202)

Table 1-2: A summary of the immunocellular and cytokine genetic knockout mice and their effect on tumor growth in the two-stage chemical skin carcinogenesis protocol. Increased resistance or susceptibility of tumor burdens is relative to WT controls used in those studies. DTA = dipheria toxin transgene

1.5 RAS oncogene

1.5.1 Signal transduction:

RAS, in its 3 isoforms, H (Harvey), K (Kirstens) and N (Neuroblastoma), activates numerous downstream signal transduction pathways and are mutated in 30% of all human cancers (203). Thus the skin carcinogenesis model has particular relevance to RAS driven human cancers. Pancreatic cancers have upwards of 90% K-Ras mutations, further support for the significance of this proto-oncogene in carcinogenesis (204). Colon, intestine and lung malignancies also have significant proportions have RAS mutations (205). Furthermore, the tyrosine kinases EGFR and ERB2 are greatly upregulated in many human tumors (including BCC and SCC). These receptors transduce their signals through RAS, creating a constitutively active RAS signal in cancer cells (206). It is the defining member of larger group of small GTPases that hydrolyze guanine nucleotide triphosphate (GTP) into the diphosphate form (GDP). RAS was originally discovered to be oncogenic by isolation and transfection of genetic elements from rat sarcoma virus (ras) infected cells that could be transformed by both the Harvey and Kirstens strains (207). N-Ras was later discovered and cloned from neuroblastoma and leukemia cell lines (208). Although all 3 three isoforms exhibit high sequence homology, their individual expression level varies in specific tissues as well as the types of cancers they are mutated in. For instance, N-Ras is most highly expressed in thymus and testis and is preferentially mutated in melanoma, myeloid leukemia, and Hodgkin's lymphoma (209-212). H-Ras transcripts are found in highest quantities in skin, brain and muscle and the oncogene is found most frequently in bladder carcinomas (213). Finally, K-Ras mutations are the most common in colon and pancreas while normal expression of the K isoform can be found most

abundantly in gut and lung (214-216). These circumstantial lines of evidence suggest that RAS isoforms do contain context specific functionality.

In normal somatic cells, RAS is frequently at the inception of signal transmission from extracellular stimuli on the cell surface to the nucleus where a myriad of necessary biological processes can begin including proliferation and cell cycle entry, cytoskeletal rearrangement, differentiation, metabolism, survival and cell death (203). RAS accomplishes this through its ability to bind GTP causing conformational activation, subsequently creating proper targets for its RAS-GTP biochemical binding partners. GTP binding is facilitated and accelerated by a GTP exchange factor (GEF) that removes bound GDP molecules on RAS. SOS is member of the GEF family and links EGFR signaling transduction to RAS activation. When GEFs remove GDP molecules, GTP binding to RAS becomes an stoichiometrically favorable event because of the 10 fold higher intracellular concentration of GTP and the higher binding affinity of GTP to RAS than GDP (217). Hydrolysis of GTP is also assisted by a GTPase activating protein (GAP) that stimulates the enzymatic activity of the small G protein. *NF1* is a RasGAP gene and its mutational inactivation is the initiating carcinogenic event found in neurofibromatosis where peripheral nerve cells cannot turn off RAS activation (218). Oncogenic mutations in RAS almost always occur at 1 of 3 amino acid positions: 12, 13, and 61 (219). These genetic alterations prevent RasGAPs from instigating GTP hydrolysis, and thus RAS remains permanently bound to the trinucleotide rendering the protein constitutively active (220). Three major signaling molecules that lay directly downstream of activated RAS are RAF, PI3K, and RalGDS (221). All 3 are required for skin carcinogenesis and their activity is also necessary for transformation in vitro when RAS is transfected or virally transduced cultured cells (222-224). The kinase RAF was the first major effector protein discovered to have been directly linked to

RAS activation. Four independent groups verified that RAS-GTP physically bound to RAF and this subsequently lead to elucidation of the downstream MAPK targets, MEK1 and ERK1/2 (225-229). ERK1/2 phosphorylation and nuclear translocation leads to nuclear activation of multiple transcription factor targets such as ETS proteins, Fos, and ELK-1 as well as other nuclear kinases like p90RSK (230). Transcriptional upregulation of genes resulting from MAPK cell signaling are pro-mitogenic and affect differentiation and actin rearrangement (231). The PI3K pathway activates multiple kinases important for cell survival and inflammation; AKT, mTOR, PLC and PKC (232). These proteins are capable of NFκb activation that in turn mediates transcription of cytokine and anti-apoptotic genes (233-235). Termination of PI3K activity is regulated by the phosphatase PTEN which dephosphorylates the second messenger PIP3, thus deactivating the pathway (236). Recently, PTEN was discovered to be the 2nd most commonly mutated protein in cancers after p53 demonstrating the significance of PI3K signaling in cellular homeostasis (237). RalGDS is a RAS GEF that links RAS GTPase to Ral GTPase (238). Activation of this pathway alone without PI3K or RAF activation proved sufficient to transform human kidney epithelial cells (239). Ral activation leads to another MAPK pathway where JNK is the ultimate kinase that leads to c-jun transcriptional activity (240). Together, activation of both NFκb and MAPK in intestinal epithelial cells were required to provoke destructive chronic inflammation in the colon that neither pathway could provoke upon singular activation alone demonstrating the significance of stimulating multiple signaling pathways during establishment of pathological inflammatory states (241).

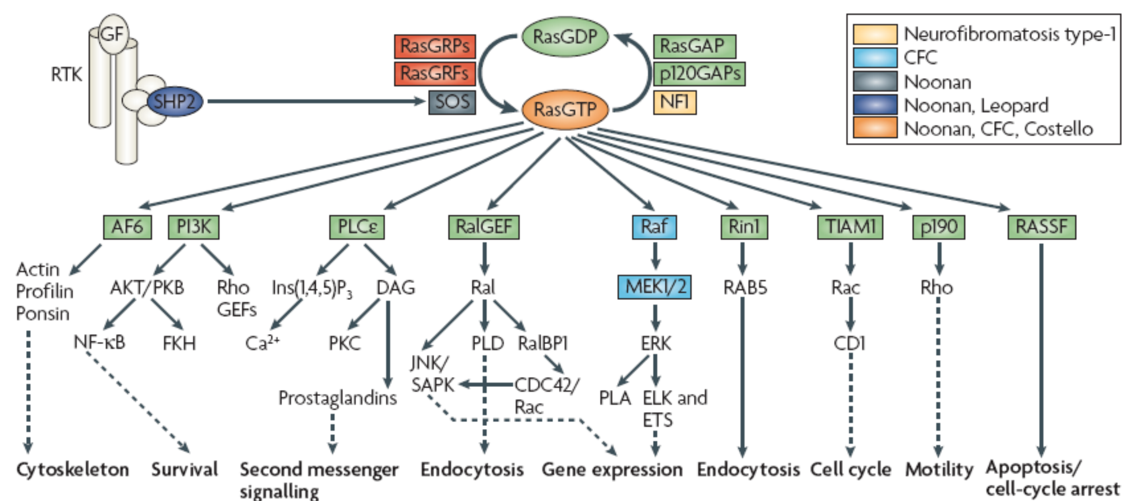


Figure 1-4: RAS activation and signal transduction. GEF mediated GTP binding of RAS initiates downstream activation of numerous molecules that control a multitude of cellular processes. When oncogenic mutations occur at residues 12, 13, or 61, RasGAPs are unable to catalyze the GTPase reaction and constitutive RAS signal transduction leads to unregulated cell cycle entry, promotion of pro-inflammatory pathways and ultimately transformation. The color coded legend indicates developmental disorders associated with mutations of the indicated genes. *Reprinted by permission from MacMillan Publishers Ltd: Nat. Rev. Mol. Cell Bio., Karnoub and Weinberg, Vol. 9, Issue 7, pp. 517-531, © 2008.*

1.5.2 RAS and inflammation

Inflammation invoked by RAS expression and oncogenic activation has only recently gained attention when control of tissue specific and conditional expression of RAS, RAS oncogenes, and RAS effectors could be achieved. In a model of non-small cell lung cancer, oncogenic K-RAS expression requires multiple inflammatory components to achieve complete tumorigenesis (242). Similarly, a K-RAS^{G12D} knock-in transgene targeted to bronchiolar epithelia provoked a macrophage and neutrophil inflammatory lung infiltrate that ultimately contributed to lung adenocarcinomas (243). Inducible H-RAS^{G12V} in xenografted tumor cells revealed that AP-1 and NFκb mediated IL-8 expression and secretion potentiated tumor associated inflammation leading to enhanced neovascularized tumor beds (244). Inflammatory contributions by macrophages, γδT cells and IL-1α are required to promote wound initiated tumors in an epidermal-specific, MEK1 skin tumor model (245-247). RAF transgene expression restricted to a K14 promoter also yielded tumors whose phenotype could be ameliorated by Gr-1

depletion (248). Pancreatitis was shown to exacerbate malignant progression of pancreatic ductal adenocarcinomas in a mouse model of $KRas^{G12D}$ expression relative to non-inflammatory conditions (249). This pancreas specific inflammation was then further demonstrated to be the mechanism underlying the ability of cancer cells to overcome K-Ras induced senescence (250). Finally, immunization with an Arg12 mutant RAS peptide intended to stimulate oncogene specific cytotoxic T cells actually enhanced tumor development in a model of RAS skin tumorigenesis suggesting, in converse, activation of RAS specific tumor promoting inflammation (251). Despite these clues, all of these studies required additional application of pro-inflammatory toxicants or engraftment of already malignant cell lines meaning the inflammatory contributions could not solely be attributed to RAS initiation. The direct contributions of RAS to inflammation have yet to be fully determined.

1.6 Immune Cells and Cancer

Nearly every bone marrow-derived immunocyte subset has been implicated in altering the biological outcomes of the aforementioned defined stages of cancer (252). Furthermore, depending on the tissue context as well as the driving mutation, each immunocyte contains the potential to contribute either tumor inhibitory or promotional properties (27). Because most immune cells are released from the bone marrow and reside in peripheral lymph tissues as differentiated, yet immature cells and maintain biologically quiescent phenotypes, they possess the ability to respond to disparate microenvironments accordingly. This in turn allows the immune system to shape a specific inflammatory response as needed. The driving force behind this is the large and distinct number of soluble factors released by immune and non-immune cell types during pathological insults. Through paracrine and autocrine stimulation of cytokine/chemokine receptors, leukocytes can tailor their phenotypes to match the requirements

for microbial elimination and/or healing. Tumors are large, metabolically active tissue masses capable of secreting copious amounts of these immune-editing factors. As previously discussed, the nature of the unrelenting positive feedback loops in tumor cells can cause acute and beneficial inflammation to often evolve into chronic and harmful inflammation. The limitless potential for phenotypical plasticity in immune cells during cancer progression has recently garnered much attention.

1.6.1 Myeloid Lineages

Neutrophils

Neutrophils (PMNs) are innate immune cells of myeloid lineage that constitute the cellular majority of first responders in any given inflammatory cascade. Their increased tissue residency normally serves to remove infectious microbes and repair damaged tissue. However, during chronic inflammation, their constitutive presence within tissues correlates with poor clinical outcomes (253-255). Activated neutrophils produce many toxic products and proteins such as hypochlorous acid (HOCl), nitric oxide (NO), free oxygen radicals (O_2-), hydrogen peroxide (H_20_2), proteases and TNF-α (256). Paradoxically, these mediators can have tumor promoting and tumor inhibiting effects. Genotoxicity of proximal cells is the most often observed abnormality as a result of these neutrophil respiratory burst products. Chronic release of these compounds by infiltrating neutrophils may generate multiple mutations required for tumor formation. Depletion of neutrophils during LPS induced inflammation reduced the genotoxic effects of HOCl on airway epithelial cells due to elimination of its catalyzing enzyme, myeloperoxidase (257). In support of this, a functionally defective polymorphism in the MPO gene is associated with a decreased risk for lung cancer (258;259). Co-culture of activated

human neutrophils with numerous cancer cell lines increased mutation rates in these cells, attributable to the phagocytes ability to alter DNA by oxidation, nitration, depurination, methylation and deamination (260). These neutrophil derived chemicals are beneficial and necessary, especially during wound healing and bacterial infection but during carcinogenesis rarely prevent tumor development (261). Conversely, high quantities of HOCl produced by PMNs can be cytotoxic to leukemic cell lines (262). Neutrophils can also trigger antibody dependent cell mediated cytotoxicity (ADCC) tumor cell lysis through FcαR recognition of IgA bound tumor cells (263) and neutrophil regulated ADCC has also been shown to occur with melanoma, neuroblastoma, and colorectal cancer cells (264-266). A model of syngeneic mammary adenocarcinoma demonstrated that PMNs can cooperate with $CD8^+$ T cells in mitigating malignant growth by enhancing immunosurveillance and tumor rejection (267). Interestingly, neutrophils can also display two entirely different phenotypes within the same tumor dependent on intrinsic cancer cell signaling. For instance, Friedlender and colleagues demonstrated that TGFβ signaling in K-RAS knock-in lung tumors controlled whether neutrophils would be tumoricidal and promote anti-tumor CD8 responses or immunosuppressive and inhibit CD8 CTL activation (268). Message RNA profiling of ALK5-inhibited neutrophils isolated from tumors revealed downregulation of arginase, a key immunosuppressive enzyme, and increased production of tumoricidal NO and H_20_2 (268). This cytotoxic phenotype was only induced upon blockade of the TGFβ pathway, supporting a critical role for this pathway during morphogenesis of cytotoxic and immunosuppressive pathways. Neutrophil depletion by use of the anti-Gr-1 depleting antibody RB6-8C5 has been shown to inhibit tumor growth (269), and in one model of xenografted H-RAS^{G12V} tumor cells this was linked to drastic reduction in CD31 positive blood vessels and inhibition of angiogenic pathways within the tumor beds (244). Other

neutrophil derived cytokines with potential immuno-modulatory effects include IL-1β, IL-6, and IL-12 (270). Perhaps the most significant tumor effecting factors that neutrophils provide are proteases. Neutrophil-derived elastase was critical in promoting proliferation of A549 cells and accelerating tumor growth of K-RAS transgenic knock-in lung adenocarcinomas by degrading intracellular cancer cell stores of IRS-1, an inhibitor of PDGF/PDGFR induced PI3K signaling (271). Also of significance in this study was the result that elastase could be transferred into tumor cells by endosomal uptake, thus mediating the protease's activity distally in a paracrine manner instead of within neutrophils themselves. Matrix metalloprotease (MMP) type 9 is also abundant in neutrophil granules and is released upon inflammatory ignition (272). Neutrophils were shown to be the dominant sources of MMP9 in the RIP-Tie2 pancreatic tumor model and this proteolytic activity contributed significantly to VEGF bioavailability and the angiogenic switch (273). CCR2 null K14-HPV16 mice exhibit reduced MMP9 expressing macrophage infiltration but have only modestly decreased dysplastic/angiogenic phenotypes due to a compensatory recruitment of neutrophils that also express MMP9 within neoplastic tissue (274). MMP9 also greatly contributes to stromal remodeling, a necessary ingredient in provoking benign lesions to become malignant carcinomas (275).

Monocytes

Monocytes maintain a more plastic state than neutrophils as they are the precursors to macrophages and dendritic cells. Furthermore, subsets of monocytes can be found in any given inflammatory cascade, a condition thought to exist specifically for macrophage or dendritic cell differentiation. In peripheral blood of mice, monocytes can be classified as either $CD11b^{+}/Ly6C^{hi}/CX_3CR1^{lo}$ inflammatory monocytes ($CD14^{+}/CD16^{-}/CCR2^{+}/CX3CR1^{lo}$ in humans) or $CD11b^{+}/Ly6C^{lo}/CX_3CR1^{hi}$ patrolling monocytes ($CD14^{+}/CD16^{+}/CCR2^{lo}/CX_3CR1^{hi}$

in humans) with distinct functions and migration patterns (276;277). A specialized $Tie2^{+}$ monocyte subset was shown in one study to provide distinct pro-angiogenic signals critical for tumor neovascularization (278). CCL2 is a critical cytokine in monocyte recruitment and is highly expressed by tumor epithelial cells and fibroblasts (279;280) and blockade of the chemokine during chronic colitis associated cancer development reduced macrophage infiltration and tumor development (281). In similar studies on prostate cancer, anti-CCL2 therapy was effective at restricting tumor burden which stringently correlated with the decreased residency of intratumoral macrophages (282;283). Since macrophages are the most abundant immunocyte subset found within nearly all solid tumors, monocyte recruitment and differentiation are critical biological checkpoints for tumor progression as well obvious avenues to exploit immunotherapy for advanced staged cancers (284).

Macrophages

Like neutrophils, macrophages are fully capable of secreting cytokines that influence and sculpt the surrounding environment upon activation. In mice, macrophages can be distinguished by their simultaneous expression of CD11b, F4/80, CD68, CSF1R, CD163 and MHCII (285). As innate antigen presenting cells, macrophages are key contributors to microbial immunity through presentation of antigen and activation of specific Th1 and Th2 immune responses. Furthermore, their ability to phagocytose dying cells, including neutrophils, is necessary to repair wounds and maintain granulocytic homeostasis. Also like neutrophils, their proteolytic tissue remodeling functions are well documented. Through expression of MMP2, 9, and 11 macrophages greatly contribute to tissue remodeling and angiogenesis during wound healing and carcinogenesis. Inhibition of MMP-9 in macrophages with zolendronic acid reduced angiogenesis in the K14-HPV16 squamous model leading to amelioration of dysplastic

progression (286). This macrophage biology is phenocopied during wound healing and eye development (287;288). Macrophages are also direct contributors of VEGF, thereby also directly stimulating blood vessel formation within tumor stroma (289). Macrophage derived EGF as a response to CSFR stimulation enhanced tumor invasion in the PyMT driven breast cancer mouse (290) establishing an CSF/EGF paracrine loop between breast cancer cells and macrophages that has been confirmed in numerous other laboratories (291-293). Perhaps most significantly, two macrophage phenotypes with distinctive and unique functional properties have spawned renewed interest in their contextual roles during carcinogenesis. Macrophages can be classified based upon their gene expression profiles as classically (M1) or alternatively activated (M2) (294). M1 macrophages infiltrate tissues in the context of bacterial and viral infections, establishing this phenotype through precursor monocyte recruitment where Th1 polarized responses are required to eliminate these foreign invaders (295). Markers of the M1 phenotype include: iNOS, TNF-α, IFN-γ, IL-12, and IL-23 (296); genes upregulated in response to LPS stimulation *in vitro*. Through these cytokines, M1 macrophages establish a pro-inflammatory phenotype, present antigen more robustly and generally favor anti-tumor responses (297). Indeed, direct tumoricidal behavior from M1 phenotypes has been demonstrated in cancer models through the activities of TNF-α (298), NO (299) and ROS (300). STAT6 deficiency skewed macrophages to an M1 bias that resulted in increased Tc cell driven immunity on 4T1 breast cancer xenografts (301). In contrast, M2 macrophages establish pro-tumor immunity through expression of Arginase (Arg1), mannose receptor, IL-10, PDGF, TGFβ, CXCL12, CXCL13, and CCL24 (302). This gene expression profile is induced largely through the activities of Th2 secreted IL-4 and IL-13 on their cognate macrophage IL4Rα receptors (303). M2 macrophages can be found in abundance within healing wounds as they are very efficient at

promoting fibrosis and angiogenesis (304;305). Furthermore, the anti-inflammatory properties of M2 macrophages are necessary to resolve inflammation following wounding so as to prevent dissemination of autoimmunity (306-308). Arg1 is the key protein that mediates this process through extracellular exhaustion of arginine, an amino acid required by T cells to properly activate (309-311). IL-10 also has significant immunosuppressive activities, specifically on $CD4^+$ Th1 directly and $CD8^+$ CTL's indirectly (312;313). Through these cytokine networks, solid tumors spawn M2 dominated phenotypes where the neoplastic microenvironment requires neovascularization, tissue remodeling and suppression of cytotoxic T cells that would seek to prevent new growth (314-316). In a genetic breast cancer mouse model M2 macrophages and Th2 cells were shown to drive the fulminant metastatic potential to lung tissue and this phenotype could be reversed with anti-CSF and anti-IL-4 therapy through a cytotoxic $CD8^+$ T cell dependent mechanism (291;317). Additionally, the CD68/CD4/CD8 ratio further correlated very stringently with disease free survival supporting a concept that an immune signature could predict clinical outcome (317). One context dependent difference of note was a study demonstrating that IFN-γ production from macrophages, a canonical M1 marker, increased melanomagenesis and migration in response to UV, an early melanocyte response preceding the onset of melanoma (318). The arrival of M2 macrophages within tissues also may be quite a distinct process from M1 tissue populating events. It was originally thought that all macrophages expanded in tissue during inflammatory processes via monocyte precursor recruitment and subsequent differentiation (319). However, a study using a helminth infection recently demonstrated that local macrophage proliferation was not only possible but actually preferred during Th2 driven immunity (320). This unique biology could certainly be extrapolated to mechanisms of macrophage expansion during carcinogenesis where Th2 and M2

cells dominate the $CD45^+$ immunocyte armament. These studies, however, have yet to be performed in the context of cancer development.

Myeloid Derived Suppressor Cells

A significant amount of cancer research has recently been devoted to immune cells that arise from myeloid progenitors and are phenotypically classified based upon their unique immunosuppressive roles. This morphologically heterogeneous population of Gr-1/CD11b double positive cells are collectively termed myeloid derived suppressor cells (MDSC), although this term can be somewhat misleading (321). The first recorded observation of immunosuppressive myeloid cells was made by Young and colleagues in a mouse model of Lewis Lung carcinoma where cancer cell derived CSF-1 stimulation of bone marrow derived monocytes inhibited T lymphocyte blastogenesis (322). The observation that these cells existed in humans was never fully appreciated until similar immunosuppressive HLA-DR^-/$CD11b^+$/Lin^-/$CD14^-$ and $CD15^+$ cells were observed in pancreatic (323), melanoma (324), renal cell carcinoma (325), breast, non-small cell lung cancer, and HNSCC bearing patients (326). These cells are not per say, a specific immune subset, with distinct lineages. More appropriately, they expand and arise from the same common myeloid progenitor stem cells as neutrophils and monocytes but extreme cytokine environments they are exposed to during hyperinflammatory states render them incapable of canonical hematopoietic differentiation. Indeed, when extracted *ex vivo* from their inflamed habitats and placed in culture with appropriate concentrations and combinations of cytokines, MDSC repeatedly differentiate into antigen presenting cells such as dendritic cells (327-329). This suggests that MDSC maintain the ability to appropriately respond to varying cytokines but resist a normal immune life cycle by remaining in cytokine-induced pluripotent phenotypes. An illustration of this comes from a study where CT26 tumor cell

conditioned media induced JAK2/STAT3 hyperactivity in myeloid cells, thus preventing normal dendritic cell maturation (330). MDSC accomplish immunosuppression of T cells by upregulation of Arg1 and/or iNOS, sapping extracellular arginine stores and producing reactive oxygen and nitrogen intermediates that induce T cell death, dysfunction and/or anergy (331). iNOS synthesis of NO was demonstrated to be a precursor step towards production of perioxynitrates that rendered TCRs from antigen specific $CD8^+$ T cells unresponsive to antigenic stimulation due to nitration of TCR tyrosine residues (332). Arg1 mediated exhaustion of tumor microenvironmental arginine resulted in T cell suppression via downregulation of the CD3 zeta chain (333). Since their initial characterization, both granulocytic ($Ly6G^+/Ly6C^{lo}$) and monocytic ($Ly6G^-/Ly6C^{hi}$) subsets have demonstrated acquisition of MDSC functionality depending on tumor type (334). These myeloid phenotypes are not solely unique to carcinogenesis but have also been observed during autoimmunity (335), infection (336) and traumatic injury (337). MDSC have also been shown to promote cancer progression through neoplastic tissue reorganization, growth factor bioavailability and angiogenic stimulation making them highly analogous to M2 macrophage phenotypes (338). However, it is still yet undetermined if monocytic MDSC subsets are just M2 macrophages precursors or if their immature states preclude them from tissue macrophage differentiation. Definitive MDSC and M2 markers ($Ly6C^+$ monocytic MDSC and $F4/80^+$ macrophages) can be distinguished by intratumoral FACS analysis but whether this represents a fluid differentiation continuum or myeloid lineage endpoints has yet to be determined.

Mast Cells

Mast cells are bone marrow derived myeloid cells that reside within the stroma of epithelial tissues such as skin, lung, and connective tissue. Their functions in the context of

mediating allergic reactions are well appreciated (339). Following allergen exposure to epithelia, mast cells rapidly release pro-inflammatory mediators from their abundant intracellular granule stores; the most well know being histamine, a potent vasodilator. Mast cell granules also contain copious amounts of proteases such as chymase, tryptase, cathepsin and MMP2/9 as well as heparin, VEGF, TNF-α, IL-1β, IL-10, TGFβ, and GM-CSF (340). Recently, the roles of mast cells during carcinogenesis are becoming well documented. Quantities of mast cells in prostate tumors is a prognostic factor for poor clinical outcomes (341) as well as in neurofibromatosis (NF1) patients (342). Furthermore, a novel mechanism of mast cell tumor promotion was discovered with an NF1 mouse model whereby NF1 heterozygosity in mast cells, but not in Schwann cell alone was required for tumor initiation by creating a hyperactivated pro-inflammatory mast cell phenotype (343). During the genesis of incipient squamous neoplasias, mast cells were required for progression to dysplastic and malignant states through the pro-angiogenic activities of mast cell derived proteases most notably MMP9 (90;344). Inhibiting mast cell degranulation during MYC induced pancreatic cancer abrogated pro-angiogenic pathways thereby blunting cancerous beta cell progression (345). Finally, mast cell derived LTB4 enhanced recruitment of MDSC, an attribute that led to increased intestinal epithelial cell proliferation and polyposis (346). Since mast cells are so effective at initiating an inflammatory response the contributions of mast cells to carcinogenesis are likely only to increase as more attention is paid to these unique cells.

Table 1. Roles of Different Subtypes of Immune and Inflammatory Cells in Antitumor Immunity and Tumor-Promoting Inflammation		
Cell Types	Antitumor	Tumor-Promoting
Macrophages, dendritic cells, myeloid-derived suppressor cells	Antigen presentation; production of cytokines (IL-12 and type I IFN)	Immunosuppression; production of cytokines, chemokines, proteases, growth factors, and angiogenic factors
Mast cells		Production of cytokines
B cells	Production of tumor-specific antibodies?	Production of cytokines and antibodies; activation of mast cells; immunosuppression
$CD8^+$ T cells	Direct lysis of cancer cells; production of cytotoxic cytokines	Production of cytokines?
$CD4^+$ Th2 cells		Education of macrophages; production of cytokines; B cell activation
$CD4^+$ Th1 cells	Help to cytotoxic T lymphocytes (CTLs) in tumor rejection; production of cytokines (IFNγ)	Production of cytokines
$CD4^+$ Th17 cells	Activation of CTLs	Production of cytokines
$CD4^+$ Treg cells	Suppression of inflammation (cytokines and other suppressive mechanisms)	Immunosuppression; production of cytokines
Natural killer cells	Direct cytotoxicity toward cancer cells; production of cytotoxic cytokines	
Natural killer T cells	Direct cytotoxicity toward cancer cells; production of cytotoxic cytokines	
Neutrophils	Direct cytotoxicity; regulation of CTL responses	Production of cytokines, proteases, and ROS

Table 1-3: **Paradoxical roles of immune cells during carcinogenesis.** *Reprinted from: Cell, Vol. 140, Grivennikov et al, Immunity, Inflammation, and Cancer, pp. 883-899, © 2010, with permission from Elsevier and Lancet..*

1.6.2 Lymphoid Lineages

γδ T cells

Lymphocytes have traditionally been thought to exclusively provide roles in anti-tumor immunity. Recently, however, this paradigm has required alteration due to multiple studies exhibiting the pro-tumorigenic activities of lymphocytes during neoplastic progression and malignancy. In that regard, the 2-stage skin chemical carcinogenesis assay has provided definitive clues into the paradoxical roles of specific lymphocyte subsets. Using this model, $\gamma\delta TCR^+$ T cells were shown to provide a protective role from papilloma development (347). Conversely, when *TCRδ -/-* bone marrow was transferred into irradiated transgenic Inv-MEK1 mice, these mice exhibited an attenuated tumor burden suggesting γδT cells provided promotional inflammatory cues towards squamous tumor development (348). The explanation

between these two models may be as simple as methods used to initiate tumors (chemical versus transgenic) or potentially the oncogene driving malignant outgrowth (RAS vs. MEK1). The intentional wounding in the transgenic model may also play a role as γδT cells are known to activate macrophages in human burn wounds (349). NKG2D receptor engagement on γδT cells stimulated cytolysis of a broad range of human epithelial cancer cells (350) and this study was supported by another where γδT cells killed H60C (a novel NKG2D ligand) expressing primary keratinocytes in vitro (351). MICA/B was expressed on a wide variety of epithelial derived human tumors and γδT cells directly lysed many cell lines derived from these tumors (352). Conversely, in a different study γδT cells actually directly suppressed cytolytic Tc anti-tumor responses (353).

αβ T cells

T lymphocytes of the αβTCR lineage also can perform dichotomous functions during tumorigenesis and malignant progression. While γδTCR$^+$ T cells were shown to provide a protective role during skin chemical carcinogenesis in FVB/n mice, genetic ablation of αβTCR$^+$ T cells revealed the exact opposite (192). To that end, αβTCR$^+$/CD4+ T helper cells display a wide diversity of functions effecting tumor outcomes. Performing the two stage protocol on a C3H/HeN mouse strain revealed a reduced tumor burden on *CD4-/-* mice relative to WT and *CD8-/-* groups (354). In a genetic model of mammary carcinogenesis that is driven the polyoma middle T antigen, CD4$^+$ T cells promoted lung metastasis but had no effect on primary tumor development (291). These CD4$^+$ T cells were characterized as strongly Th2 biased, mediating their effects through IL-4. Similarly, the K14HPV16 squamous tumor model supported these conclusions by demonstrating that *CD4* null mice had decreased dysplastic lesions through a mechanism of reduced skin recruitment of MMP9 expressing Gr-1/CD11b cells (355).

Conversely, antibody depletion of CD4 cells during UVB exposure, potentiated neutrophil infiltration into the skin and enhanced tumorigenesis suggesting a protective role for $CD4^+$ T cells (356). In the transgenic RIP-Tag2 pancreatic tumor model, $CD4^+$ T cells were shown to induce proliferation and recruitment of antigen specific $CD8^+$ T cells that ultimately led to cytolysis of the cancerous islet cells (357). Finally, total $CD4^+$ T cells were necessary and sufficient to implement sustained tumor regression upon MYC inactivation, a process that involved activation of senescence pathways (358).

$CD4^+$ T cells

Studies on specific CD4 lineages further implicate their tumor promoting effects. Perhaps the most famous and well characterized functions for in $\alpha\beta TCR^+$ T cells in cancer is the role of $CD4^+$ T regulatory cells (Tregs) in dampening anti-tumor immunity and enhancing solid tumor progression; now a heavily exploited avenue of cancer immunotherapy (359). Tregs accomplish their immunosuppressive effects through TGFβ and IL-10 and are greatly expanded in lymphatic and tumor tissue usually during later stages of malignancy where the cytokine polarizing requirement of TGFβ is broadly available (360). Again, like many of the dysfunctional inflammatory processes discussed, Treg expansion is necessary for normal immunological resolution but through multiple mechanisms, the presence of Tregs within solid tumors is chronically reinforced producing harmful consequences (361;362). Depletion of Tregs by anti-CD25 treatment culminating in enhanced cytotoxic immunity was an effective therapy for numerous mouse tumors, irrespective of tumor type, and provides rational for targeting Tregs as an immunotherapy or adjuvant therapy for human cancers (363-366). IL-17 producing $CD4^+$ T cells, commonly known as Th17 cells, expand greatly under pro-inflammatory conditions in which the concerted actions of TGFβ and IL-6 simultaneously induce upregulation of the

transcription RORγT and IL-17 expression (367). The role of Th17 cells in driving autoimmune diseases, including psoriasis, is well documented (368;369). Additionally, Th17 cells regulate neutrophil hematopoiesis (370) and can potentiate angiogenesis within tumor stroma (371) and their presence within human cancers reveals complex functional duality (372). In the 2-stage skin carcinogenesis model inactivation of IFN-γ signaling during TPA promotion either with a neutralizing anti-IFN-γ antibody or through the use of *IFNGR* knockout mice suppressed tumor formation and this was linked to reduced Th17 pro-inflammatory activity (201). In agreement with this, Th17 cells exhibited strong recruitment and activation properties for Gr-1/CD11b MDSC populations that ultimately enhanced lymphoma, prostate, and melanoma xenograft tumor growth (373). Furthermore, chemically induced skin tumor formation is greatly attenuated in IL-17 knock-out mouse, a result attributed to both cancer cell autonomous and extrinsic factors (200). In corroboration with this study, B16 melanoma and MB49 bladder carcinoma growth were blunted in *IL-17-/-* mice due to reduced IL-6-STAT3 signaling (374). The anti-tumor effects mediated by Th17 cells have also been observed. Their high rates of differentiation within human prostate tumors and mouse pancreatic cancers is a positive prognostic factor (375;376). Using the same B16 melanoma tumor model, Martin-Orozoca et al observed that Th17 cells actually enhanced $CD8^+$ CTL anti-tumor immunity thereby preventing B16 lung nodules through a mechanism of increased CCR6+ dendritic cell recruitment to tumor sites (377). Another group demonstrated the efficacy of adoptively transferred in vitro polarized Th17 cells in debulking large, established B16 melanomas underscoring their anti-B16 immune responses (378). Moreover, neutralization of IL-17 reversed the anti-tumor immune effects of TGFβ mediated squamous tumor regression punctuating the functional plasticity of this unique T helper phenotype (379).

CD8$^+$ T cells

Differing from the CD4 lineage, αβTCR$^+$/CD8$^+$ T cells are traditionally thought to contain a more limited phenotypic diversity, providing tumor antigen specific cytotoxicity and indeed, there is ample evidence to support this paradigm (380-383). Furthermore, many of the immune-targeted therapeutic efforts have been aimed at stimulating sustained CTL responses in advanced stage cancer patients either through vaccination or adoptive transfer of autologous CD8$^+$ T cells (384). Researchers have even explored the therapeutic viability of transferring CTLs transduced with chimeric antigen receptors whose TCR's bind tumor antigen with up to 1000 fold greater affinity than their endogenous counterparts (385;386). Unfortunately, these attempts have been met with limited success (387), mostly against CD19$^+$ chronic lymphocytic leukemias (388). On the contrary, recent research has begun to show CD8$^+$ T cells also possess the capacity for pro-tumorigenic functionality. Following up on their result that *TCRβ-/-* mice yielded reduced tumor volumes and prolonged carcinoma development in FVB/n mice, Girardi and colleagues identified a definitive role for pro-tumorigenic CD8$^+$ T cells by using the skin carcinogenesis protocol on *CD8 -/-* mice (194). Here complete ablation of CD8$^+$ T cells recapitulated results using the *TCRβ-/-* mouse. Reconstitution with purified splenic/lymph node CD8β$^+$ T cells into adults or neonatal reconstitution with *CD4-/-* fetal liver hematopoietic cells (FLHC) into *TCRβ-/-* recovered WT tumor growth and malignant conversion (194). This unique CD8$^+$ T cell biology was subsequently characterized as co-expressing IFN-γ, IL-10, and IL-17 while simultaneously being deficient for perforin production, a key cytolytic protein (389). A similar cytokine profile was observed in human HCC patients where the frequency of IL-17$^+$/CD8$^+$ T cells (Tc17) within cancerous liver tissue correlated with a poor prognosis (390). Also, Tc17 cells, and not Th17 cells increased within psoriatic lesions on human skin, supporting

a pathogenic role for immune cells rarely observed during homeostatic conditions (391). The *in vitro* requirements for IL-17 expression in $CD8^+$ T cells polarizing them to a Tc17 phenotype seem to be identical to that of Th17 cells and adoptive transfer of these Tc17 conditioned cells caused disease exacerbation of a diabetes model (392). Other chronic inflammatory syndromes where Tc17 cells have been found in high proportions and contributed to disease progression are multiple sclerosis (393), pulmonary inflammation (394) and allergic dermatitis (395). In contrast to these studies, another group using B16 melanoma xenografts discovered transferring in vitro generated, antigen specific Tc17 cells actually enhanced anti-tumor CTL immunity in part by stimulating cytotoxic neutrophils, thereby inhibiting tumor growth (396). Heinrichs and colleagues supported these conclusions with similar B16 melanoma study of their own (397). All of these studies indicate that the pathogenic roles of $CD8^+$ T cells may be more complex during chronic inflammatory processes including carcinogenesis and demands more creative reanalysis when considering $CD8^+$ T cell-targeted clinical intervention.

B cells

B lymphocytes are a necessary component to immunological activation and prevention of infectious disease dissemination through establishment of humoral immunity. Antibody production is essential in fighting bacterial and viral infections by stimulating innate immune cells through complement and FcR activation. B cells also express MHCII molecules, providing T cells with a 3rd antigen presenting option. However, researchers have also implicated B lymphocytes in the pathogenesis of numerous inflammatory related diseases. As proof of principle, Rituximab, anti-CD20 therapy originally approved for lymphoma patients, has also been approved to ameliorate the disease course of rheumatoid arthritis and systemic lupus erythematosus (398;399). Anti-CD20 treatments ameliorated tumor burden in 50% of treated

colorectal cancer patients (400) and a B cell deficiency enhanced the efficacy of a melanoma vaccine in mice (401). Abnormally high quantities of immune complexes (IC) within neoplastic tissues is correlates with increased disease progression and poor cancer patient survival in head and neck, breast, and genitourinary malignancies and is considered a risk factor within pre-malignant, chronically inflamed organs (402). Furthermore, a recent flow cytometric and histochemical analysis of leukocyte composition in human breast cancer tissue revealed a heavy and significant B cell infiltrate that decreased in stage matched patients receiving chemotherapy (403). Genetic manipulation of mouse cancer models has also allowed researchers to unequivocally identify a causal role for B cells in promoting squamous cancer development, most notably in the K14-HPV16 transgenic model of incipient squamous neoplasia. Here, IgG-stimulated FcγR$^+$ myeloid cell recruitment was required for neoangiogenesis and matrix remodeling leading to pre-malignant progression (404). Total B cell repletion as well as repeated injections of serum from transgenic mice into B cell deficient *JH-/-* or *Rag1-/-* mice faithfully phenocopied incipient neoplastic progression in B cell competent K14HPV16 littermates demonstrating immunoglobulin production alone was sufficient to initiate disease (405). Similarly, using the two-stage CC model, adoptively transferred IL-10 secreting B regulatory cells were capable of partial recovery of WT skin tumor burdens in *JH-/-* and *Rag1-/-* mice again suggesting a crucial role for B cells in the development of non-melanoma skin cancer (189). A comprehensive study using multiple cancer cell xenografts on *IgM-/-* mice revealed greatly enhanced CTL activity that led to tumor resistance in the B cell deficient mice (406). Moreover, transfer of IL-10 competent, CD5$^+$/CD1d^{hi} B regulatory cells (Bregs), even in relatively small numbers, limited anti-CD20 therapy and fully restored tumor growth in a lymphoma model (407). Finally, B cells were required to mediate the inflammatory promotion

of castrate resistant prostate cancer through the paracrine effects of lymphotoxin (408). Consistent with the paradoxical theme of leukocytes and cancer, B cells also have been shown to contribute to anti-tumor immunity. High rates of intratumoral B cell infiltration is positively associated with survival and lower relapse rates in humans with node-negative breast cancer (409), ovarian cancer (410), non-small cell lung cancer (411), and cervical cancer (412). In mice, CD20 depletion abrogated CD4 and CD8 immune responses during syngeneic B16 tumor growth, allowing for greater tumor volume and lung metastasis (413). A leukemia mouse model induced by Friend Leukemia virus also had reduced T cell responses in B cell deficient mice (414) and direct cytotoxicity of tumor by B cells was observed through IFN-α inducible TRAIL expression (415). Along with parallels drawn from autoimmune disorders, these recent results in cancer patients and murine models clearly point to multiple B cell driven mechanisms of disease onset and progression during carcinogenesis.

Lymphocyte/Myelocyte Interactions

Rarely do lymphocytes exert their pro-tumorigenic functions directly upon cancer cells in advanced stage tumors or pre-malignant tissue. Most often, they act indirectly through complex cell to cell interactions such as stimulating the chemotaxis of myeloid cells or skewing the tumor-promoting phenotypes of other leukocytes. The vast majority of research on myelocyte and lymphocyte interactions during carcinogenesis is related to macrophage phenotype and function. For instance, the studies previously discussed involving the promotional roles of Th2 cells in PyMT and B lymphocytes in K14-HPV16 transgenic mice both pointed to mechanisms of macrophage recruitment bearing a strong M2 bias (317;404). In the MMTV mammary model, anti-IL-4 treatment or IL-4Ra deficiency reversed the pro-metastatic effects from M2 macrophages. In the HPV16 skin model, FcγR stimulation by HPV16 induced immune

complexes promoted gene expression of a number of M2 genes. Correlatively, the transferred $CD5^{+}/CD1d^{hi}$ Bregs that provided a tumor protective response to anti-CD20 therapy in a model of Burkett's lymphoma, also promoted differentiation of macrophages to the M2 phenotype (407). These regulatory B cells achieved this through the effects of IL-10 dampening the phagocytic capabilities of monomyelocytic cells, thus rendering anti-CD20 therapy ineffectual because anti-CD20 ADCC is mediated by FcγR expressing phagocytes (416). Indeed, the effects of IL-10 on monocytes/macrophages include downregulation of MHCII, CD80, CD86, IL-12, IL-1, iNOS, IL-23, TNF-α and phagocytic mechanisms, and the upregulation of IL-10, IL-1Rα, soluble TNFαR, FcγR, and favors macrophage differentiation over dendritic cells from monocytic precursors (417). The tumor promoting $CD4^{+}$ Th cells in HPV16 mice worked through a mechanism of stimulating MMP9 expressing $Gr\text{-}1^{+}/Mac\text{-}1^{+}$ cell recruitment to enhance tissue remodeling and dysplasia in the E6/E7 transgenic skin model (418). The cell killing activities of neutrophils contributed to dwindling melanoma tumor sizes through the paracrine effects of in vitro conditioned Tc17 cells adoptively transferred into mice bearing B16 xenografts (419). Consistent with the positive feedback loops in cancer inflammation, M2 macrophages themselves robustly express chemokines that attract Th2 polarized immunity such as CCL17, CCL22, and CCL24 (420). IL-4 and IL-13, Th2 produced cytokines are strong M2 polarizing cytokines, secreted in copious amounts within solid tumor masses and IL4Rα knockout mice have reduced MDSC phenotypes (421). In addition, IL-4 increased cathepsin expression in macrophages that influenced pancreatic adenocarcinoma invasion properties (422). IL-17 was shown to stimulate recruitment of MDSC that ultimately contributed to tumor growth, a phenotype reversed in IL-17R null mice (423). A similar study with the PyMT mouse showed that MDSC recruited to *TGFBR2* null breast carcinomas reciprocally provided Th17 polarizing

IL-6, TGFβ, and IL-23 cytokines and the immunosuppressive properties of MDSC were reversed by IL-17 neutralization in tumors (424). The promotional effects of Th17 cells on tumor growth were also observed by Wang and colleagues in a melanoma and bladder carcinoma model where IL-17 potentiated tumor cell IL-6 production thereby activating STAT3 mediated pro-survival and pro-angiogenic pathways in stromal inflammatory cells (425). Human monocytes cultured in the presence of $CD4^{+}/CD25^{+}$ Tregs favor an M2 like phenotype, that secrete low amounts of TNF-α and high amounts of IL-10 (426). Furthermore, four independent groups showed the distinct positive relationship between MDSC and Tregs where expansion of one immunocyte subset promoted differentiation of the other (427-430).

1.7 Hypothesis and objectives

These previous studies in mice and humans establish a precedent for the complex relationships between lymphocytes and myeloid cells that cooperatively orchestrate and reinforce tumor promoting inflammatory activity. Most evidently, the work involving genetic ablation of lymphocytes during multistage skin carcinogenesis models with either a basal keratinocyte targeted E6/E7 transgene or chemical initiation of an H-Ras oncogene followed by inflammatory promotion strongly indicate that B cells and $CD8^{+}$ T cells provide necessary pro-inflammatory signals for fulminant squamous tumor formation. Additionally, since RAS is a common oncogene in many other epithelial derived malignancies, and a few other groups have circumstantially linked RAS oncogene to inflammatory tumor promotion, a logical approach to studying this biology would be to directly target an oncogenic RAS transgene to the skin. By removing the requirement of phorbol ester application, we can implicate a direct link between RAS oncogene and tumor promoting inflammation. Other groups have previously demonstrated

this approach and its usefulness in studying squamous tumor development but only as it relates to tumor cell autonomous effects (431-435). To that end we have employed two inducible and skin restricted H-RAS^{G12V} transgenic mouse models to answer this question. We hypothesize that leukocytes provide critical signals to RAS transformed keratinocytes and the pre-malignant stroma that promote tumor expansion and progression.

Chapter 3 reveals a crucial role for CD8$^+$ T cells in enhancing acute myeloid inflammation in response to RAS induction. This inflammatory response enhances keratinocyte proliferation ultimately leading to increased tumor formation. The diverse myelocytic infiltrate encompassed neutrophils, mast cells and macrophages of which neutrophil and mast cell residency were the most critical for squamous tumor development. Furthermore, CD8$^+$ T cells provoke neutrophil inflammation through the paracrine actions of IFN-γ.

Data in chapter 4 implicates the necessity of B cells for CD11b$^+$/Ly6C^{hi} monocytes to acquire immunosuppressive functionality in response to basal layer RAS activation. In addition, I show how the level of oncogene expression is directly proportional to the severity of inflammation and development of MDSC populations.

In chapter 5, the specific inflammatory differences between basal layer/stem cell and suprabasal layer driven RAS expression are highlighted as a potential mechanism behind the increased risk for malignant progression in tumors arising from initiated basal/stem cell layer keratinocytes. I provide evidence for inherent RAS signaling properties unique to proliferating and terminally differentiated keratinocytes and propose this leads to specific cytokine expression patterns.

These studies are not only important in elucidating immune-mediated mechanisms of epithelial carcinogenesis but also mimic chronic inflammatory environments observed in

cutaneous tissues. These data contribute new insight into how RAS induced inflammation affects disease outcome and should be taken into consideration by clinical researchers when designing novel immune-therapies for the treatment of solid tumors.

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Chapter 2: Materials and Methods

2.1 Animal Studies

InvtTA (1) or K14rTA (2) transgenic mice were crossed with the homozygous tetOHRAS^{G12V} line (3) to yield littermates of tetOHRAS^{G12V} (ST) and double transgenic (DT) InvtTA/tetOHRAS^{G12V} (InvRas) or K14rTA/tetOHRAS^{G12V} (K14Ras) offspring. Genotypes were determined by PCR with specific primer sets. Transgene expression in InvRas mice was suppressed by doxycycline (10 µg/ml) administered *ad libitum* in drinking water of breeding and weaned mice and was induced in K14Ras mice by varying amounts of dox chow. Unless otherwise noted, all K14Ras and control mice in those studies were given the standard maximal dox dose of 1 g/kg *ad libitum*. Specific leukocytes were depleted with 500 µg of RB6-8C5 (α-Gr-1), GK1.5 (α-CD4), YTS169.4 (α-CD8β), and HB94 (α-IgG) monoclonal antibodies administered intraperitoneally every other day (α-Gr-1) or once weekly (α-CD4, α-CD8β) coincident with dox removal. B cells were depleted with 150 µg α-CD20 mAb (5D2, Genentech) 7 days prior to dox induction and control groups received equal amounts of appropriate α-Ragweed isotype control. *Rag1 -/-* mice were reconstituted by retro-orbital transfer of 5 million negatively selected FACS and/or MACS® (Miltenyi Biotec) purified T or B cells isolated from inguinal lymph nodes and spleen of non-transgenic FVB/n mice (1:4 ratio) 2 days prior to transgene induction. Purities of > 95% were routinely achieved during cellular purification and efficiency of depletion and repletion was validated by flow cytometry. Differential leukocyte counts were determined on a Mascot™ Hemavet 950FS blood analyzer (Drew Scientific Inc.). For cytokine neutralization, 500 µg of neutralizing α-IFN-γ, XMG1.2 (BioXcell) and α-IL-17A, TC11-18H10.1 (Biolegend) antibodies were administered IP every 3 days beginning on day 0 through one day before animal sacrifice. To induce tumors, shaved DT

mice were switched to 250 ng/ml dox (InvRas) or fed 0.01 g/kg dox chow for 2 weeks followed by 0.025 g/kg dox for 1-2 weeks (K14Ras). One hour prior to sacrifice for all mice, 6 mg/mouse of 5-Bromo-2'-deoxyuridine (BRDU; Sigma-Aldrich) was injected IP for incorporation of BRDU into proliferating cells in S phase. Wright-Giemsa stains (Ricca Chemical Company) were performed on cardiac blood smears and sorted cytospins. All mice were on FVB/n background and animal studies were performed in compliance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals following protocols approved by The Pennsylvania State University IACUC.

2.2 Primary keratinocyte studies

Primary keratinocytes were harvested from newborn offspring of K14Ras breeder pairs as previously described (4). Following isolation, keratinocytes were cultured in LoCal medium (0.05mM Ca^{2+}) in 6 well dishes until confluent. To induce differentiation, medium was switched in select groups to 0.12 mM or 0.5 mM Ca^{2+} (HiCal) for 24 hours. Doxycycline (dox) was added to culture medium (1 μg/ml) to induce transgene expression for 24 hours. The MEK1/2 inhibitor U0126 (Calbiochem) was added to cultures at a 10 μM concentration for 8 hours following dox treatments to block ERK1/2 activation. To block AP-1 and NFκb activity, keratinocytes were infected with replication deficient adenoviral vectors (MOI 10) expressing dominant negative proteins 24 hours prior to further experimentation. The AP-1 DNA binding mutant A-FOS (5) and the degradation resistant IκBα mutant IKBsr (6) have been previously described. Following treatments, keratinocytes were scraped in RIPA buffer for protein lysates or Trizol for RNA isolation and further processed as described below.

2.3 Tissue Histology and Analysis

Immediately following sacrifice of mice, skin sections were immersed in 10% neutral

buffered formalin (NBF) or 70% ethanol (EtOH). Following 24 hours fixation, NBF fixed sections were switched to 70% EtOH and stored until paraffin embedding. Five micron sections were deparrafinized (Histochoice) and rehydrated through a graded series of alcohols (100%, 90%, and 70%) into PBS. Immunohistochemistry (IHC) for CD45, F4/80, and p-ERK1/2 was performed on NBF fixed sections by heat mediated antigen retrieval (95˚ C, 15 minutes, 10 mM Citrate/0.01% Tween/pH 6.2). Myeloperoxidase (MPO), CD3, and BRDU were detected using EtOH fixed sections. For BRDU staining sections were first treated with 4N HCL, 7 minutes at room temperature. For all IHC stains, 3% H_20_2 was used to quench endogenous peroxides, and VECTASTAIN® ABC (Vector Laboratories) and ImmPACT™ DAB (Vector Laboratories) were employed for streptavidin/peroxidase immunodetection. Epidermal thickness was quantitated by photographic capture of 10 random fields of view (FOV) on at least 5 sections/experimental group and then averaging 10 vector measurements/FOV from the basal layer of the epidermis to bottom of the stratum corneum using Spot Advanced imaging software (Diagnostic Instruments Inc.). Percent $BRDU^+$ cells were calculated by counting the number of positively stained cells in the epidermis and normalizing to the number of keratinocytes in the basal layer/FOV. Metachromatic toluidine blue staining (0.1%) was used to distinguish mast cells which turn violet in a pH of 2.0-2.5.

2.4 Cytotoxicity Assay

An experimental assay for detecting cellular mediated cytotoxicity by neutrophils was modified from similar cytotoxicity assays (7) using the KDalert ™ GAPDH Assay Kit (Applied Biosystems) to quantitate GAPDH. Percent cytotoxicity was calculated using the following equation: % killing = $[GAPDH_{Co\text{-}culture} – (GAPDH_{effectors} + GAPDH_{target})]/GAPDH_{total\ effector\ lysates} * 100$.

2.5 Suppression Assay

A method to measure suppression of *in vitro* activated T cell proliferation was adopted and modified from similar studies (8;9). Following 4 days of dox chow administration in K14Ras mice or 7 days of dox removal in InvRas mice, single cell suspensions from spleens or skin were prepared and stained with α-CD11b, α-Ly6G, and when noted, α-Ly6C and α-F4/80. Specific myeloid populations were FACS sorted on a Cytopeia Influx Sorter (BD Biosciences) and collected in RPMI 1640 1x (CellGro) media. One day prior to sorting, flat-bottomed 96 well tissue culture plates (Griener CellStar) were coated with 50 µl of 10 µg/ml α-CD3 (15A2, eBioscience) and 10 µg/ml α-CD28 (37.51, BD Biosciences). Following cell sorting, syngeneic, non-transgenic splenocytes were harvested into a single cell suspension, counted and the appropriate amount of splenocytes were stained with 2.5 µM CFSE (Molecular Probes) in 1xPBS/0.1% BSA at a cell concentration of 10×10^6 c/ml for 15 min. at 37˚C. Following staining, the remaining unbound CFSE was quenched with 5 volumes of complete RPMI media (10% heat inactivated FBS, 1mM Na-P, 1% Pen/Step, 50µM β-ME, and 20mM Hepes buffer). 2×10^5 CFSE labeled splenocytes were then admixed with sorted myeloid subsets at a 1:1, 1:2, or 1:4 $CD11b^+$ cell: splenocyte ratio and placed in PBS washed CD3/CD28 coated tissue culture plates with 200 µl complete RPMI media and co-cultured for 60-72 hours in a 37˚C/5% CO_2 incubator. Following incubation, individual wells were harvested, washed and stained with α-CD4/PE and α-CD8/PE-Cy5 for FACS analysis. All groups were co-cultured in triplicate. In some assays, the Arg1 inhibitor Nor-NOHA (500 mM) or the iNOS inhibitor L-NMMA (500 mM) was added to co-cultures to attempt to inhibit T cell suppression.

2.6 Antibodies

Unconjugated antibodies used for IHC were as follows: Rabbit α-MPO (1:500, Dako

Cytomation), Mouse α-BRDU (1:50, BD Biosciences), Rat α-F4/80 (C1:A3-1, 1:500, Biolegend), Rat α-CD45 (30-F11, 1:500, eBioscience), Rabbit α-CD3ε (1127, 1:150, Santa Cruz), Rabbit a-p-ERK1/2 T202/Y204 (1:500, 4370, Cell Signaling). Fluorescently conjugated antibodies used for flow cytometry and cell sorting were as follows: Rat α-Gr-1/FITC (RBC-8C5, BD Biosciences), Rat α-CD45/APC-EFluor780 and FITC (30-F11, eBioscience), Rat α-Ly6G/PE (1A8, BD Biosciences), Rat a-Ly6C/FITC (AL-21, BD Biosciences), Rat α-CD11b/APC (M1/70, eBioscience), Rat α-IL-17/PE (17B7, eBioscience), Rat α-IFN-γ/PE-Cy7 (XMG1.2, BD Biosciences), Rat α-IL-10/APC (JES5-16E3, BD Biosciences), Rat α-FoxP3/PE (FJK-16s, eBioscience), α-TCRβ/FITC (H57-597, BD Biosciences), Rat α-CD4/PE, PE-Cy5, and FITC (GK1.5, eBioscience), Rat α-CD8α/PE-Cy5 and FITC (53-6.7, eBioscience), Rat α-CD44/PE-Cy5 (1M7, eBioscience), Rat α-CD62L/PE (MEL-14, BD Biosciences), Rat α-B220/PE-Cy7 (RA3-6B2, BD Biosciences), Rat α-CD19/FITC or PE-Cy7 (6D5, Biolegend), Rat α-CD1d/PE (1B1, Biolegend), Rat α-CD5/FITC (53-7.3, Biolegend). The eBioscience Treg staining set was used to detect FoxP3 positive cells. The Red Live/Dead® Fixable Dead Cell Stain kit (Molecular Probes) was used to distinguish live and dead cells in skin preps.

2.7 FACS analysis

Single cell suspensions were prepared for flow cytometric staining by gentle mechanical dissociation of lymphoid and skin tissue or simple syringe collection of blood by cardiac puncture. Liberated cells in lymph nodes, spleen, and blood were subsequently passed through a 70 micron mesh filter (BD Biosciences) and erythrocytes lysed using a hypotonic buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.3). Skin leukocytes were isolated from mechanically minced whole skin tissue by collagenase digestion [complete RPMI, 0.825 mg/ml collagenase I, 2.5 mg/ml collagenase IV (Worthington), 0.25 mg/ml hyaluronidase IV-S (Sigma),

1000 units/ml DNase (MP Biomedicals)] for 1 hour @ 37˚ C with constant agitation. Following enzymatic treatments, tissue suspensions were passed thoroughly through a 16 gauge syringe and needle and strained through 70 micron mesh filters and washed with PBS. To distinguish live and dead cells, we used the Red Live/Dead® Fixable Dead Cell Stain kit (Molecular Probes). All single cell suspensions regardless of tissue origination were first blocked with α-CD16/CD32 prior to primary antibody staining and 1% BSA/1x PBS was used as a common wash and stain buffer. To fix and permeabilize cells for intracellular cytokine detection, 4% fresh preparations of paraformaldehyde (fixation) and 0.2% saponin/1% BSA/1x PBS (permeabilize) were applied to cells already stained for extracellular surface antigens. To facilitate FoxP3 staining, the eBioscience Treg staining set was used. Fluorescently stained single cell suspensions were analyzed on an FC500 (Beckman Coulter) or an LSRFortessa (BD Biosciences) cytometer.

2.8 Biochemical and Molecular Analysis

Total skin protein lysates were made by homogenization of stone ground snap frozen tissue pieces in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% Sodium deoxycholate, 0.1% SDS, 1 mM EDTA), rotated for 1 hour at 4°C and centrifuged at 14,000 RPM for 10 min to separate insoluble biomaterial. Total keratinocyte protein lysates were harvested by direct addition of RIPA buffer to PBS washed keratinocytes and cell scraping. Cytosolic and nuclear fractionation was carried out using two buffers. First, cells were scraped in buffer A (cytosolic fraction): 0.33 M sucrose/10 mM Hepes pH 7.4/1 mM $MgCl_2$/0.1% Triton X-100; and incubated 15 min. on ice followed by 2 washes with Buffer A. The remaining fraction was incubated with buffer B (nuclear fraction): 0.45 M NaCl/10 mM Hepes pH 7.4, 1 mM $MgCl_2$; 30 min. on ice with agitation and centrifugation to remove insoluble debris. Total protein concentrations were determined using a colorimetric BCA detection kit (Sigma). 15-30

μg of protein lysates were electrophoresed onto an 8-12% acrylamide gel, transferred to nitrocellulose and blotted with primary antibodies against H-Ras (Santa Cruz), p-ERK1/2 (Cell Signaling), p-MEKK4 (Cell Signaling), p-MKK3/6 (Cell Signaling), p-AKT (Cell Signaling), total AKT (cell signaling), p50 (1190, Santa Cruz), p65 (8008, Santa Cruz), and β-actin (Millipore) for protein loading control. Total RNA was isolated from skin using the same method as protein isolation except Trizol reagent (Invitrogen) was used instead of RIPA buffer. RNA was then reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega). mRNA transcript levels of specific cytokine/chemokine genes were determined by qRT-PCR using PerfeCTa™ SYBR® Green SuperMix for iQ™ (Quanta BioSciences) and normalized to level of the house keeping gene GAPDH.

2.9 Statistical Analysis

Student's *t* tests calculated p values between experimental groups of two only. For groups of 3 or more, one way Anova was used along with Tukey's post-analysis *t* tests. GraphPad Prism 4.0 was employed to format all figures and calculate significance. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

2. 9 Bibliography

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Chapter 3: Proinflammatory CD8$^+$ T cells promote Ras-induced cutaneous inflammation and squamous tumor formation

3.1 Abstract

CD8$^+$ T cells primarily function in anti-tumor immunity but a potential role in tumor promotion is not well understood. Here we show that proinflammatory CD8$^+$ T cells co-expressing IFN-γ and IL-17A infiltrate squamous tumors formed by inducible expression of an epidermally targeted and inducible *H-RAS*^{G12V} oncogene. Depletion of CD8$^+$ T cells prior to *RAS* induction led to reduced tumor burden, delayed tumor onset, and blunted intra-tumoral inflammation. The initial response to *RAS* expression in the normal epidermis was an increase in cutaneous IFN-γ and IL-17A co-expressing CD8$^+$ T cells and Th17 cells, as well as systemic neutrophilia and intraepidermal infiltration of cytotoxic Ly6G$^+$ cells. Depletion of CD8$^+$ but not CD4$^+$ T cells at this early stage reduced cutaneous and systemic inflammation, suppressed the RAS-induced increase in cutaneous Th17 and IL-17A$^+$ γδ T cells, and ameliorated epidermal hyperproliferation similar to that observed on a *Rag1*-/- background. Reconstitution of *Rag1-/-* inducible RAS mice with purified CD8$^+$ T cells restored neutrophilic inflammation and epidermal hyperproliferation but did not recover tumor formation or tissue mast cell infiltration, indicating potential cooperation with other lymphocyte subsets. Neutralization of IFN-γ but not IL-17A in CD8$^+$ T cell reconstituted *Rag1*-/- mice blocked CD8-mediated skin inflammation and epidermal hyperproliferation. These observations support a tumor promoting role for proinflammatory CD8$^+$ T cells in the pathogenesis of squamous cell cancer mediated primarily through the paracrine effects of IFN-γ on myeloid and adaptive immune cells.

3.2 Introduction

Chronic inflammation is now widely considered a risk factor for cancer development (1;2). Opposing the pro-tumorigenic effects of chronic inflammation is lymphocyte-mediated tumor immunosurveillance and anti-tumor immunity (3). However, recent clinical and experimental studies have also documented the protumorigenic roles of specific lymphocyte subsets. For instance, $CD4^+$ T regulatory cells have been shown to dampen anti-tumor immunity (4-6), while $CD4^+$ T cells expressing IL-17 (Th17 cells) can exacerbate recruitment of myeloid cells that aid in malignant progression (7;8). Similar studies in experimental breast cancer show a link between Th2 cells and recruitment of macrophages that promote metastasis (9). In contrast to disparate functions of $CD4^+$ lineages, the majority of clinical and experimental evidence show that $CD8^+$ T lymphocytes primarily have a role in preventing solid tumor development (10-13). However more recent studies implicating IL-17 and IFN-γ co-expressing $CD8^+$ T cells in the pathogenesis of inflammatory diseases such as psoriasis (14;15), COPD (16) and human HCC (17) suggest that $CD8^+$ T cell functionality during chronic inflammation and carcinogenesis may be more complex.

In the mouse 2-stage skin carcinogenesis model, activating mutations in the H-*Ras* gene are caused by the carcinogen 7, 12-dimethylbenze[a]anthracene (DMBA) (18), and repetitive 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment induces chronic inflammation and proliferative stimuli that drive squamous papilloma outgrowth, some of which progress to SCC (19). In this model skin resident $\gamma\delta TCR^+$ T cells suppress papilloma development while $\alpha\beta TCR^+$ T cells enhance premalignant progression (20), with the latter linked to tumor infiltrating, IL-17/IFN-γ/IL-10 expressing $CD8^+$ αβ T cells with a reduced cytotoxic phenotype (21). Genetic ablation of B cells also reduces tumor formation in the 2-stage model (22), indicating that

multiple lymphocyte lineages can promote squamous tumors. Despite these studies it is not clear if the inflammatory microenvironment caused by RAS activation is entirely tumor cell autonomous or is modulated by specific components of the adaptive immune system, especially in early neoplastic stages, in part because the two stage model itself requires chronic treatment with a potent inflammatory stimulus. Here we have directly examined the effects of the innate and adaptive immune system on RAS-induced inflammation using a doxycycline suppressible bitransgenic mouse model in which human tetO*HRAS*^{G12V} (23) expression is driven by the suprabasally expressed Involucrin promoter of the epidermis linked to the tetracycline transactivator (24). Our results show that the primary inflammatory response to oncogenic RAS expression is dependent on CD8$^+$ but not CD4$^+$ T cells, and the myelocytic infiltrate correlating with maximal papilloma formation is mediated in part through IFN-γ. This work elucidates and supports a previously unappreciated relationship between CD8$^+$ T cells and squamous tumor development that underscores the need to manipulate the immune microenvironment in order to achieve successful therapeutic intervention.

3.3 Results

CD8$^+$ T cells drive skin tumor development and enhance intra-tumoral inflammation

To determine the effects of the adaptive immune system on RAS driven skin tumor formation we switched 7 week-old InvtTA/tetORAS^{G12V} (DT) on a *Rag1*+/+ and *Rag1*-/- background from a maintenance dose of 10 μg/ml doxycycline (dox) that completely suppressed RAS^{G12V} transgene expression to 250 ng/ml dox. Within 10 days focal tumors began to form on dorsal and ventral skin and on tails, lips and feet (Fig. 3-1A). The average maximal tumor burden in DT*Rag1*+/+ mice was 10 papillomas/mouse after approximately 26 days, but on a

Rag1-/- background tumor onset was delayed and total tumor burden was attenuated to 2 tumors/mouse (Fig. 3-1A). Tumors that formed in both groups were exophytic papillomatous lesions (Fig. 3-1D). To assess the role of $CD8^+$ T cells during Ras-induced tumorigenesis, we injected DT mice with the depleting anti-CD8β monoclonal antibody YTS169.4 or corresponding isotype control once weekly throughout the duration of the tumor study. CD8-depleted mice had a similar tumor latency and initial kinetics of tumor formation as the DT*Rag1-/-* mice (Fig. 3-1A), but at later timepoints the total tumor burden in the CD8-depleted group surpassed that of DT*Rag1*-/- mice. Furthermore, although there appeared to be an initial stimulation of tumor formation, transfer of naïve $CD8^+$ T cells into DT*Rag1*-/- mice did not sustain tumor development to that of DT*Rag1+/+* animals (Fig. 3-1A). Tumor cell infiltration of $CD3^+$ lymphocytes in CD8-depleted mice confirmed the presence of remaining T cell subsets as well as the recovery of $CD8^+$ T cells in CD8-repleted DT*Rag1*-/- mice (Fig. 3-1E). Lymph nodes harvested from CD8-repleted mice also revealed significant $CD8^+$ lymphocyte populations (data not shown). The majority of tumor infiltrating $CD8^+$ T cells isolated from pooled DT tumors and $CD8^+$ T cell reconstituted *Rag1-/-* mice expressed both IL-17A and IFN-γ (Fig. 3-1B). Interestingly, the reconstituted $CD8^+$ T cells exhibited increased proportions of IFN-γ^+ and IFN-γ^+/IL-17^+ phenotypes relative to endogenous tumor infiltrating $CD8^+$ T cells in wildtype tumors indicating Tc cells may possess altered proinflammatory activity in the absence of other adaptive immune cells. We compared myeloid cell infiltration in tumors taken at 1 month after RAS induction to determine if this was linked to CD8-dependent tumor formation. As expected there was abundant infiltration of MPO^+ (myeloperoxidase) neutrophils and mast cells (toluidine blue) within the stroma of DT tumors (Fig. 3-1C). However, in the DT*Rag1-/-* and CD8-depleted DT*Rag*1+/+ tumors that formed, the pronounced infiltration of neutrophils and mast

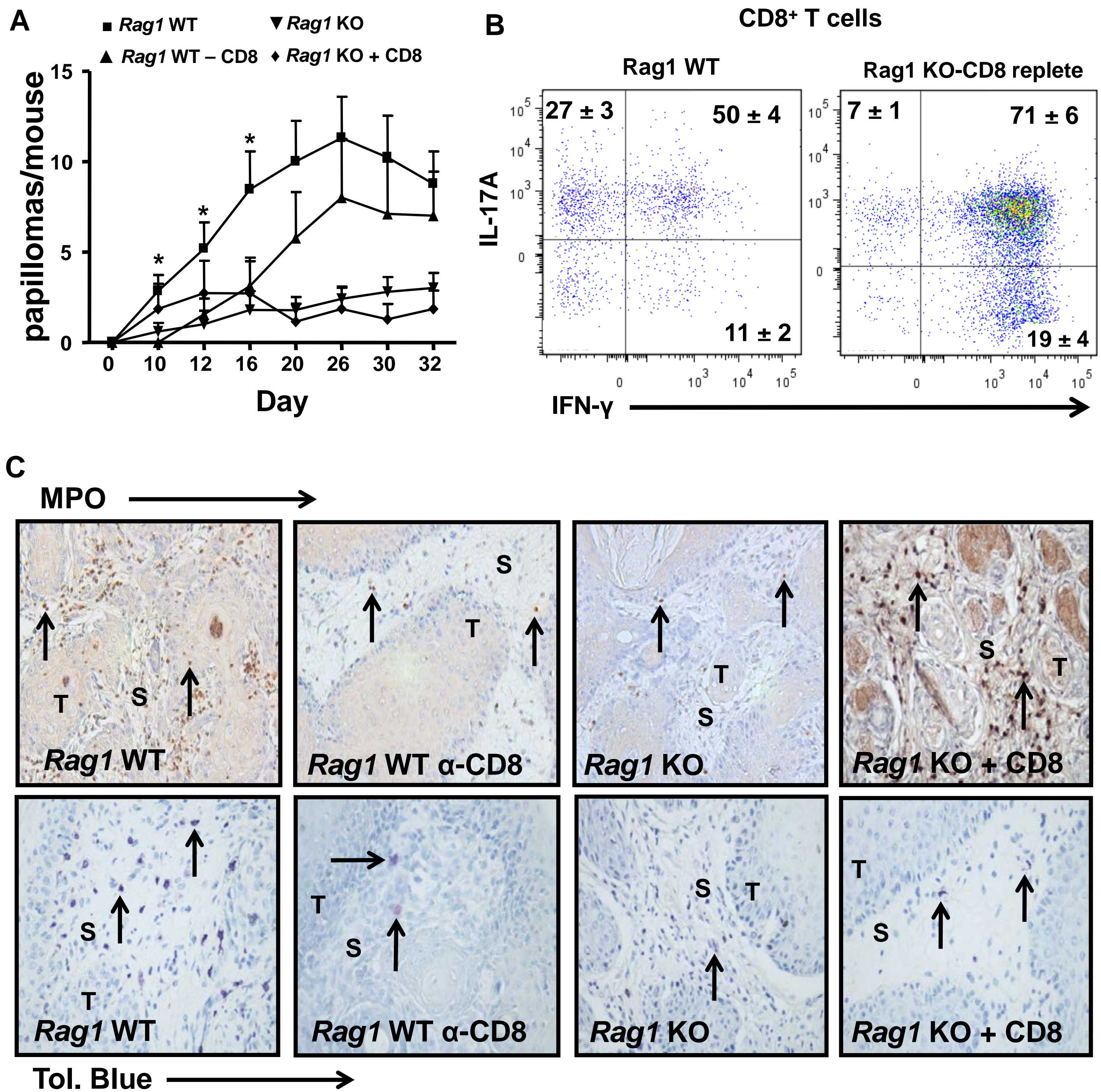


Figure 3-1

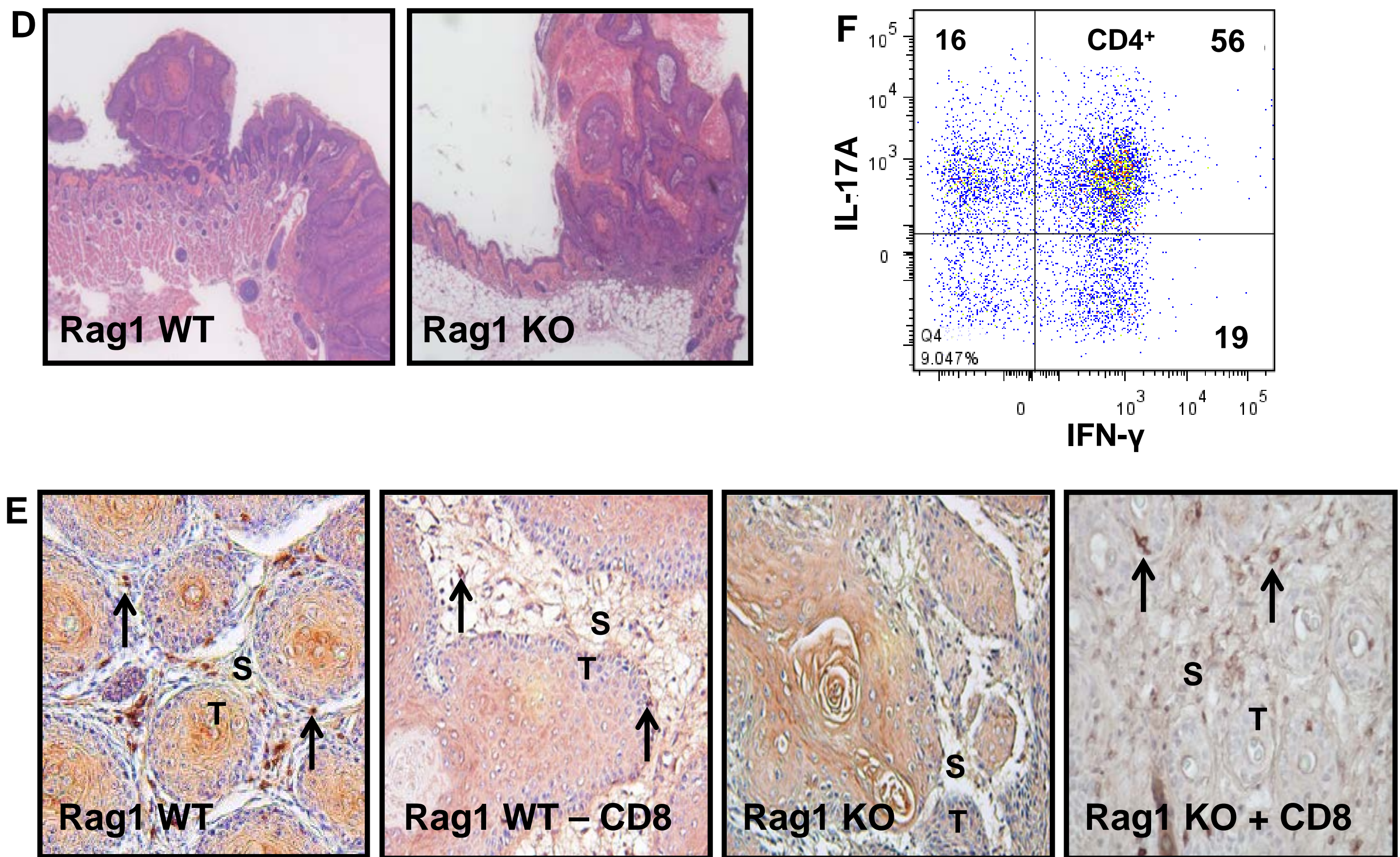
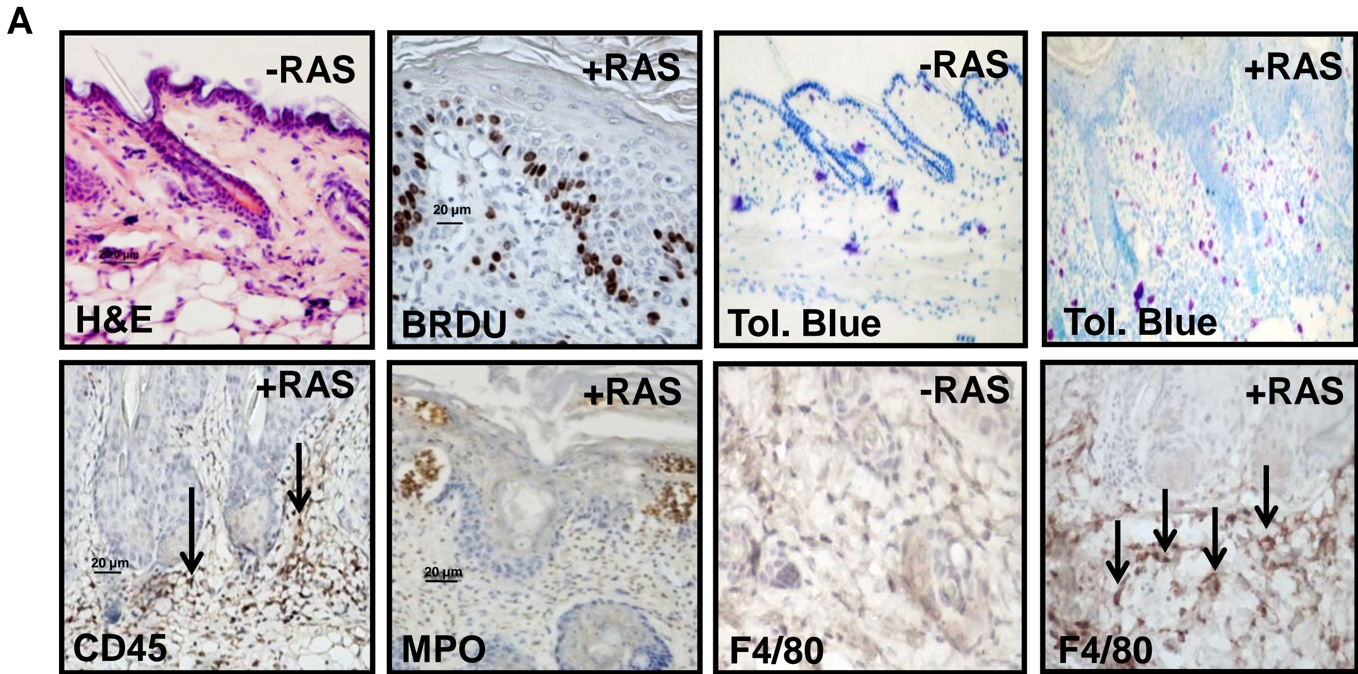


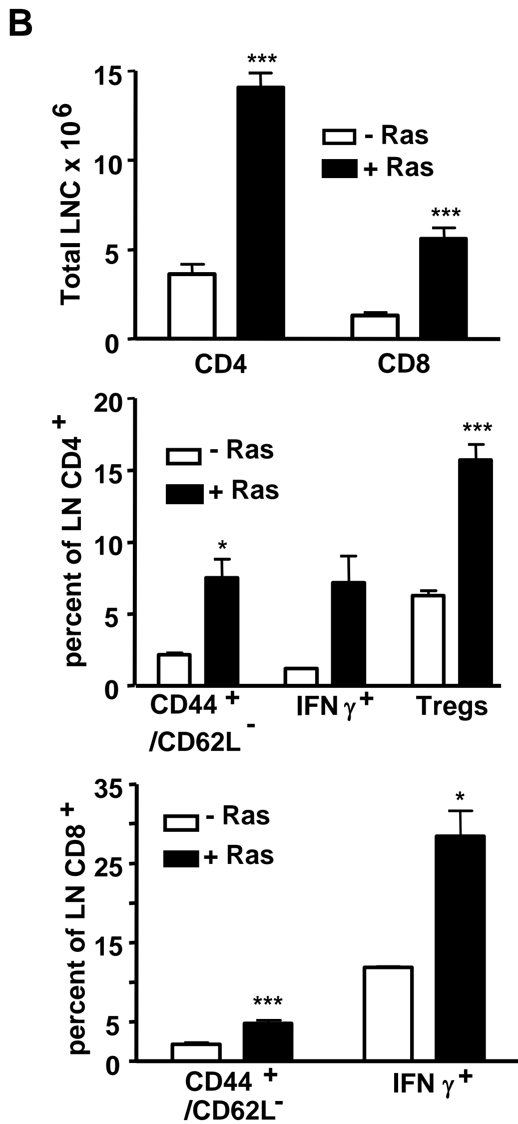
Figure 3-1: CD8⁺ T cells accelerate squamous tumor development: A) Development of skin tumors in DT*Rag1WT (+/+) (■),* CD8-depleted DT*Rag1WT (▲),* DT*Rag1KO (-/-) (▼),* and CD8-repleted DT*Rag1KO (♦)* mice determined at the timepoints indicated by quantitating papilloma development from skin of the entire animal. Mice were sacrificed at day 32 following switch to reduced dox dose. Control mice were untreated or α-IgG treated DT/*Rag1+/+* mice. WT control n = 11, WT α-CD8 n = 9, KO control n = 15, KO + CD8 n = 7. Graph displays results averaged from 2 independent experiments. * = significantly different from CD8 depleted DT and DT*Rag1KO* respectively. B) Intracellular cytokine FACS for IL-17A and IFN-γ on PMA/ionomycin stimulated single cell suspensions isolated from tumors quantitated in A. $CD8^+$ flow profiles were gated on live/$CD45^+$/$TCR\beta^+$/$CD8^+$ populations. Individual tumors were pooled to perform FACS and means and SEM's are calculated from tumors of 5 different mice. C) Representative tumor sections from the indicated groups stained for neutrophils (anti-MPO, left, magnification x20) or mast cells (toluidine blue, right magnification x40). Arrows indicate positively stained cells. D) Representative tumors from DT*Rag1WT and DTRag1KO mice* exhibiting benign papilloma histology, magnification 4X. E) Representative images of tumor sections from indicated groups stained for anti-CD3ε; magnification 40x. Arrows point to $CD3^+$ cells. T = tumor, S = stroma. F) Intracellular cytokine FACS analysis on live/$CD45^+$/$CD4^+$ T cells isolated from end stage tumors of DT/*Rag1WT* mice.

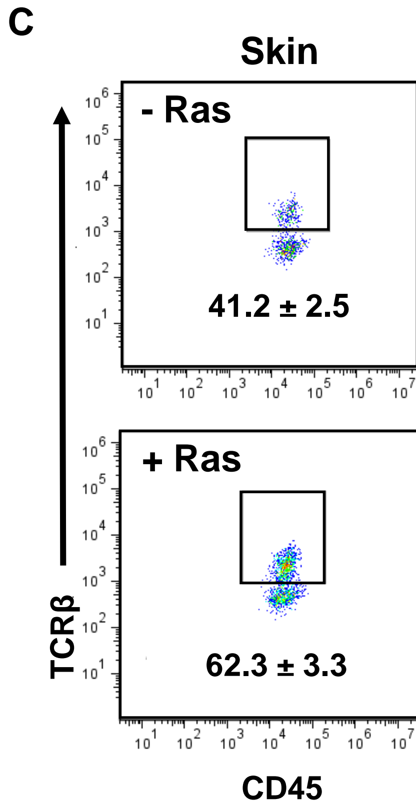
cells was significantly reduced (Fig. 3-1C). Transfer of $CD8^+$ T cells to DT*Rag1-/-* mice restored levels of neutrophil tumor infiltration but did not restore mast cell infiltration (Fig. 3-1C). Together these data indicate that proinflammatory, tumor infiltrating $CD8^+$ T cells are necessary but not sufficient for early phases of tumor outgrowth and this may be linked to their ability to provoke infiltration of tumor promoting myeloid subsets.

Conditional expression of H-RAS^{G12V} in the epidermis activates both innate and adaptive immune response.

To determine how $CD8^+$ T cells could influence early stages of RAS-driven tumor formation we next examined the acute inflammatory responses to transgene expression in the epidermis. Three days after dox removal DT mice had a scruffy hair coat, scaling, and inflamed ears and tails coinciding with expression of the RAS transgene (Fig. 3-3A) with no change in single transgenic (ST) littermates (Fig. 3-2A). Dorsal skin sections examined after 7 days revealed severe epidermal acanthosis, hyperkeratosis, hyperemia, basal layer hyperproliferation and extensive dermal $CD45^+$ immune infiltrates (Fig. 3-2A) as well as increased cutaneous expression of proinflammatory cytokines and chemokines (Fig. 3-3B). As expected, expression of the hematopoietic cytokines G-CSF and GM-CSF in RAS-expressing skin correlated with a systemic increase in $Gr1^+/CD11b^+$ myelocytes (Fig. 3-3D), and the majority of these cells had a polymorphonuclear morphology (Figure 3-3C). In the skin, there was a significant increase in $Ly6G^+/CD11b^+$ cells (Fig. 3-3E) and this intense infiltrate rendered formation of small to moderately sized microabscesses containing MPO^+ neutrophils within the hyperplastic epidermis (Fig. 3-2A), as well as increased dermal residency of polychromatic mast cells and $F4/80^+$ cells (Fig. 3-2A). Neutrophils isolated from RAS-expressing mice, but not control mice, were







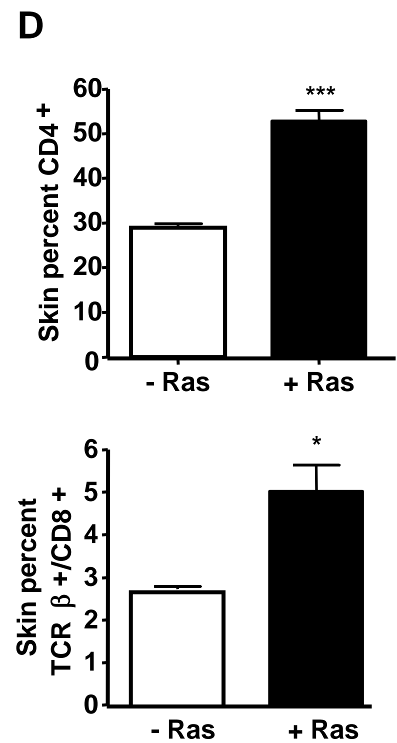


Figure 3-2

Figure 3-2: Conditional expression of *H-RAS*^{G12V} causes acanthosis, hyperkeratosis, hypereamia, and intraepidermal inflammation. A) Representative skin sections from control DT mice on dox or ST mice off dox (-RAS) or DT mice removed from dox (+RAS) for 7 days. Sections were stained with hematoxylin and eosin (H&E), anti-BRDU (BRDU) for cell proliferation, anti-CD45 (CD45) for leukocytes, anti-myeloperoxidase (MPO) for neutrophils, Toluidine blue (Tol. Blue) histochemical stain for mast cells, and anti-F4/80 (F4/80) for macrophages arrows indicate positivity, magnification 20X. B) *Top:* Total lymph node cell number after RAS induction by trypan blue exclusion. *Middle and Bottom*: FACS analysis of inguinal lymph node cells for $CD4^+$ and $CD8^+$ T cell subsets gated on $CD45^+$/$CD3^+$ and the surface markers listed. Intracellular cytokine staining for IFN-γ and the transcription factor FoxP3 for regulatory $CD4^+$ T cell populations was done on at least 7 mice and repeated in 2 experiments independent of each other. C) Representative FACS profiles gated on viable $CD45^+$ cells/lymphocyte SSC demonstrating $TCR\beta^+$ staining from ST (- Ras) and DT (+ Ras) dorsal skin. Results are average from 5 ST and 6 DT mice and repeated independently twice. D) Quantitative FACS on skin single cell suspensions for viable CD45+/lymphocyte SSC/$CD4^+$ and $CD45^+$/$TCR\beta^+$/$CD8^+$ lymphocytes. Means and SEM were collected from the same number of mice as in C.

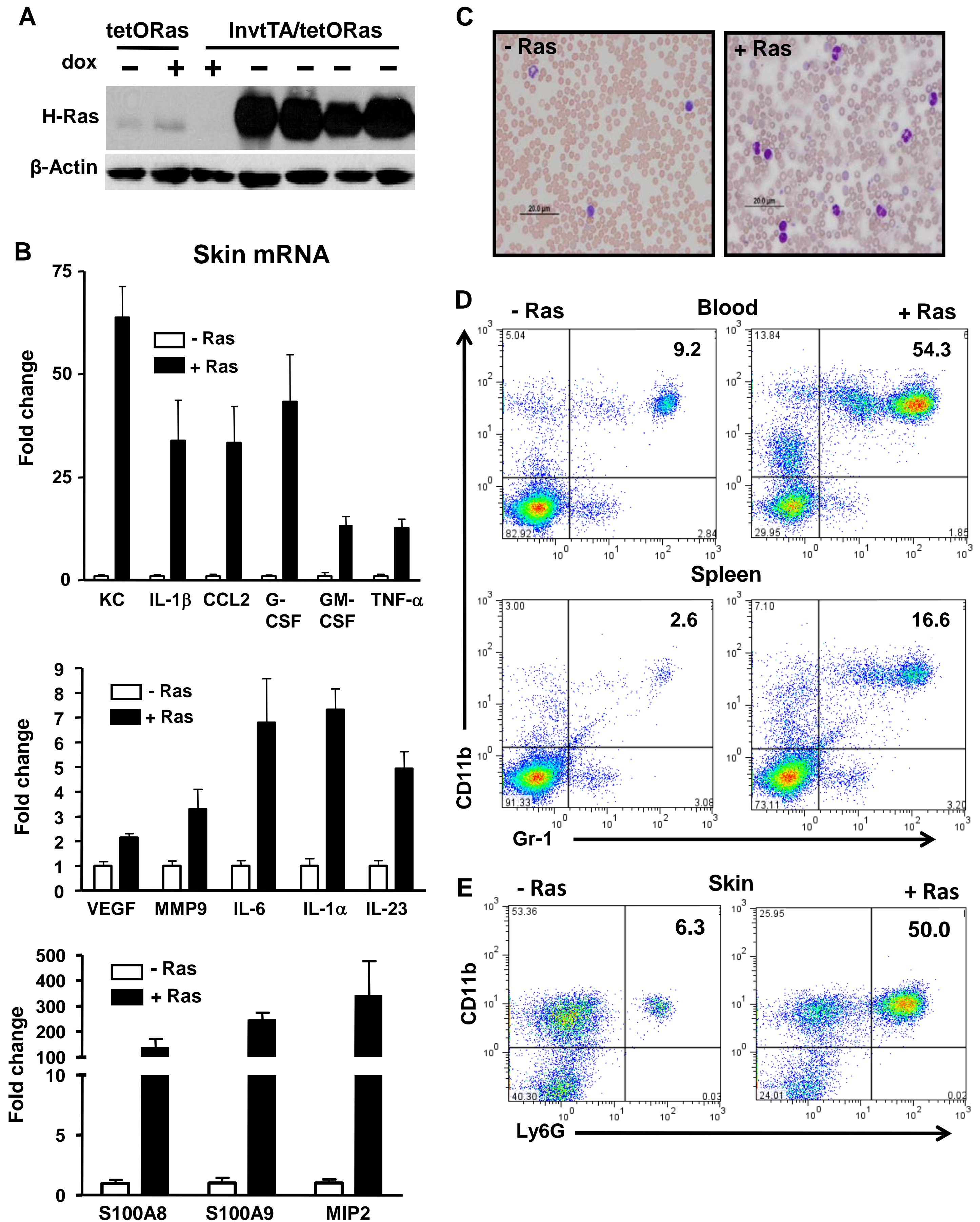


Figure 3-3

Figure 3-3: Epidermal RAS expression causes systemic neutrophilia and cutaneous inflammation through proinflammatory cytokine gene expression. A) Immunoblot on total skin lysates probed with α-H-Ras and α-β-actin. B) QRT-PCR analysis of cDNA synthesized from total skin RNA. Although not indicated, all fold increases were statistically significant below a p value of 0.05. C) Giemsa stains of peripheral blood from ST and DT mice off dox for 7 days. D) FACS profiles from blood (top 2) and spleen (bottom 2) of ST and DT mice off dox for 7 days gated on $CD45^+$ cells depicting CD11b/Gr-1 staining. E) FACS profile from single transgenic ST (- Ras) and DT (+ Ras) dorsal skin gated on live/CD45 cells and plotted for Ly6G/CD11b. Mice were off dox for 7 days. Means and SEM were calculated in blood and spleen from at least 12 and 5 mice respectively.

cytotoxic in a dose dependent manner *in vitro* for primary mouse keratinocytes and the papilloma tumor cell line, SP-1 (Fig. 3-6C).

Inguinal skin draining lymph nodes from DT mice were enlarged and there was a significant increase in total cell number relative to ST littermates indicating activation of an adaptive immune response (Fig. 3-2B). There were significant increases in $CD4^+$ and $CD8^+$ T cell effector memory populations ($CD44^+$/$CD62L^-$), as well as IFN-γ-secreting $CD8^+$ and $CD4^+$ cells and $CD4^+$/$Foxp3^+$ Treg cells (Fig. 3-2B). Skin residency of $TCR\beta^+$ lymphocytes increased from 41% to 62% of total $CD45^+$ lymphocytes in RAS expressing skin, comprised of both $CD4^+$ and $CD8^+$ T cells but we observed undetectable B cell infiltration (Fig. 3-2C, D, and data not shown). Both subsets of $TCR\beta^+$ lymphocytes produced significant amounts of IFN-γ and IL-17A relative to ST littermates (Fig. 3-4A and B), but $TCR\beta^+$/$CD8^+$ lymphocytes were the primary producers of IFN-γ in the skin following RAS induction (Fig. 3-4B). In addition to co-expression of these cytokines, $CD8^+$ T cells that infiltrated the skin in response to RAS also expressed IL-10 (data not shown) similar to the tumor promoting $CD8^+$ phenotype found in DMBA/TPA induced late stage skin papillomas and SCC (25).

Lymphocytes augment RAS-induced epidermal hyperplasia and inflammation.

To determine if lymphocytes contributed to these early RAS-induced inflammatory changes and epidermal hyperproliferation we compared effects of RAS induction on a *Rag1*+/+ and -/- background. Seven days after dox removal cutaneous inflammation and epidermal microabscesses were strikingly attenuated in DT*Rag1*-/- mice (Fig. 3-5A). Neutrophil and mast cell infiltration was reduced from 50% to 30% of $CD11b^+$ cells and ~2.5 fold respectively (Fig. 3-5B) and the level of the CXCR2 ligands KC and MIP2 were diminished ~4 fold in DT*Rag1*-/-

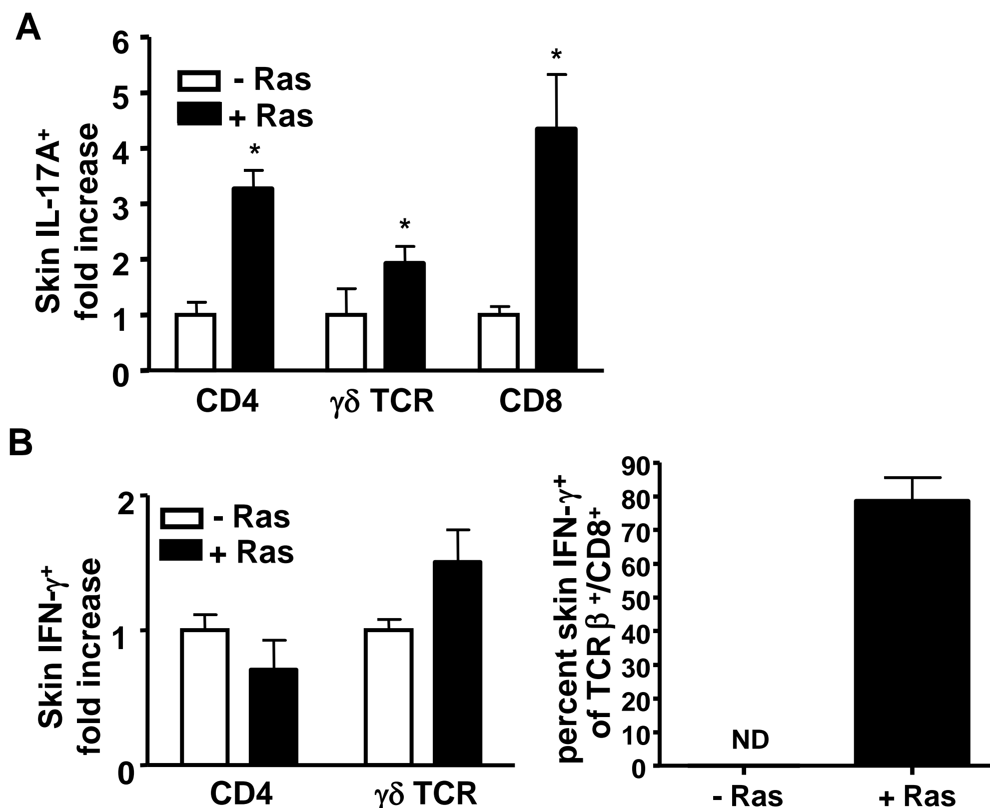


Figure 3-4: RAS increases IL-17A and IFN-γ expressing lymphocytes in skin. A) Intracellular cytokine FACS analysis for IL-17A obtained from ex vivo stimulated skin single cell suspensions as percent positive of viable/CD45$^+$ cells and the lymphocyte marker indicated, and expressed as fold increase relative to control mice (- Ras); means and SEM from at least 6 mice per group. B*) Left panel:* Intracellular cytokine FACS analysis for IFN-γ obtained from *ex vivo* stimulated skin single cell suspensions as percent positive of viable/CD45$^+$ cells, and expressed as fold increase relative to control mice; means and SEM from at least 6 mice per group. *Right panel:* Percent of skin infiltrating CD8$^+$ T cells expressing IFN-γ, measured by intracellular cytokine FACS analysis from *ex vivo* stimulated skin single cell suspensions gated on viable/CD45$^+$/TCRβ$^+$ cells*.* IFN-γ$^+$/CD8$^+$ T cells in control mouse skin were not detectable (ND).

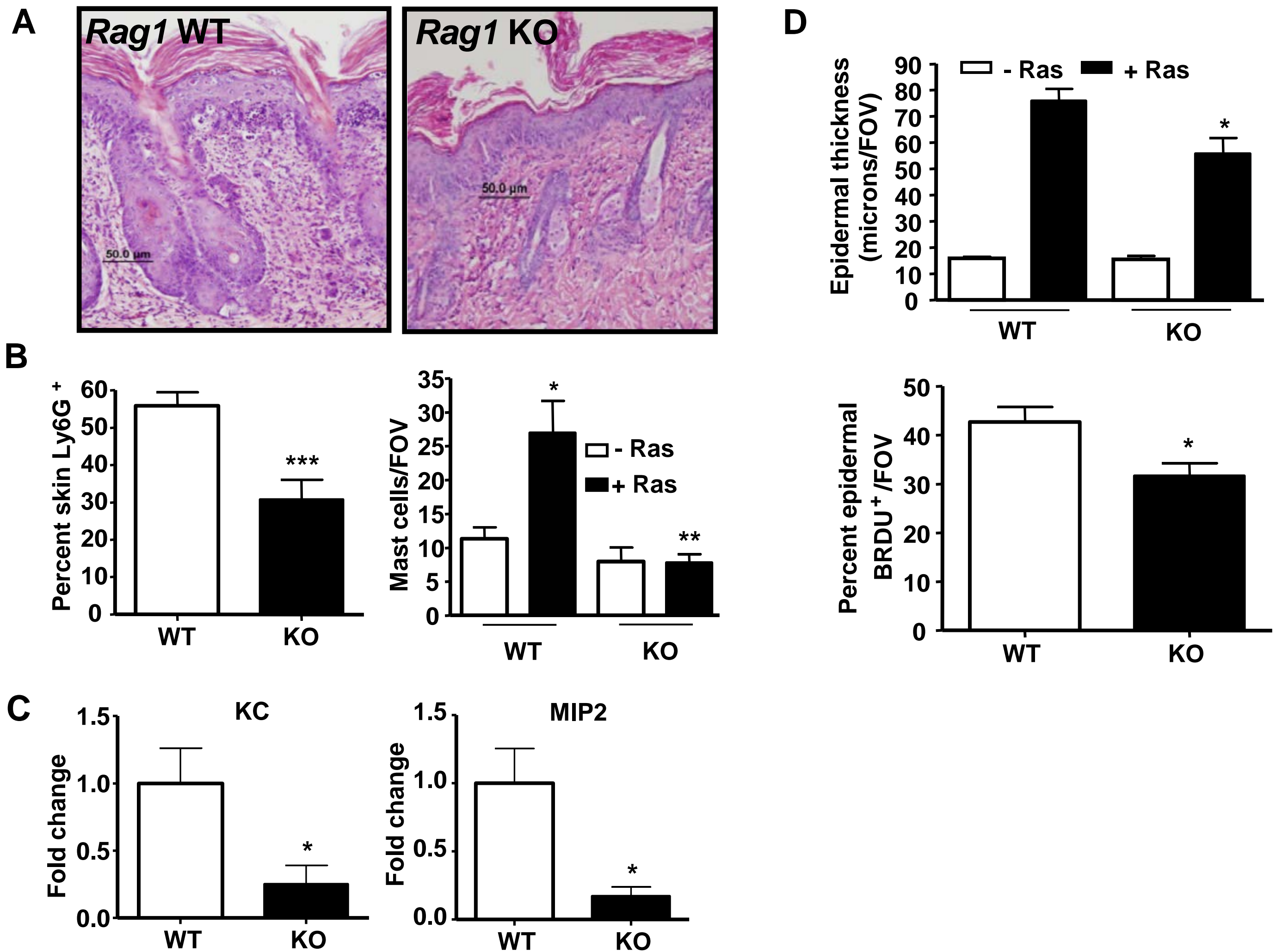


Figure 3-5: Lymphocyte ablation ameliorates RAS-induced epidermal proliferation and intra-epidermal inflammation. A) Representative H&E images (20x) of DT/*Rag1WT* and DT/*Rag1KO* dorsal skin, off dox for 7 days. B) *Top:* Quantitative FACS analysis displaying Ly6G$^+$ neutrophils stained from dorsal skin single cell suspensions of DT /Rag1WT or KO mice gated on the live CD45$^+$/CD11b$^+$ population. *Bottom:* Mast cell counts in dorsal skin sections as determined by differential toluidine blue staining. * signifies relative increase from *Rag1WT* ST and DT mice; ** signifies relative decrease between DT mice. C) *Top:* Quantitation of hyperplasia from H&E stained dorsal skin sections of ST and DT *Rag1* WT and KO mice. Means and SEM were collected from 3 ST mice and at least 8 DT mice. *Bottom:* Basal layer epidermal proliferation analysis was determined by BRDU incorporation between ST and DT*Rag1*WT and KO mice. Statistical significance was calculated between DT groups. BRDU+ cells were counted on 5 random fields of view (FOV) from 5 sections per both WT and KO groups. D) QPCR for KC and MIP-2 amplified from total skin RNA harvested from RAS expressing DT *Rag1KO* and *Rag1WT* mice after 7 days. N = 5/group.

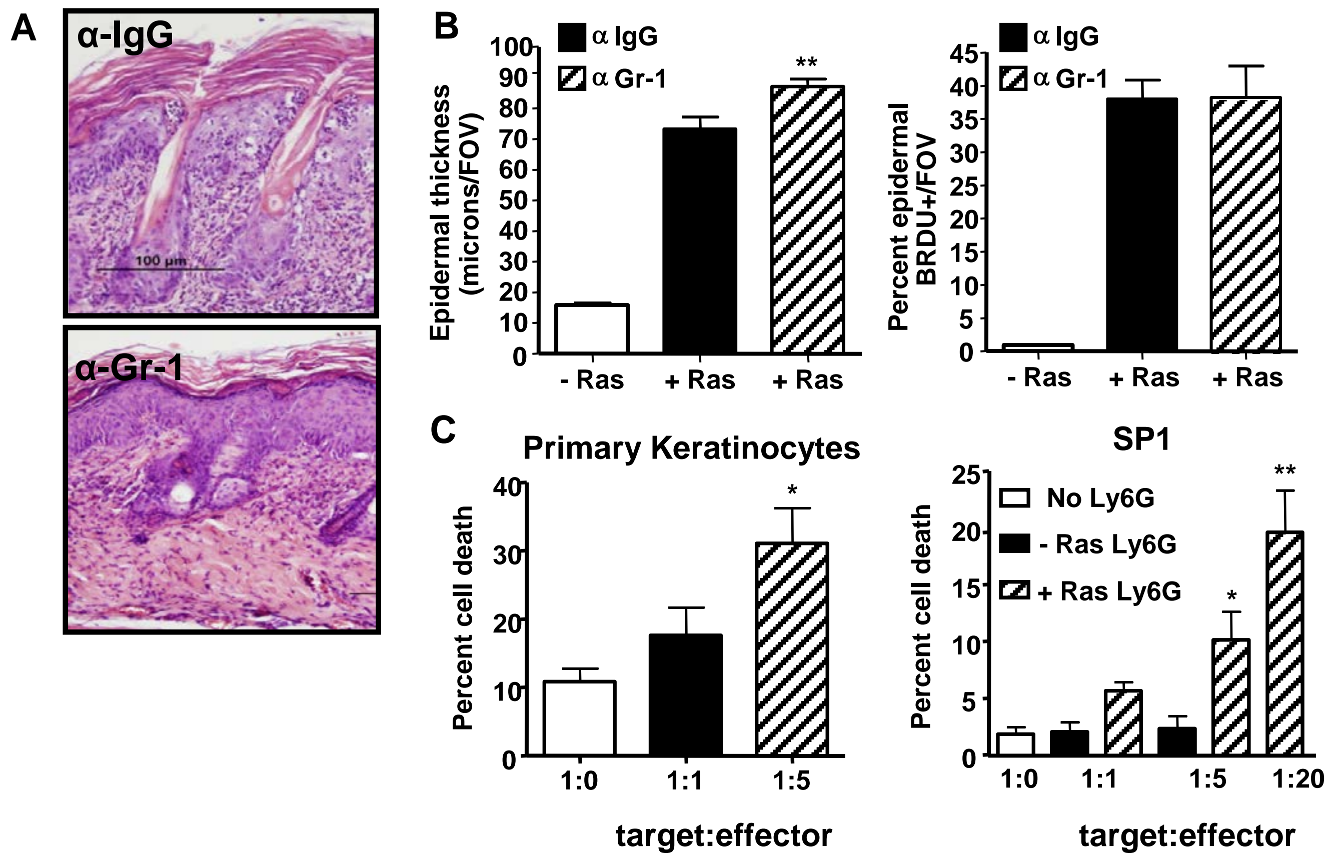


Figure 3-6: Gr-1 depletion of Ras-induced cytotoxic neutrophils does not block epidermal hyperproliferation. A) H&E stained dorsal skin sections from DT*Rag1+/+* mice receiving isotype IgG or α-Gr-1 by intraperitoneal injections every other day for 7 days beginning at day 0. B) Quantitation of epidermal thickness and proliferation of dorsal skin sections from indicated mice. All p values indicate significance between RAS-expressing groups calculated from 10 mice. C) Cell death was measured in co-cultures between either primary keratinocytes (left) or the mouse skin papilloma cell line SP1 (right) and sorted splenic Ly6G+/CD11b+ neutrophils from ST and DT mice off dox for 7 days at the indicated target: effector ratio. Percent cell death was determined as stated in material and methods. Data is presented as the mean and SEM from 3 independent experiments performed in triplicate co-cultures. P values indicate significance of co-cultures with neutrophils from Ras-expressing groups versus non-Ras-expressing groups.

skin tissue (Fig. 3-5C). Significantly, in the absence of lymphocytes, RAS-induced keratinocyte proliferation was reduced as was epidermal thickness (Fig. 3-5D). When Gr-1^{+} cells were depleted concomitantly with RAS induction for 7 days, epidermal microabscess formation was blocked (Fig. 3-6A) and there was a small but significant increase in epidermal hyperplasia but no significant change in keratinocyte proliferation (Fig. 3-6B). Thus, lymphocytes are required for cutaneous myeloid inflammation and maximal epidermal proliferation caused by oncogenic RAS expression, while Gr-1^{+} myelocytic inflammation alone may be immediately dispensable for RAS driven epidermal hyperproliferation.

CD8^{+}, but not CD4^{+} T cells, are necessary for cutaneous inflammation and keratinocyte proliferation.

We next determined the contribution of CD4^{+} and CD8^{+} T cells to the RAS-induced inflammatory response using antibody-mediated depletion immediately prior to RAS induction. Figures 3-7A and B show that in the CD8-depleted but not CD4-depleted mice systemic neutrophilia was reduced 2-fold, cutaneous CD11b^{+}/Ly6G^{+} cells decreased from 47% to 27% and microabscesses were suppressed. Interestingly, in RAS-expressing CD8-depleted mice reductions were also observed in CD4^{+} T cell effector memory cells in skin draining lymph nodes, CD4^{+} skin infiltration and skin Th17 differentiation and IL-17 production by cutaneous γδ T cells (Figs. 3-7C, D). Increases in mast cell residency were also reduced in CD8-depleted but not CD4-depleted DT mice to numbers similar to that found in the DT*Rag1* -/- mice (Fig. 3-7E). Importantly, these losses in myeloid inflammation again correlated distinctly with suppression of RAS-induced epidermal proliferation from 42% to 28% BRDU^{+} keratinocytes/FOV in α-CD8β treated DT groups (Fig. 3-7F). These data suggest that CD8^{+} T cells are necessary to generate

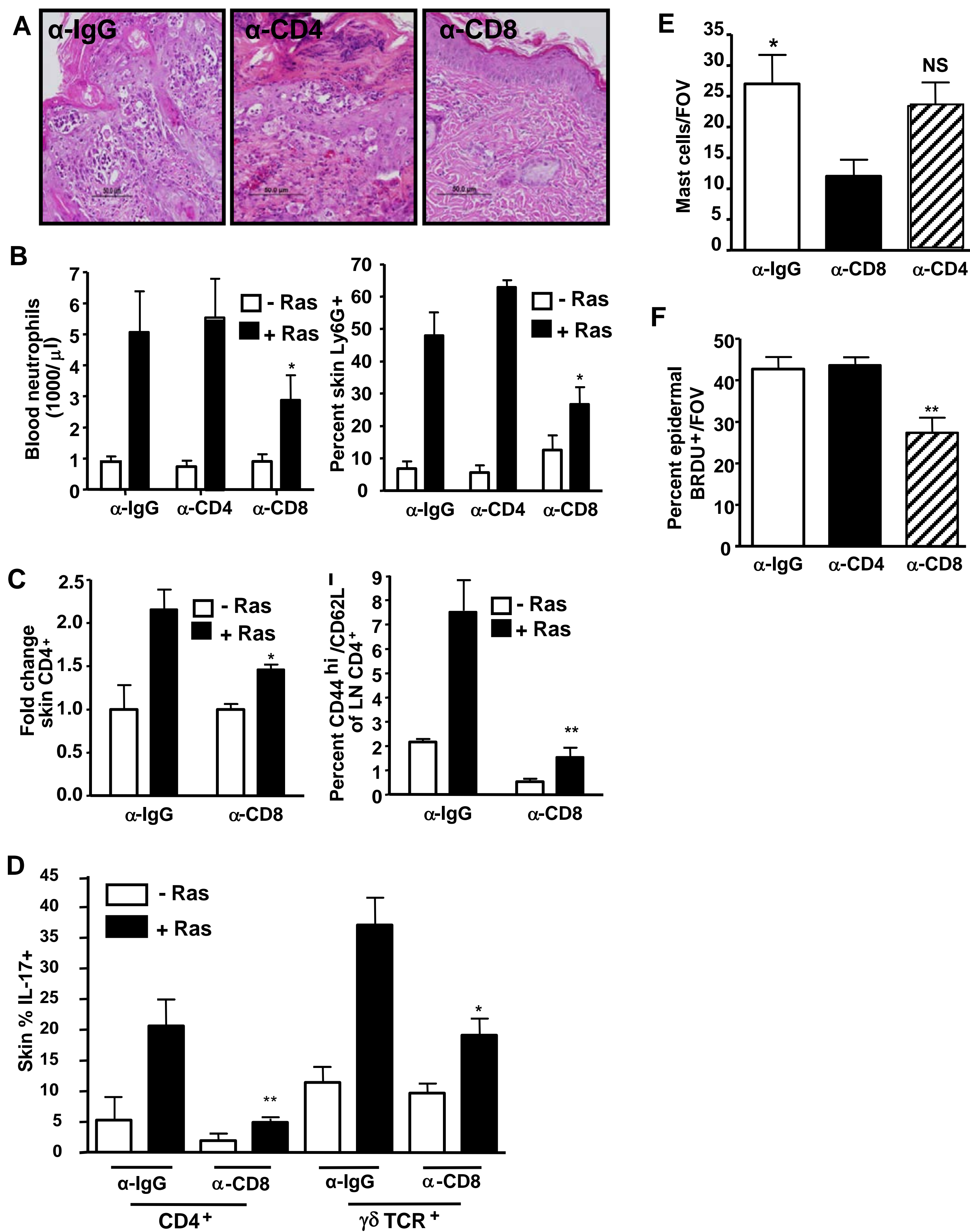


Figure 3-7

Figure 3-7: Depleting CD8+ but not CD4+ T cells diminishes RAS-induced cutaneous inflammation and keratinocyte proliferation. A) Representative H&E images (20x) from dorsal skin sections from α-CD4, α-CD8β or IgG DT*Rag1 +/+ mice.* B) *Left:* Total blood neutrophil counts from mice receiving IP injections of α-CD4, α-CD8β or IgG isotype control antibody removed from dox for 7 days. *Right:* FACS analysis on skin Ly6G+ cells gated on the viable CD45+/CD11b+ population. Data was collected from 7 mice in each treatment group and repeated twice. C) Expression of CD4 activation markers or total CD4+ cells gated on lymphocyte scatter respectively from mice injected with IgG or α-CD8 antibody. Means and SEM were calculated from 5 mice in each group. D) *Ex vivo* stimulated skin single cell suspensions from ST or DT, and IgG or CD8 depleted mice without dox for 7 days. Percent fold increase in IL-17A+ cells is relative to ST groups after gating on a live/CD45+ population and either CD4+ or γδTCR+ subset. E) Mast cells/FOV were quantitated from IgG, a-CD4, or a-CD8 dorsal skin sections on 5 separate sections/group. NS = not significant. F) Epidermal proliferation quantified by anti-BRDU immunohistochemical from dorsal skin sections. Determined from 5 random fields of view (FOV) from 5 sections per group.

the multiple pro-inflammatory components that may contribute to maximal RAS-induced epidermal proliferation.

CD8$^+$ T cells are sufficient to recover neutrophilia, intra-epidermal tissue damage and hyperproliferation.

Despite that CD8$^+$ T cell transfer alone was not sufficient to restore tumor formation in DT*Rag1-/-* mice (Fig. 3-1A) we tested if CD8$^+$ lymphocytes were sufficient to restore the acute phenotypes of RAS-induced myeloid inflammation and keratinocyte proliferation as they may still potentially be important at conditioning a permissive neoplastic microenvironment during hyperplastic stages. To achieve this, DT*Rag1* -/- mice were reconstituted with purified, total CD3$^+$ or CD8$^+$ T cells, and RAS expression induced for 7 days. Skin from both total T cell and CD8$^+$ cell-repleted mice had increased percentages of CD11b$^+$/Ly6G$^+$ infiltrates compared to control mice as well as restoration of systemic neutrophilia to near wild type levels (Fig. 3-8B and E). Furthermore, both lymphocyte reconstitutions yielded intraepidermal chemotaxis of cytotoxic neutrophils (Fig. 3-8A and D). Of note, while depletion of CD8$^+$ T cells decreased dermal mast cell numbers, they were not restored after RAS-induction in CD8$^+$ T cell transferred mice (Fig. 3-8G). This suggests that multiple lymphocytes could be important in RAS-induced mast cell infiltration although further kinetic and adoptive transfer studies are required to determine this. Transfer of both CD3$^+$ and CD8$^+$ T cells also increased RAS-induced epidermal proliferation from 30% to 40% and 28% to 45% BRDU$^+$ keratinocytes/FOV respectively indicating that the CD8-dependent Gr-1$^+$ myeloid inflammation was sufficient to enhance RAS-activated proliferation of keratinocytes (Fig. 3-8E). Significantly, lymphocyte transfer into ST*Rag1*-/- littermates had no effect on inflammation and proliferation excluding the possibility

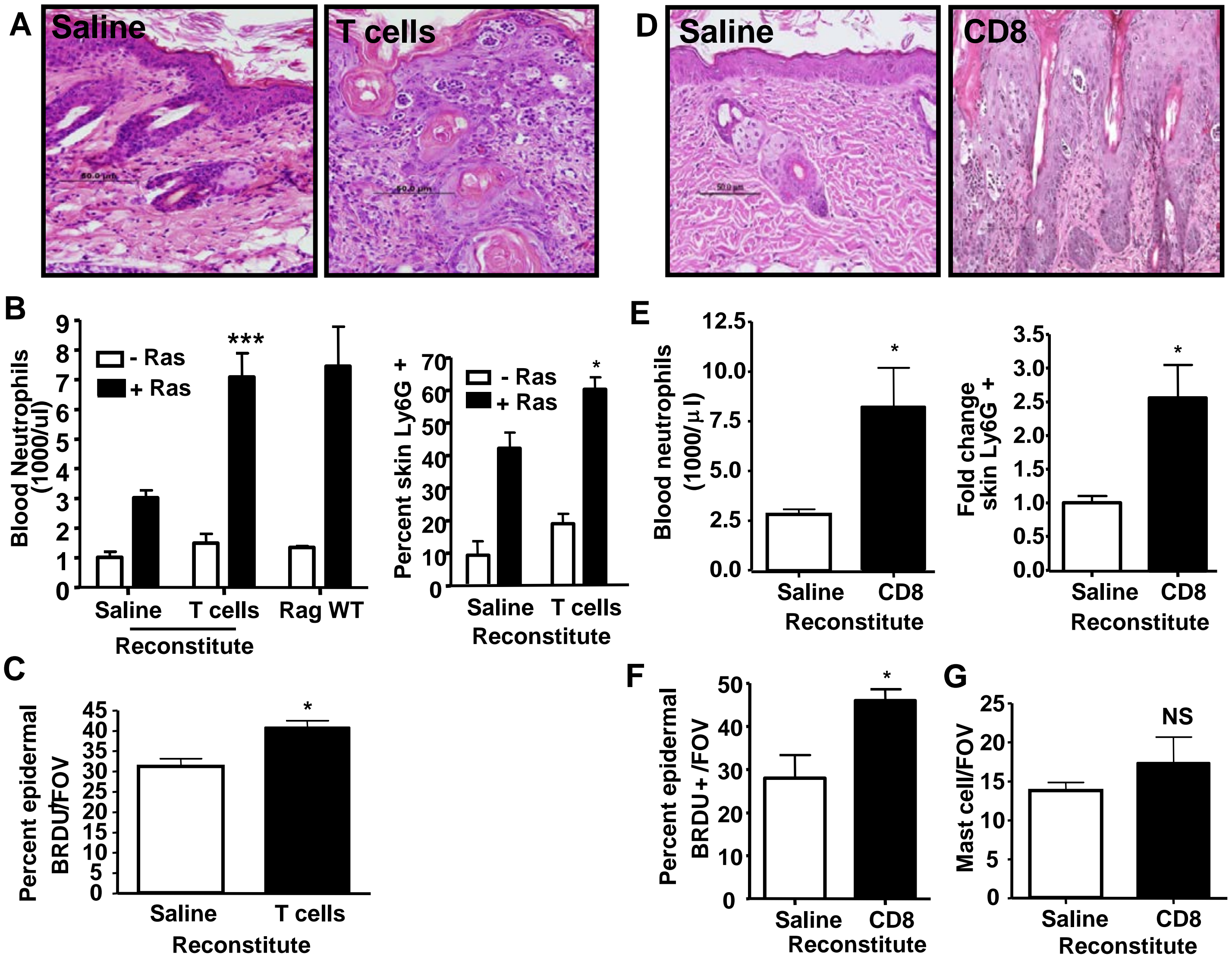


Figure 3-8: Reconstitution with total CD3⁺ and CD8⁺ T cells recovers neutrophilia, cytotoxic Ly6G⁺ skin inflammation and epidermal proliferation in DT/*Rag1 -/-* mice. A) Representative H&E images (20x) from dorsal skin of DT*Rag1KO* mice repleted with saline or total splenic/lymph node CD3+ T cells. B) *Left:* Total neutrophils in whole blood isolated from ST and DT mice either on DT*Rag1WT* or DT/*Rag1KO* backgrounds mock reconstituted or with naïve T cells *Right:* Quantitation of skin Ly6G⁺ neutrophils isolated from DT/*Rag1KO* repletion groups gated on viable CD45⁺/CD11b⁺ cells. Data are from at least 6 mice in each group, repeated twice. C) Epidermal proliferation in dorsal skin of mock (saline) or naïve T cell reconstituted DT*Rag1KO* mice off dox for 7 days. D) Dorsal skin histology from mock or CD8-repleted DT/*Rag1KO* mice expressing transgene for 7 days. E) *Left:* Total neutrophil counts in whole blood of *DTRag1KO* mice reconstituted with saline or naïve splenic and lymph node CD8⁺ cells. *Right:* Fold increase of skin infiltrating Ly6G+ neutrophils in viable CD45⁺/CD11b⁺ cells. CD8-reconstitution was done on 6 (saline) and 7 (CD8) mice from 3 independent experiments. F) Epidermal proliferation in mock or CD8 repleted DT*Rag1KO* mice off dox for 7 days. BRDU⁺ basal keratinocytes were counted on 5 random fields of view (FOV) from 5 sections per group. G) Mast cell numbers determined from toluidine blue staining of indicated repletion groups in DT*Rag1KO* mice, 5 sections per group. NS = not significant.

of CD8 mediated skin autoimmunity (data not shown). Thus, although $CD8^+$ T cells were incapable of recovering total tumor burden, they were sufficient to initiate the pro-proliferative effects of RAS activated inflammation.

IFN-γ but not IL-17 mediates $CD8^+$ T cell driven inflammation

Since IFN-γ and IL-17A expressing $CD8^+$ T cells were present in squamous tumors and inflamed skin of RAS-expressing mice we tested if they were responsible for the CD8-driven inflammatory response. Figure 3-9 shows that neutralization of IFN-γ in $CD8^+$ T cell repleted DT*Rag1-/-* mice completely ablated $Ly6G^+$ skin infiltration (Fig. 3-9A), and strongly reduced epidermal hyperplasia (Fig. 3-9B) and keratinocyte proliferation (Fig. 3-9C). In contrast, IL-17A neutralization had no effect on $Ly6G^+$ skin infiltration or keratinocyte proliferation (data not shown). Together these data suggest that IFN-γ production by $CD8^+$ T cells precedes and drives the acute inflammatory process during hyperplastic stages of RAS-induced neoplasia. In turn, these early events may contribute to skin tumor promotion and development.

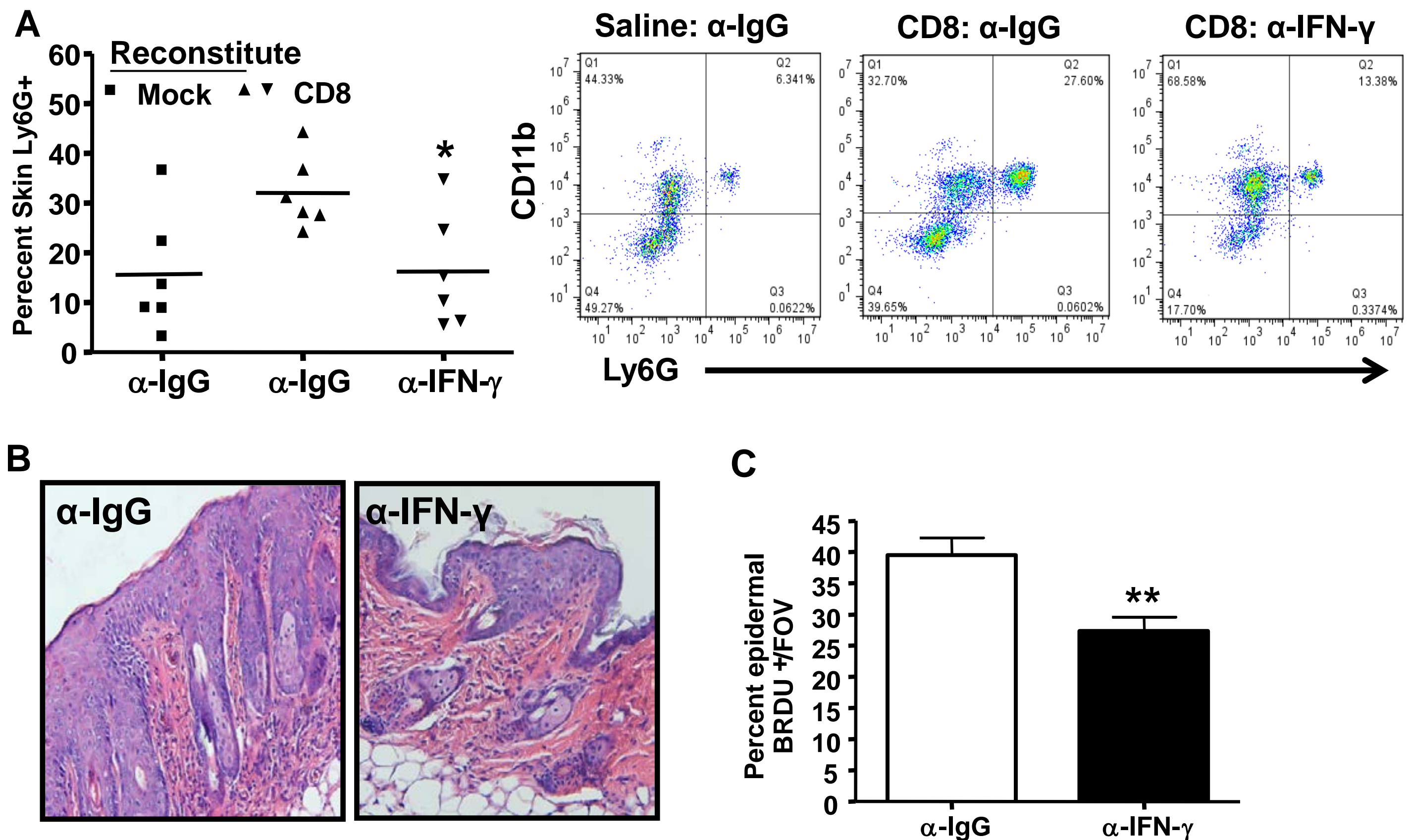


Figure 3-9 IFN-γ neutralization blocks CD8-driven skin inflammation in response to RAS. A) *Left*: Ly6G$^+$/CD11b$^+$ percentages in live/CD45$^+$ skin cells were compiled from DT/Rag1KO mice mock repleted (■) or CD8 repleted (▲▼) receiving either α-IFN-γ neutralizing antibody or relevant isotype control as indicated. *Right*: Representative flow profiles from the histogram on left indicating Ly6G positivity from mock repleted, CD8-repleted isotype control, and CD8-repleted IFN-γ neutralized mice. Data compiled from 6 mice/group. B) Representative histology from CD8 repleted DT*Rag1KO* mice receiving IP antibody treatments. C) Epidermal proliferation determined by α-BRDU immunohistochemistry. Determined from 5 random fields of view (FOV) from 5 sections per group.

3.4 Discussion

While $CD8^+$ T cells are most often viewed as an arm of anti-tumor immunity (26) our results demonstrate that RAS-induced epidermal inflammation and tumor formation is initially driven by skin infiltrating IFNγ/IL-17/IL-10 co-expressing $CD8^+$ T cells. Recent studies on human cancers and autoimmune diseases demonstrate a pro-inflammatory phenotype of $CD8^+$ T cells with cytokine profiles similar to that described here, including $IL\text{-}17^+/CD8^+$ T cells (Tc17) that correlated with poor clinical prognosis in HCC (27;28), tumor promoting $CD8^+$ T cells generated by high TPA doses in the 2-stage skin carcinogenesis model (29) and Tc17, but not Th17, cells that significantly increase in human psoriatic skin lesions (14). Although numerous clinical and experimental studies implicate Th17 cells as critical mediators in the pathogenesis of inflammatory skin disorders (30-33), and we observed increased $CD4^+$ activation and infiltration of Th17 cells into RAS expressing skin, depletion of $CD8^+$ but not $CD4^+$ T cells reduced RAS-induced cutaneous inflammation. However, we find that neutralization of IFN-γ, but not IL-17A, blocked the ability of transferred $CD8^+$ T cells to drive inflammation in response to RAS, indicating that IFN-γ is the primary driver of cutaneous inflammation in this model of RAS-driven squamous cancer. We cannot definitively exclude the possibility that other IL-17 isoforms may be contributing to acute skin inflammation but several other studies support our claims. Elevated serum IFN-γ has been linked to pathological severity in several skin inflammatory diseases (34-36), and high expression levels of IFN-γ is coincident with promotion of chemical carcinogenesis (37). Interestingly, neutralization of IFN-γ or *IFNGR1* deficiency suppressed papilloma formation in the 2-stage model in part through reduced IL-17 expression and Th17 frequencies (38). Thus the apparent lack of effect of IL-17A neutralization we observe may reflect the initial stages of the inflammatory response that are dependent on IFN-γ while

later stages of tumor outgrowth may also require IL-17. In support of this hypothesis, skin tumor burdens on IL-17 null mice are greatly attenuated during chemical carcinogenesis (39) and we also observed copious amounts of IL-17 secreting CD4$^+$ T cells that infiltrated *Rag1*WT and CD8 depleted *Rag1*WT tumors (Fig. 3-1F). While CD8$^+$ T cells are the likely source of IFN-γ for promotion of epidermal squamous tumors, IFN-γ from skin infiltrating macrophages also promote pre-malignant inflammatory activity in a mouse model of melanomagenesis (40). Thus, depending on the tumor context IFN-γ produced by different cell types may have similar tumor promoting roles.

Depletion of CD8$^+$ T cells reduced Th17 and γδ-17 tissue residency, total CD4$^+$ skin infiltration and tumor formation but CD8 transfer into DT*Rag1-/-* mice did not recover tumor formation to wildtype levels. These results suggest that Th-17 and γδ-17 cells may participate in tumor promotion but require a CD8$^+$ T cell trigger. Conversely, depleting CD4$^+$ T cells enhanced CD8$^+$ T cell activation (data not shown), and adoptively transferred intratumoral CD8$^+$ T cells produced higher IFN-γ and IL-17 amounts in the absence of other lymphocytes (Fig. 3-1B) suggesting an even more dynamic CD4$^+$/CD8$^+$ cellular interaction in the skin. The inability to reduce epidermal proliferation by depletion of Gr-1$^+$ cells alone in DT*Rag1+/+* mice also contrasted with the recovery of acute epidermal proliferation and Gr-1 inflammation in CD8-repleted DT*Rag1-/-* mice. This suggests that Gr-1$^+$ cellular inflammation may indeed be pro-proliferative but that other non-Gr-1$^+$ myeloid cells that increase following RAS induction are also key contributors to cutaneous inflammation and are augmenting epidermal proliferation and tumor formation. Supporting this concept, Gr-1$^+$ cells have been shown to exhibit various pro-tumorigenic properties in multiple tumor models (41-43). Given that squamous tumor formation in B cell null mice was only partially reduced compared to *Rag2 -/-* mice, and B cells isolated

from DMBA/TPA treated mice and transferred into *Rag2 -/-* mice only partially recovered wild type tumor numbers (44) these results strongly suggest that both B cells and $CD8^+$ T cells are required either independently or interdependently for maximal inflammatory responses that drive squamous tumor development. The link between B cells and mast cell infiltration in squamous cancer (45;46) suggests that in this Ras-driven tumor model B cell mediated mast cell skin infiltration may be the missing myeloid component that is critical for collaboration with $CD8^+$ T cells for tumor development although further studies will be needed to confirm this. Indeed, $CD8^+$ T cells alone were incapable of recovering acute mast cell infiltration (Fig. 3-8G), consistent with the requirement of IgG production by B cells to stimulate chemotaxis and activation of non-Gr-1^+ myelocytes into pre-malignant tissue (47). Differences between these models and that described here could reflect both tissue compartment of oncogenic expression and engagement of multiple downstream effectors by activated RAS, such as JNK and p38 MAPK signaling, which previous studies have shown can lead to qualitatively distinct skin inflammatory responses (48;49).

Activating mutations in RAS occur in approximately 30 percent of all human cancers (50). K-RAS mutations can cause severe chronic inflammation that potentiates neoplastic progression (51;52), and in some cases this has been directly linked to expression of specific proinflammatory cytokines (53-55). Although tumor promoting roles of $CD4^+$ T cells and B cells have been demonstrated, our work shows for the first time that RAS-induced inflammation and squamous tumor development also requires $CD8^+$ T cells through the action of IFN-γ on infiltrating inflammatory myeloid cells. These results highlight the potential of targeting pro-inflammatory $CD8^+$ T cells for cancer prevention or as adjuvant cancer therapy to suppress tumor promoting inflammation.

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Chapter 4: B cells are required for immunosuppressive activity in Ly6C^{hi} monocytes during inflammation initiated by epidermally restricted *H-RAS* expression

4.1 Abstract

B lymphocytes are critical contributors to immunological activation and prevention of infectious disease dissemination. More recently human and mouse studies have identified a role for B cells in promoting squamous cancer development, most notably in a transgenic model of incipient squamous neoplasia of the skin and the classical 2-stage skin carcinogenesis model. To further elucidate the pro-tumorigenic inflammatory roles of B cells, we created a doxycycline-inducible bi-transgenic mouse model in which human Harvey (H)-RAS^{G12V} expression is restricted to basal layer epithelia by a keratin 14 (K14) promoter-driven reverse tetracycline transactivor (rTA). Double transgenic (DT) K14rTA/tetORas^{G12V} mice given doxycycline (dox) rapidly develop systemic and cutaneous inflammation that includes expansion of a specific Ly6C^{hi}/Ly6G^{neg}/CD11b^{+} monocytic population that exhibits nitric oxide (NO) dependent immunosuppression *in vitro* and *in vivo*. When DT mice were crossed onto a *RAG1* null background, immunosuppressive activity in the same population was completely ablated. Despite significant tissue infiltration of TCRβ^{+} T cells but not B cells, depletion of CD4^{+}, CD8^{+} or CD4^{+} and CD8^{+} T cells did not affect the immunosuppressive phenotype in bitransgenic *Rag1* +/+ mice. In contrast, reconstitution with B cells alone into bitransgenic *Rag1* null mice was sufficient to recover monocytic mediated immunosuppression of polyclonal stimulated splenocytes. Depletion of B cells was ineffective at alleviating the immunosuppressive functionality of Ly6C^{hi} monocytes due to persistence of IL-10 producing Breg cells resistant to α-CD20 therapy. Taken together, these results demonstrate for the first time a requirement of B cells to directly stimulate immunosuppressive activity in a monomyelocytic subpopulation during an inflammatory process. This novel information may be useful for clinicians seeking to

combine chemotherapy with adjuvant therapy targeted at ameliorating tumor promoting inflammation.

4.2 Introduction

B lymphocytes are the sole producers of immunoglobulin in the human body. Through the secretion of these glycoproteins they serve are important modifiers of adaptive humoral immunity and can secrete numerous cytokines that influence innate immune cells and tailor specific immune responses to infection. The roles of B cells in sustaining chronic inflammation during autoimmunity is now well appreciated (1). Recently, the role of B lymphocytes during tumor promotion and carcinogenesis has also been elucidated in numerous cancer models. B cell infiltration into cancerous human mammary tissue was increased relative to normal adjacent tissue and could be decreased following chemotherapy treatment (2). Neoplastic progression of E6/E7 oncogene driven squamous carcinogenesis was greatly attenuated in B cell null mice (3) and the promotional roles were later demonstrated to involve a mechanism of mast cell and macrophage activation by immune complex/FcγR ligation (4). Moreover, enhanced Th1 and Tc anti-tumor immunity in *IgM-/-* B cell knockout mice led to the rejection and/or slow onset of multiple transplanted tumor grafts (5). A similar model of squamous skin cancer by DMBA/TPA application produced parallel observations in B cell knockout mice where production of IL-10 by B cells was critical to yield the tumor promoting effects of TNF-α (6). In agreement with this, B1 cells ($CD5^+$) co-cultured with macrophages influenced macrophage phenotype through the paracrine effects of IL-10 by downregulating pro-inflammatory TNF-α, IL-1β and CCL3 which ultimately led to permissive B16 xenograft growth (7). Tedder and colleagues demonstrated the unique requirement of $CD5^+/CD1d^{hi}$ Bregs in dampening

monocyte/macrophage phagocytosis of anti-CD20 treated lymphomas providing experimental evidence for Rituximab resistance (8). Furthermore, lymphotoxin from B cell sources induced castration resistance in prostate cancer bearing mice by stimulating IKKα and STAT3 activity in cancer cells, provoking metastasis (9). Cancer patients do develop specific antibody responses (10) but these elevated serum immunoglobulin levels correlates poorly with disease outcome (11). All of these studies point to a putative and disease-potentiating role for B lymphocytes during carcinogenesis of solid and fluid malignancies.

Myeloid derived suppressor cells (MDSC) is a term given to a phenotypically heterogeneous group of bone marrow derived myelocytes that share common immunosuppressive functionality (12). Most MDSC co-express Gr-1/CD11b surface markers and systemically expand during processes of intense or chronic inflammation including cancer development, sepsis, infection, autoimmunity and trauma (13). Nearly every myeloid lineage, depending on pathological context, has been characterized as MDSC (14-16). The acquisition of immunosuppressive activities has been shown to possibly result from cytokine quality where the type or combination of cytokine(s) influences MDSC outcomes (17) or cytokine quantity where the amount of a single cytokine may determine immunosuppressive acquisition (18). In this instance, it was clearly demonstrated that Gr-1/CD11b immature myeloid cells (iMC) expanded during both GM-CSF adjuvant treatments but their effects on tumor development were dichotomous. Results from previous studies indicated that these MDSC phenotypes may represent iMC halted in an undifferentiated state by constitutive cytokine receptor signaling (19). Removing these iMCs from this environment or by blocking cytokine signaling caused MDSC to lose many of their classical phenotypes and complete their intended life cycles (20-22). In vitro induction of MDSC from bone marrow derived c-Kit progenitors required the presence of GM-

CSF in multiple studies (23-25) and hyperactivation of STAT3 is responsible for deregulated dendritic cell differentiation (26;27).

Based on these previous studies and our observations made in a similar model of RAS-driven skin carcinogenesis and acute inflammation, we hypothesized there may exist a pathological role for B cells by influencing the phenotypes of expanded Gr1/CD11b populations. Using a keratin 14 (K14) tetON regulated transgenic mouse, we show for the first time a direct causality between the activities of IL-10 expressing Breg cells and the pre-malignant expansion of Ly6C^{hi}/Ly6G^{-}/CD11b^{+} immunosuppressive monocytic cells responding to expression of an H-RAS^{G12V} oncogene. The findings can be extended to increasing the therapeutic options for the treatment of solid tumors where chronic depression of anti-tumor responses prevents sustained cancer remission.

4.3 Results

K14-RAS expression causes systemic heterogeneous expansion of immature myelocytes

Oncogenic H-RAS^{G12V} expression was restricted to basal layer epithelia and controlled by a K14rTA transgenic mouse (28) crossed to a second tetOH*RAS*^{G12V} (29) mouse. When double transgenic (DT) mice were fed doxycycline chow (1g/kg), transgene induction led to immediate and drastic epidermal hyperplasia and infiltration of CD45^{+} leukocytes (Fig. 4-1A and B). Immunohistochemical and FACS staining of single cell suspensions from skin revealed that many of the tissue resident immune infiltrates also expressed the macrophage marker F4/80 and the Ly6G neutrophil marker (Fig. 4-1A and C). Correlatively, FACS analysis of peripheral blood and spleen revealed systemic expansion of Gr-1/CD11b double positive myeloid cells and upregulation of numerous chemotactic and hematopoietic cytokines that correspond with the

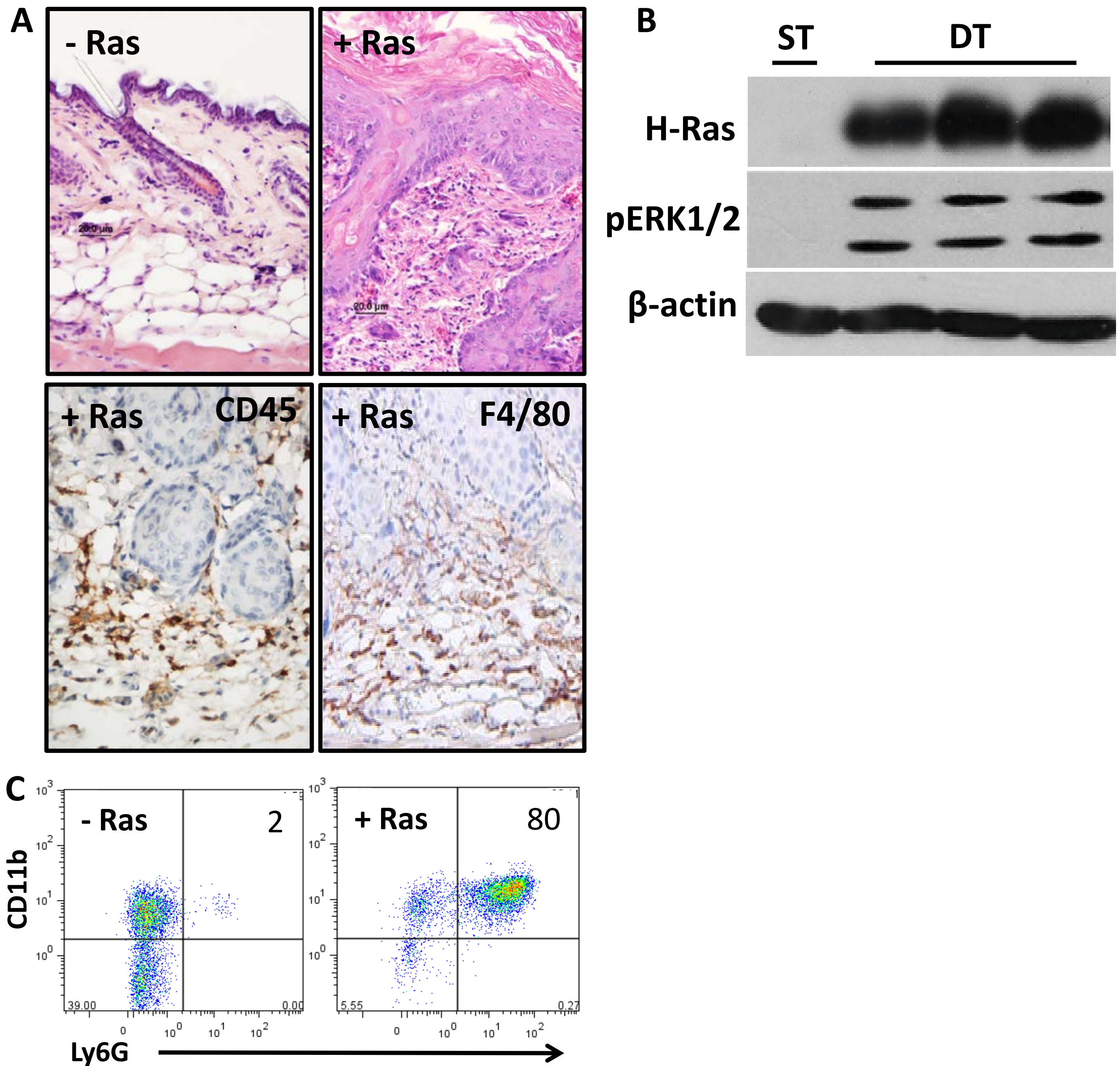

Figure 4-1: **Basal layer epidermal RAS induction provokes acanthosis and infiltration of myeloid-derived leukocytes.** A) *Top*: H&E staining of dorsal skin sections from single transgenic (ST) tetoHRAS^{G12V} (- Ras) or double transgenic (DT) K14rTA/tetoHRAS^{G12V} mice (+ Ras) on doxycycline chow (1g/kg) for 5 days. *Bottom*: Immunohistochemical (IHC) detection of the leukocyte specific marker CD45 (left) or the macrophage/dendritic cell marker F4/80 (right). B) Immunoblot on total skin protein lysates from ST + dox or DT + dox mice probed with α-H-Ras, α-pERK1/2, and α-β-actin as a loading control. C) FACS analysis of total skin single cell suspensions from +/- Ras mice stained with α-CD45/CD11b/Ly6G gated on the live/CD45$^+$ population.

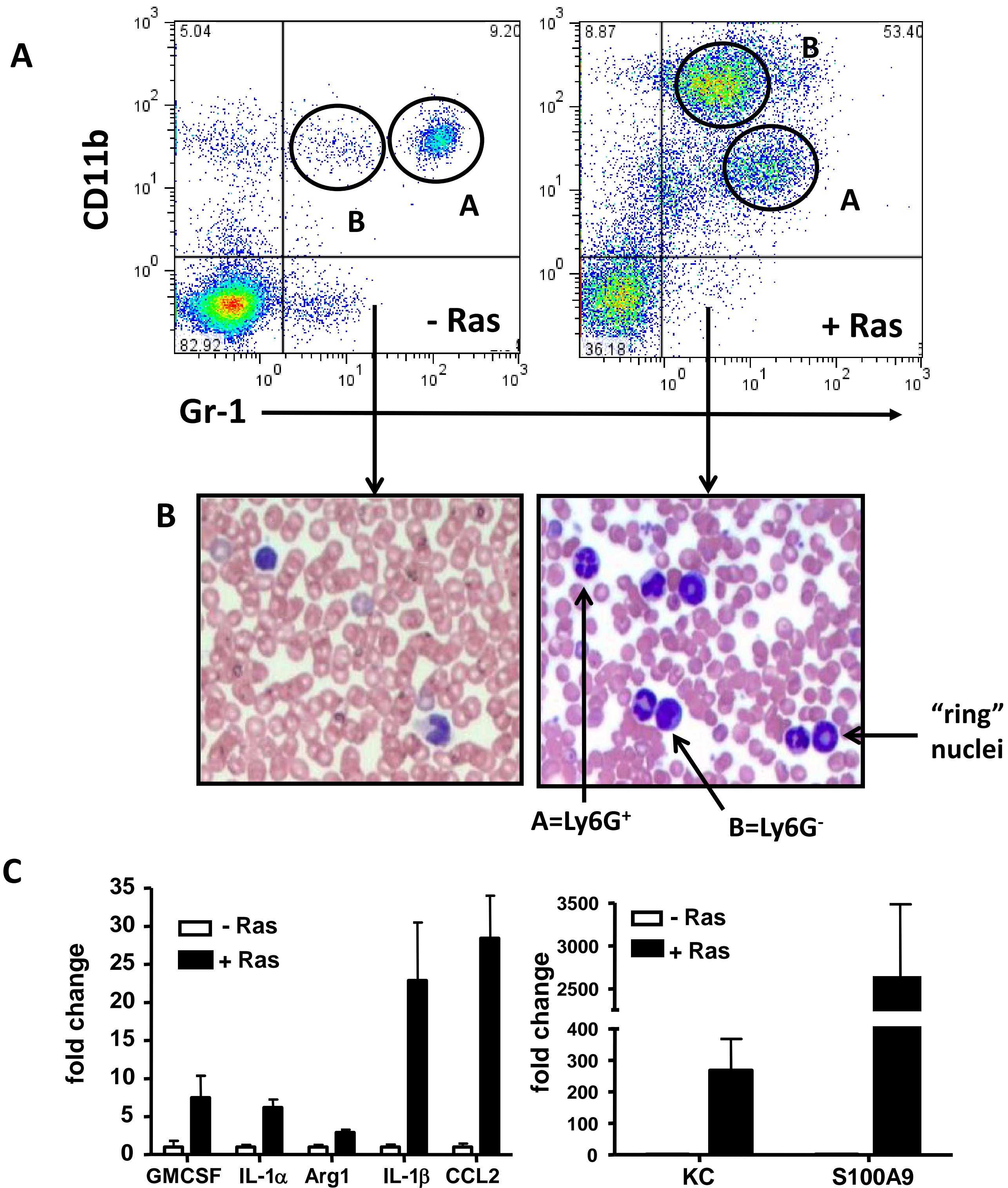

Figure 4-2: **RAS transgene expression causes systemic expansion of morphologically heterogeneous myelocytes.** A) Peripheral blood FACS analysis stained with α-CD45/CD11b/Gr-1 and gated on CD45^{+} cells. Population "A" = Ly6G^{+}; population B = Ly6G^{-}. B) Giemsa stains of blood smears collected from ST or DT mice 4 days on dox chow. Arrows point to the population of myeloid cells indicated. C) mRNA expression from total skin RNA for the genes indicated as measured by q-RT-PCR. All fold changes in + Ras mice are statistically significant relative to - Ras mice which is normalized to 1.

systemic and cutaneous inflammation (Fig. 4-2A and C). The Gr1/CD11b cells could be further characterized based on their Ly6G expression patterns where CD11b^{+}/Ly6G^{+} cells were neutrophilic in morphology and CD11b^{+}/Ly6G^{-} cells displayed mononuclear features (Fig. 4-2B). Interestingly, cells with ring shaped nuclear morphology also expanded systemically indicative of immature myeloid (iMC) morphology (Fig. 4-2B). These ring shaped iMC have been described to arise in both monocytic and granulocytic lineages constituting nearly 50% of bone marrow cells but are nearly absent in the periphery during steady state (30). Expanding iMC with ringed nuclei have also been described in periods of intense inflammation during infection (31), shock (32), trauma (33), myeloproliferative diseases (34) and malignancy (35).

Ly6C^{hi}/Ly6G^{-}/CD11b^{+} monocytes from RAS expressing mice inhibit T cell proliferation through nitric oxide production

MDSC populations, depending on tumor type, have been described as either arising from the granulocytic lineage or monocytic lineage where nuclear morphology would mirror that of their respective leukocytes (36). Based on the rapid systemic expansion of both Ly6G^{-} and Ly6G^{+} myeloid cells with immature morphology, we then used these markers to sort and collect these populations to determine their relative immunosuppressive properties and any defining characteristics. Cytospins of the purified cell preps revealed both subsets contained iMC with ring shaped nuclei suggesting global myeloid differentiation defects (Fig 4-3A). After 4 days of dox administration, the purified Ly6G^{-} population isolated from spleens of K14Ras mice greatly inhibited proliferation of in vitro anti-CD3/CD28 stimulated naïve splenocytes (Fig. 4-3B). There was no T cell specificity in inhibiting proliferation as both CD4^{+} and CD8^{+} subset proliferation decreased equally (Fig. 4-3B). Conversely, the Ly6G^{+} neutrophilic population was

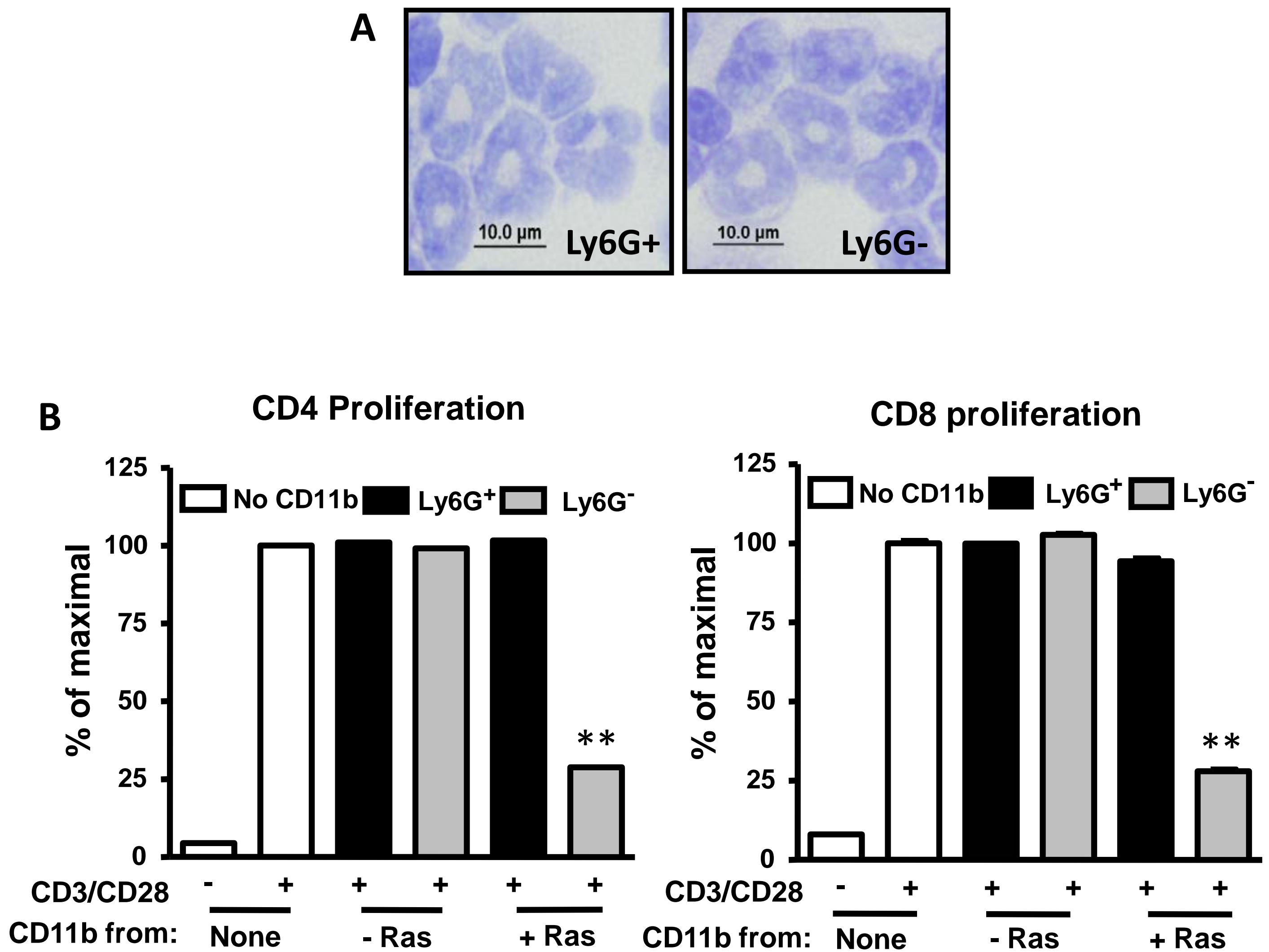

Figure 4-3: **Ly6G⁻/CD11b⁺ cells isolated from RAS expressing mice suppress CD3/CD28 stimulated proliferation of T cells.** A) Cytospins of FACS purified Ly6G+/- fractions demonstrating nuclear heterogeneity and ring morphology in both fractions. B) FACS sorted CD11b⁺ Ly6⁺/⁻ cells from spleens of +/- Ras mice on dox for 4 days were co-cultured with CFSE labeled syngeneic splenocytes from non-transgenic mice in α-CD3/CD28 coated wells. Following 72 hours in culture, cells were isolated and stained for CD4 (left) and CD8 (right) and measured for dilution of CFSE fluorescence by flow cytometry. Data is expressed as a percent relative to the maximal proliferation achieved of CFSE labeled splenocytes cultured alone. ** = $p < 0.01$.

completely incapable of inhibiting T cell proliferation. Ly6G^{-} cells isolated from ST littermates on dox and DT littermates off dox contained no ability to suppress T cell proliferation confirming the requirement of RAS oncogene expression to mediate these effects (Fig. 4-3B).

We further characterized the Ly6G^{-} population using the Ly6C marker as the Gr-1 molecule consists of both Ly6G and Ly6C molecules and Ly6C has been shown to be expressed on multiple leukocytes. This FACS gating strategy revealed that Ly6C^{+}/Ly6G^{-}/CD11b^{+} cells actually contained two distinct Ly6C expression patterns, Hi and Low (Fig. 4-4A). These myeloid subsets were confirmed to be primarily eosinophilic (lo) and monocytic (hi) in nature although iMC nuclear morphology was again present in both fractions (Fig. 4-4B). Additionally, sorted Ly6C^{hi} but not Ly6C^{lo} cells were capable of suppressing polyclonal stimulated T cells in vitro (Fig. 4-4C). This inhibition took place in a dose dependent manner and could be reversed by the addition of L-NMMA, a pseudo iNOS substrate, but not the pseudo Arginase substrate Nor-NOHA (Fig. 4-4D). As iNOS and Arginase have been shown to be the primary mechanisms behind MDSC mediated immunosuppression, these results suggest that these expanding Ly6C^{hi}/Ly6G^{-}/CD11b^{+} myelocytes fit many of the characteristics of inflammatory monocytes that have acquired an immunoregulatory MDSC phenotype through the production of nitric oxide intermediates (14).

Levels of RAS oncogene dictate the acquisition of an immunosuppressive phenotype

Much speculation has arisen as to the nature and cause of existing MDSC populations within a pro-inflammatory environment. One postulate is that the intensity of inflammation may determine the phenotypic outcome of responding innate cells where cytokine quantity may overrule the complementary actions of multiple secreted products in a given tissue

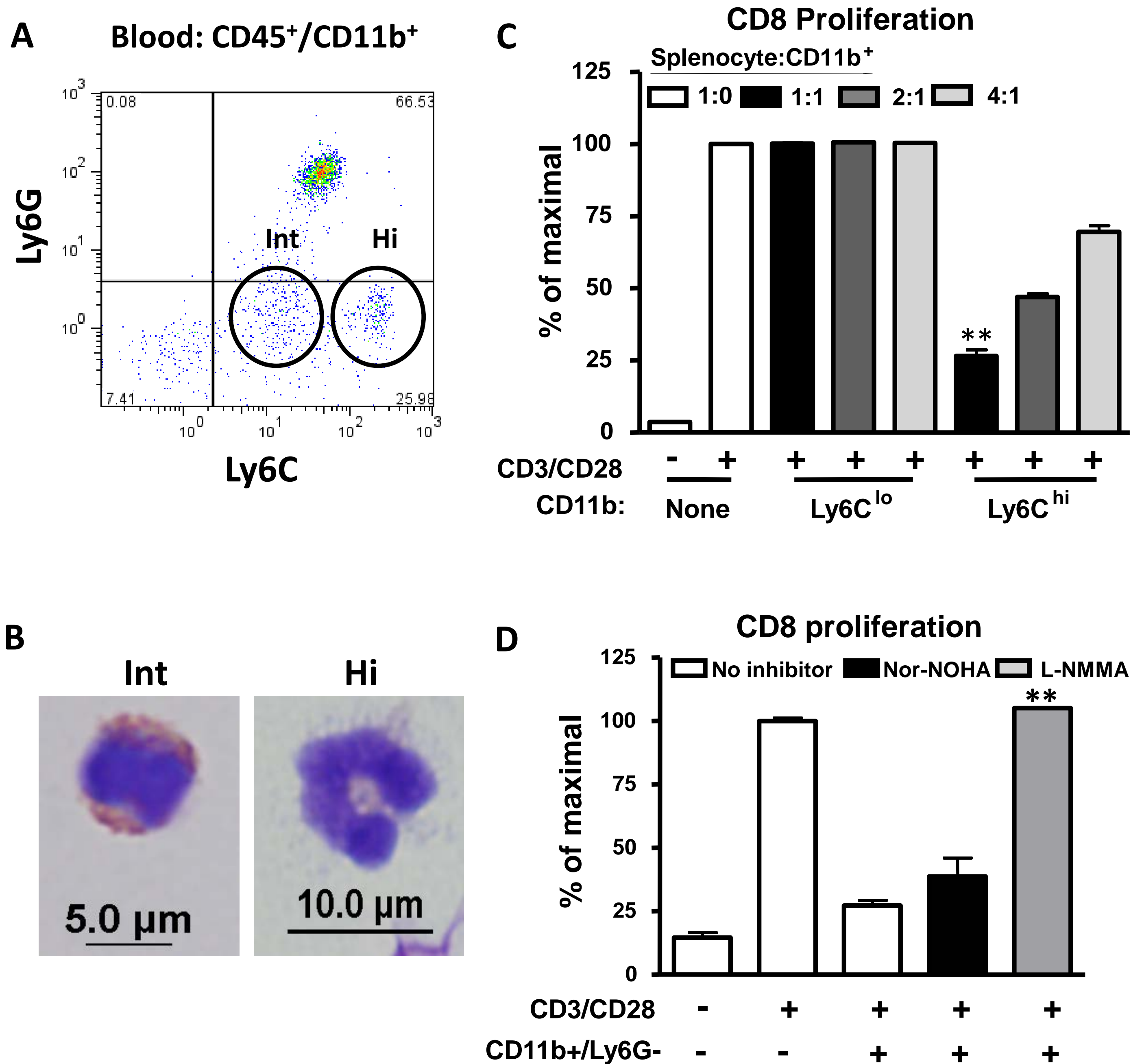

Figure 4-4: RAS induced MDSC are a population of Ly6C^{hi}/Ly6G^{-}/CD11b^{+} inflammatory monocytes that suppress T cell proliferation by iNOS. A) Blood FACS profile gated on CD45/CD11b cells and displayed as a quadrant gate for Ly6G and Ly6C expression. B) Giemsa stain on Ly6C^{hi} and Ly6C^{lo} sorted cells displaying eosinophilic (left) and monocytic (right) morphological features. C) Suppression assay was carried out as before co-cultured with Ly6C^{lo} and Ly6C^{hi} splenic myeloid populations. P value is relative to Ly6C^{lo} group. D) T cell suppression assay co-cultures were cultured Ly6C^{hi} cells and either Nor-NOHA (500mM), L-NMMA (50mM) or no inhibitor. Significance is relative to the no-inhibitor treatment alone.

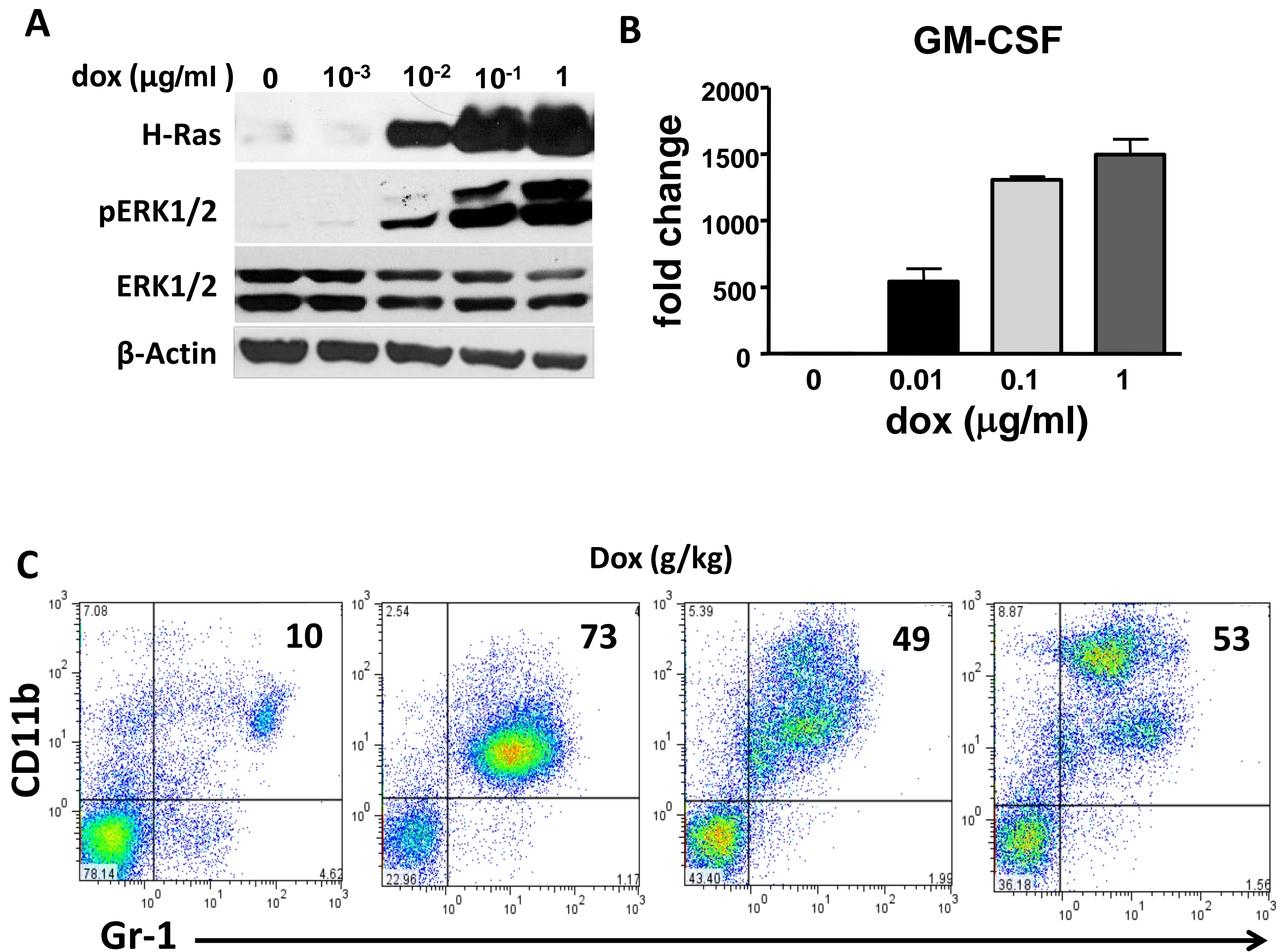

Figure 4-5: Titration of RAS transgene correlates with cytokine expression levels and the nature of systemic Gr1/CD11b expansion. A) Immunoblot for H-Ras, pERK1/2, total ERK1/2 and the loading control B-actin on protein lysates from DT primary keratinoctyes cultured in the presence of the indicated doxycycline concentrations for 24 hours. B) Q-RT-PCR for GM-CSF expression was performed on cDNA made from primary keratinocytes cultured with increasing dox doses for 24 hours. C) Gr1/CD11b FACS profiles from peripheral blood collected from DT mice + dox chow at the indicated doses fed for 4 days.

microenvironment. We addressed the potential of this hypothesis in DT/RAS mice by taking advantage of the dose responsive nature of the rTA/tetO system where quantitative transgene levels can be manipulated correspondingly with titration of doxycycline amounts (37). Figure 4-5 demonstrates that increasing dox concentrations *in vitro* correlated well with upregulation of GM-CSF in primary keratinocytes, a known inducer of MDSC activity (38;39) (Fig. 4-5B). Blood FACS analysis revealed a proportional decrease in $CD11b^+/Ly6G^-$ cells relative to the total $Gr\text{-}1^+/CD11b^+$ population indicating a preferential hematopoietic response to specific myeloid subsets (Figure 4-5C). Furthermore, sorting and co-culturing $Ly6G^-/CD11b^+$ cells from K14Ras mice receiving various dox doses yielded a reduction in suppressive activity on a per cell basis (Fig. 4-6A). Corresponding to this loss of immunosuppression, the splenocyte T cell numbers were inversely proportional to increasing dox doses suggesting an increase in local immunosuppression *in vivo* with higher transgene expression levels (Fig. 4-6B). Taken together, we interpret these results to mean that levels of oncogene expression can reflect cytokine quantities that potentially determine the qualitative outcome of a given inflammatory cascade. However, an alternative explanation to the reduction in T cell numbers that we cannot presently exclude is that the increase in the severity of inflammation produces an abundance of cell death inducing cytokines such as TNF-α and FAS ligand.

A non-$CD4^+/CD8^+$ lymphocyte is required for suppressive activity in $Ly6C^{hi}$ monocytes

Based on these data and previous studies clearly demonstrating a link between regulatory lymphocyte populations and immunosuppressive myeloid phenotypes (40;41) we hypothesized that there may exist a requirement of lymphocytes for the development of MDSC in our model. To assess this, we crossed both K14rTA and tetORAS transgenic mice onto the lymphocyte

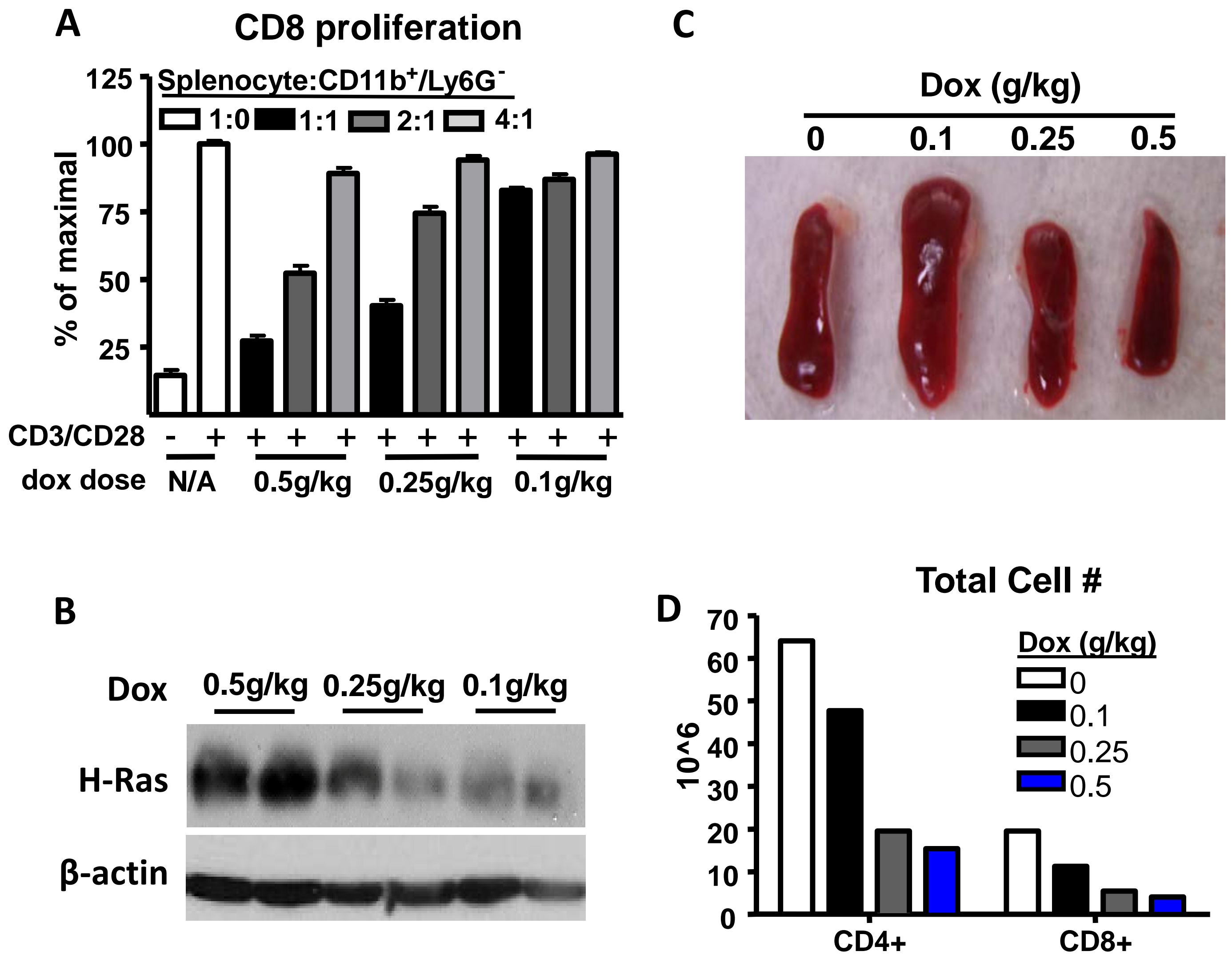

Figure 4-6: RAS expression levels dictate acquisition of immunosuppressive capability. A) Inhibition of T cell proliferation was measured as before in co-cultures of CFSE labeled splenocytes and Ly6G-/CD11b+ cells isolated from DT spleens fed decreasing concentrations of dox chow for 4 days. B) Immuno-staining for H-Ras transgene and B-actin loading control on protein lysates collected from total skin of mice used in "A". C) Representative spleens harvested from mice receiving titrated dox doses exhibiting changes in splenic size with increasing dox concentrations. D) Total $CD4^{+}$ and $CD8^{+}$ T cell counts were performed on spleens from dox titrated DT mice by hemocytometer and FACS staining.

deficient *Rag1-/-* background to yield DT*Rag1-/-* mice. In the absence of lymphocytes, RAS transgene expression provoked similar cutaneous inflammation and skin hyperplastic pathology (Fig. 4-7A). Strikingly, however, when putative Ly6C^{hi} monocytic MDSC were sorted from RAS expressing DT*Rag1-/-* mice and co-cultured in the suppression assay these cells demonstrated a complete inability to suppress T cell proliferation (Fig. 4-7B). This led us to further examine the lymphocyte subset responsible for instructing Ly6C^{hi} MDSC to acquire their immunosuppressive capabilities. We examined this by administering depleting antibodies against CD4^{+} and CD8β^{+} T cells prior to RAS induction and subsequently cultured Ly6C^{hi}/CD11b^{+} cells in the suppression assay. Interestingly, neither removal of CD4^{+} or CD8^{+} T cells alone or in combination had any effect on Ly6C^{hi} suppression of T cell proliferation (Fig. 4-7C). These data indicate that a lymphocyte is required to induce the T cell suppressive actions of Ly6C^{hi} monocytes but that lymphocyte is not of CD4/CD8 T cell origin.

B cells restore the suppressive capability of RAS induced Ly6C^{hi} MDSC

In light of these findings and previous studies using B cell null mice, we hypothesized B cells may provoke these immunosuppressive traits in monocytic MDSC populations. We tested this by reconstituting DT*Rag1-/-* mice with 5 million MACS purified splenic/lymph node B cells 2 days prior to dox administration. In stark contrast to CD4/CD8 depletion in DT*Rag1+/+* mice, B cell repletion of DT*Rag1-/-* fully restored Ly6C^{hi} inhibition of α-CD3/CD28 T cell stimulation. This strongly suggests that B cells were the lymphocyte orchestrating Ly6C^{hi} acquisition of MDSC phenotypes during RAS-induced inflammation (Fig. 4-8A). To confirm the necessity of B cell presence in mediating MDSC activity, we followed up on these observations by performing a suppression assay with Ly6C^{hi} cells purified from spleens of anti-CD20 depleted

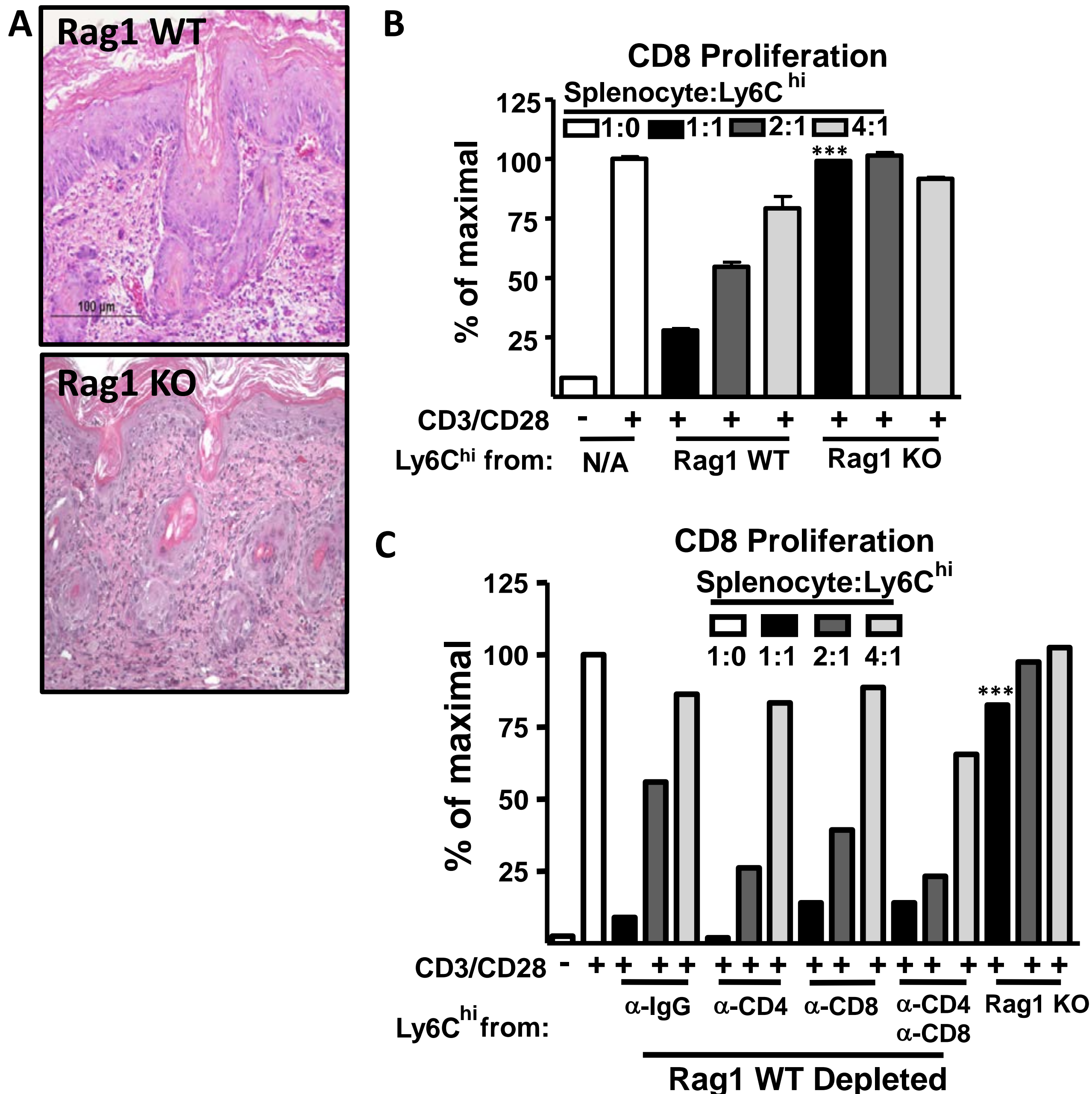

Figure 4-7: A specific non-CD4+/CD8+ lymphocyte is required for Ly6Chi cells to be immunosuppressive. A) Representative skin histology from DT/*Rag1+/+* (WT) and DT/*Rag1 -/-* (KO) mice on dox for 4 days. B) Ly6Chi/CD11b+ cells were sorted from spleens of DT/*Rag1WT* mice or DT/*Rag1KO* mice that were fed dox chow for 4 days and co-cultured in the suppression assay as before. C) Two days prior to dox administration, DT WT mice were IP injected with depleting antibodies against CD4, CD8, irrelevant isotype control (IgG) or CD4 and CD8. After 4 days on dox, Ly6Chi/CD11b+ cells were again purified by FACS and assessed for their inhibition of T cell proliferation. Cells sorted from DT KO mice were used as a negative control for suppressive activity. Indicated significance is relative to IgG treated Rag WT suppressive activity.

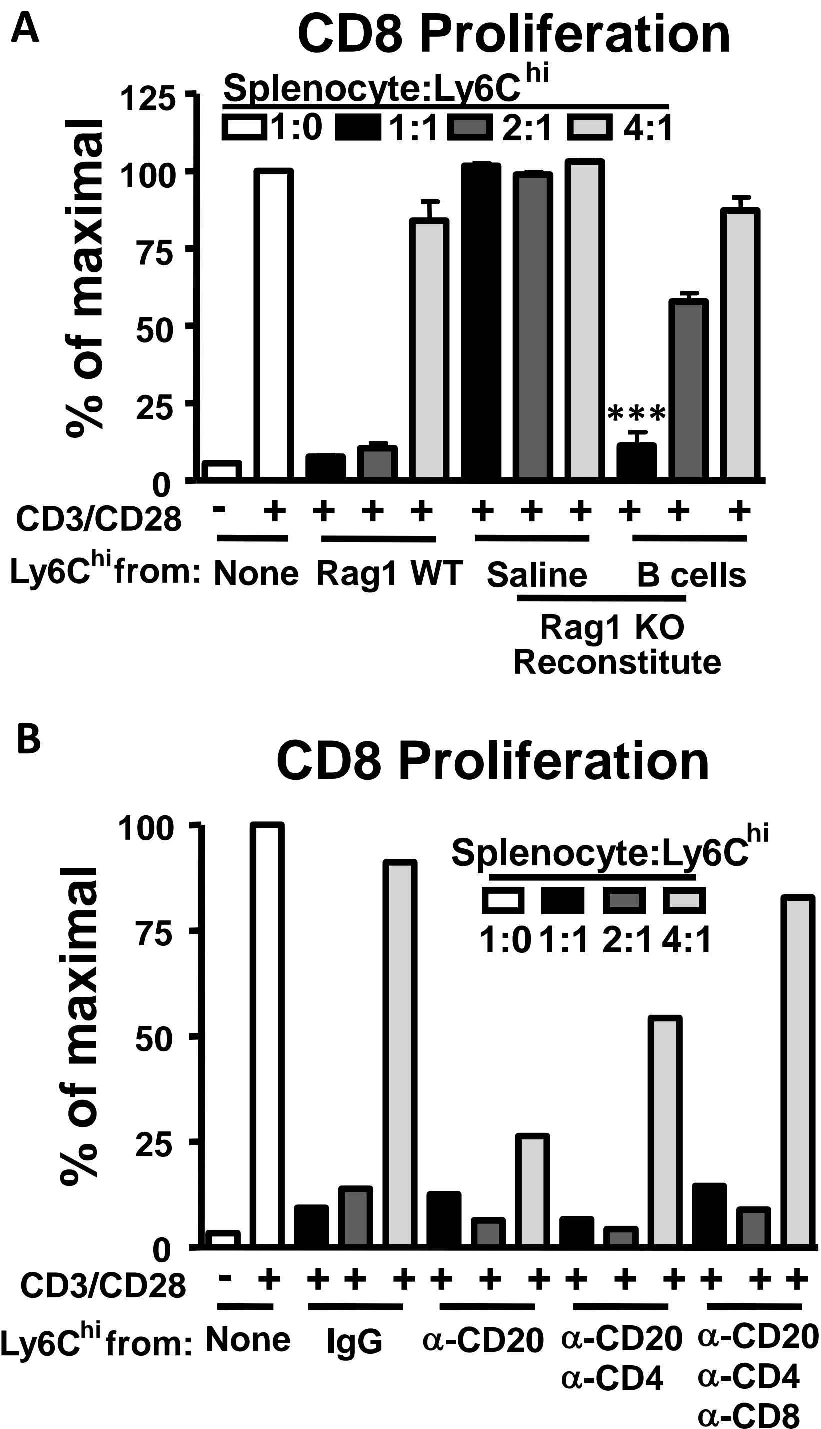

Figure 4-8: B cell reconstitution rescues MDSC phenotype but α-CD20 depletion does not ablate it. A) T cell suppression assay co-cultures with Ly6C^{hi} cells sorted from spleens of DT/Rag1 WT or DT/Rag1 KO mice reconstituted with saline or total splenic/lymph node B cells on dox for 4 days. *** = significant relative to Ly6C^{hi} cells from mock reconstituted Rag1 KO mice. B) DT/Rag1 WT mice were IP injected with depleting antibodies as indicated either 2 days (CD4 and CD8) or 7 days (CD20) prior to Ras induction by dox chow. Ly6C^{hi} cells were then sorted from spleens after 4 days on dox and co-cultured in the T cell suppression assay.

DT*Rag1+/+* mice 7 days prior to RAS expression. Surprisingly, B cell depletion had no effect on immunosuppressive activity of Ly6C^{hi} monocytes even in combination with CD4 and CD8 depletion suggesting there was not a compensatory T cell mediated mechanism of MDSC induction in the absence of B cells (Fig. 4-8B). When verifying complete B cell depletion we discovered that although peripheral blood and lymph node CD19^{+}/B220^{+} lymphocytes had been completely depleted, a definitive CD19^{+}/B220^{lo} splenic B cell population persisted (Fig. 4-9A). This likely did not represent increased B cell hematopoiesis in RAS expressing mice as α-CD20 treatments on normal ST + dox littermates mirrored remaining B cell percentages in DT + dox mice, even after IP dosing DT mice twice during the study, or varying the duration allowed for full B cell depletion to occur (Fig. 4-9B).

IL-10 expressing B regulatory cells likely cause MDSC phenotypes

Previous studies have shown that the anti-CD20 clone MB20-11 is capable of depleting splenic B cells to similar percentages as we observed but not to the extent as for CD4 and CD8 monoclonal depletion (Fig. 4-9) (42). To that end, we wanted to characterize the α-CD20 resistant population to see if any B cell subsets were preferentially increased after the treatments. The ~8% remaining CD19^{+}/B220^{lo} cells displayed significantly higher proportions of CD5^{+}/CD1d^{hi} and CD5^{+}/CD1d^{lo} phenotypes, again consistent with residual B cell populations following similar CD20 depletion studies (Fig. 4-10 and Fig. 4-11A) (8). These B cell subsets have been shown to possess immunoregulatory properties through the expression of IL-10, classified as B-regulatory (Bregs) cells (43). More specifically, IL-10 competent CD5^{+}CD1d^{hi} Bregs have been termed B10 cells and CD5^{+}/CD1d^{lo} as B1-a cells (44). This led us to hypothesize that Ly6C^{hi} monocytes in RAS expressing mice gain immunosuppressive behavior

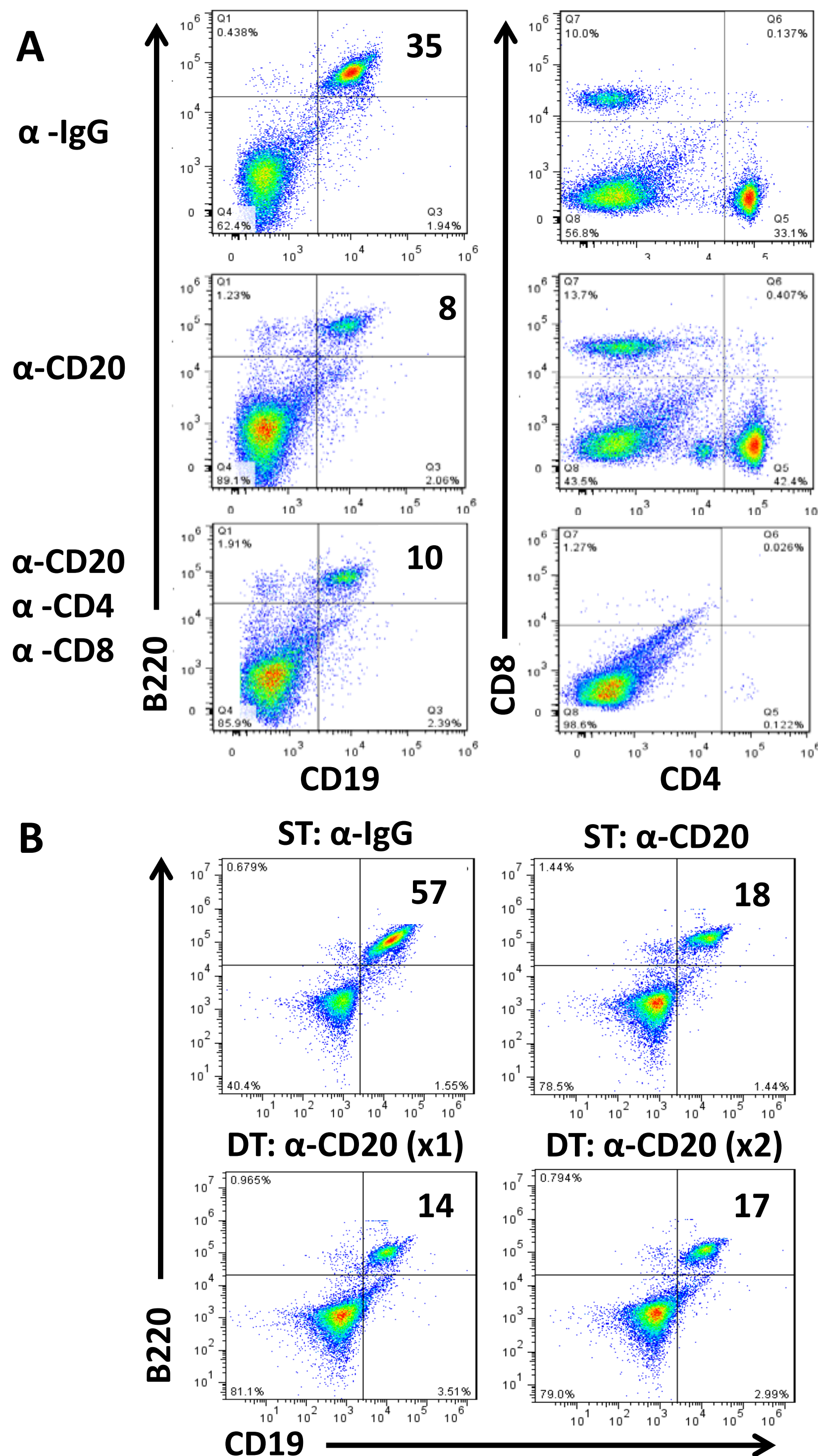

Figure 4-9: A resistant B cell population persists following depleting α-CD20 treatment regardless of dosage, RAS expression, or duration following depletion. A) Flow cytometry of spleen single cell suspensions CD19⁺/B220⁺ B cells and CD4⁺ or CD8⁺ T cells from DT mice on dox for 4 days, gated on CD45⁺ population. Anti-CD20 antibodies were injected 7 days prior and α-CD4/CD8 antibodies 2 days prior to dox chow feeding. B) ST or DT mice were given α-CD20 or α-IgG IP injections 2 days prior to feeding dox chow for 4 days and a separate DT group was given a 2nd IP injection 2 days after dox administration. Spleens were then harvested and stained for CD45, CD19 and B220 for flow cytometric analysis.

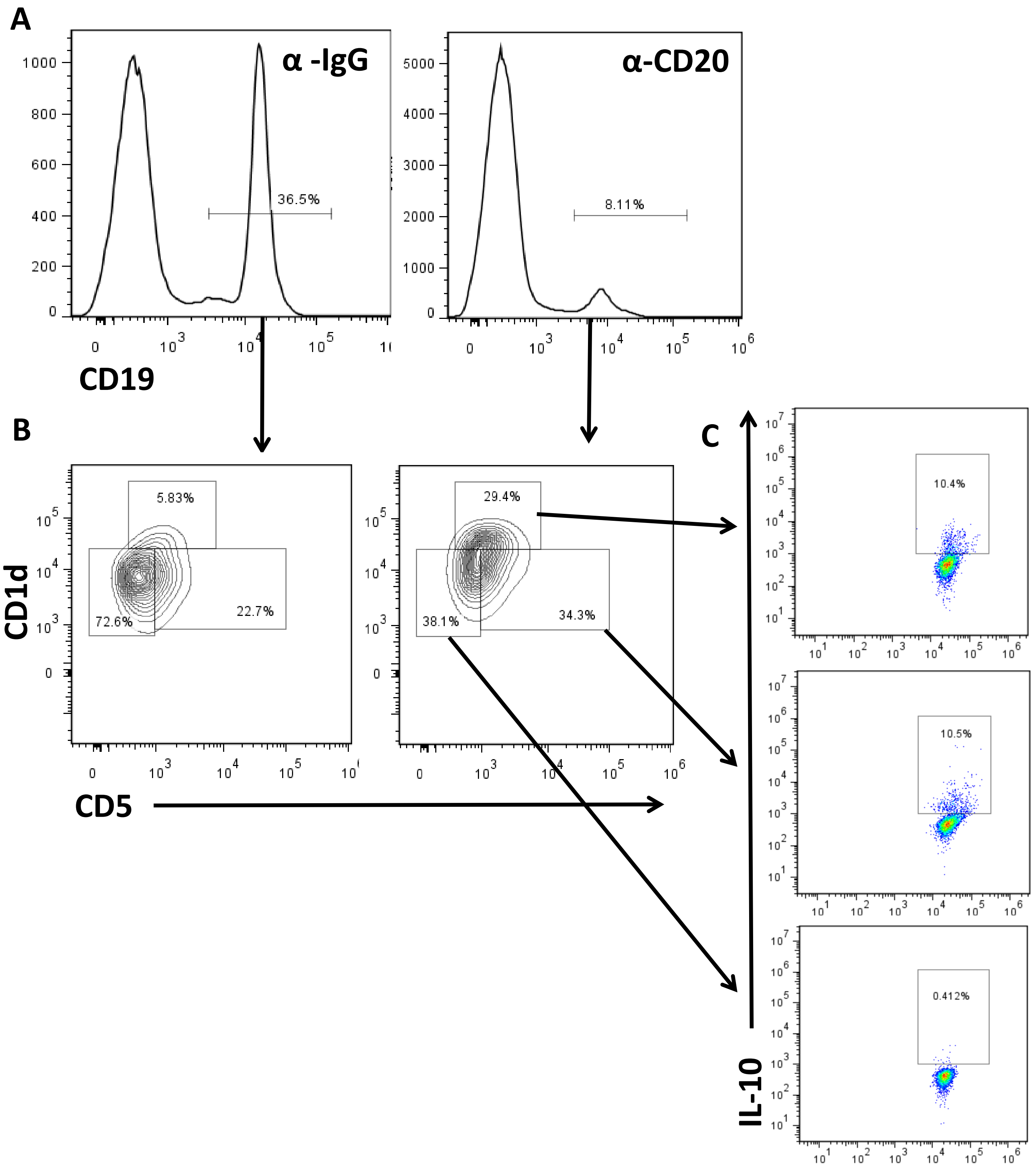

Figure 4-10: The majority of remaining B cells following α-CD20 depletion are IL-10 expressing B regulatory cells. A) Splenic $CD19^+$ population remaining 7 days following α-CD20 injection. B) $CD19^+$ population in "A" gated on CD5 and CD1d co-expression. The three gates are defined as $CD5^+/CD1d^{hi}$, $CD5^+/CD1d^{lo}$, and $CD5^-/CD1d^{lo}$. C) The three B cell populations in "B" were examined for IL-10 expression by intracellular staining of PMA/ionomycin/LPS stimulated spleen cells.

through the effects of Breg derived IL-10. Following 4 days of dox, IL-10$^+$ B cells increased in the spleens of DT mice (Fig. 4-11A). Negligible IL-10 production was observed in the CD19$^+$ fraction in blood or inguinal lymph nodes as determined by intracellular FACS staining (data not shown). Acute RAS expression did not, however, have any effect on the ratio of Breg differentiation (data not shown). Furthermore, IL-10 positivity increased within the remaining B10 and B1-a subsets after α-CD20 administration indicating a proportional decrease not only in non-regulatory B cells but also in α-CD20 sensitive Bregs devoid of IL-10 competency (Fig. 4-11C). Thus, α-CD20 depletion is ineffective at reducing immunosuppressive MDSC functions because of a specific α-CD20 resistant B cell subset that may be driving these myeloid phenotypes. These data elucidate a crucial role for Bregs in the development of MDSC during oncogene activation that may depend on key regulatory signals from IL-10.

4.4 Discussion

To our knowledge, this is the first report directly linking lymphocytes to the acquisition of immunosuppressive functionality in MDSC populations. Contrary to this, Gallina *et al* observed that total CD11b cells isolated from spleens of syngeneic, tumor bearing Rag2-/-γ_c-/- mice maintained the ability to thwart specific T cell lysis when co-cultured in an MLR assay (14). The differences in that study and ours include the method of inflammatory provocation (transgenic oncogene vs. syngeneic malignant cell engraftment), the mouse strain used (FVB/n vs. C57BL/6 and Balb/c), NK cell functional deficiency in γ_c-/- mice, and the malignant stage at which MDSC were studied (pre-malignant vs. advanced staged tumors); all variables which can produce significant differences in MDSC biology. The causal role of B cells in shaping aspects of innate immunity is not a new concept, and that which is pertaining to monomyelocytic biology

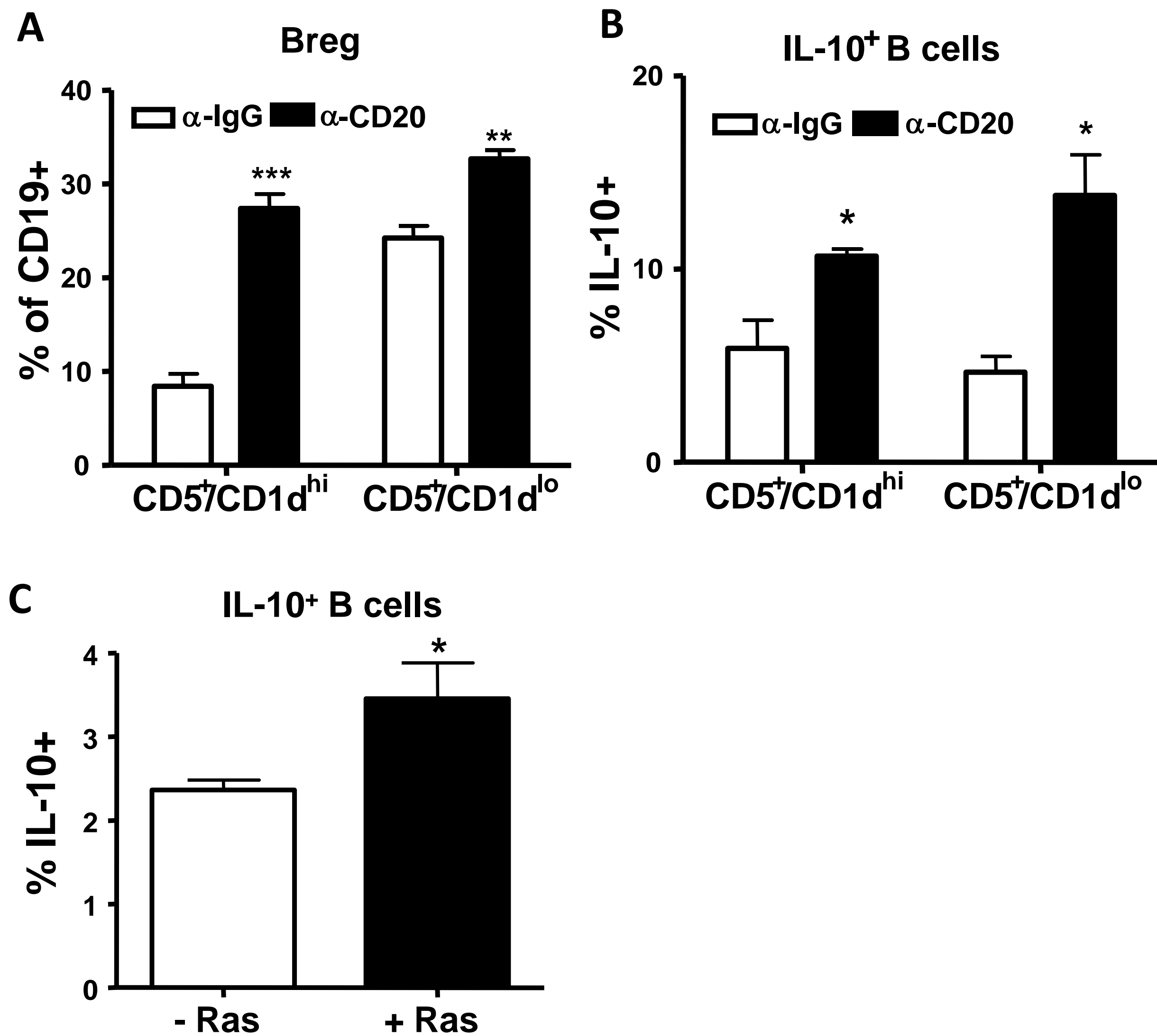

Figure 4-11: CD20 depletion preferentially depletes non-IL-10 expressing B cells and RAS expression promotes B10 cell differentiation. A) Graphical compilation of data from figure 3-10. Spleens were harvested from DT mice following single IP injection of α-IgG or α-CD20 7 days prior to 4 days of dox chow feeding. FACS percentages are expressed as percent positive of the markers listed gated first on the $CD45^+/CD19^+$ population. B) As in "A", the Breg subsets indicated were examined for their IL-10 expression following PMA/ionomycin/LPS stimulation and intracellular flow staining. C) Cytoplasmic IL-10 expression in total $CD19^+$ spleen cells of – Ras or + Ras mice on dox chow for 4 days examined by FACS as previously described. N = 6 in all panels shown.

is most relevant to these results. The three major mechanisms B cells can influence monocytes/macrophages are: interactions of IgG with FcγR, immune complex/complement activation, and cytokine secretion. Our results combined with a number of research studies causes us to favor a hypothesis involving cytokine mediated mechanism of B cell induced MDSC activation in our model. Co-culturing B1 cells, but not B2, with macrophages pushed their gene expression profiles towards M2 phenotypes and dampened M1 gene expression; a phenomenon dependent on B cell derived IL-10 (45). The aforementioned study by Schioppa *et al* discovered that one of the mechanisms behind skin tumor resistance in *TNF-α-/-* mice was a lack of IL-10 production in B cells (6). This also involved a mechanism of increased skin macrophage infiltration correlating with decreased CTL responses. Moreover, co-culturing total splenocytes from C57BL/6 WT or *μT -/-* B cell knockout mice with various irradiated tumor cell lines resulted in increased IFN-γ production from $CD8^+/CD3^+$ cells in B cell null co-cultures. This involved, in part, a CD40L/CD40 interaction and the increased production of tumor cell stimulated IL-10 in B cells (46). Accordingly, *IgM -/-* mice also exhibited enhanced IFN-γ responses and anti-tumor immunity to EL-4, B-16, and MC-38 transplantable cancers and B cell reconstitution reversed these effects (5). All of these studies circumstantially implicate IL-10 production by B cells in mediating the immune inhibitory responses of alternatively activated macrophages. However, one novel study implicated C5R signaling as a novel mechanism for the accumulation of MDSC into tumors and their T cell inhibitory properties (47). FcγR deficiency, as previously discussed, greatly attenuated the pro-angiogenic effects of macrophages leading to defective cutaneous dysplastic progression (4) and direct IgG ligation of FcγR I/III on macrophages inhibited IL-12 and upregulated IL-10 expression, a hallmark M2 trait (48). Interestingly, the most abundant immunoglobulins produced in response to transgene expression

in the K14-HPV16 model did not target non-self E6/E7 antigens but were autoantibodies with specificity to type 1 collagens; (4;49). Secretion of autoantibodies is biology unique to $CD5^+$ B1 subsets. These studies indicate that there exist multiple mechanisms of monomyelocytic regulation by B cells during skin carcinogenesis. Because FACS defined B cell subsets are capable of different functions, and the induction of MDSC biology is not specific to one independent process (12), it is plausible that MDSC expansion, chemotaxis, immunosuppression, and pro-angiogenic behaviors can be regulated independently of one another by a distinct B cell mediated mechanism.

The influential role of IL-10 on monomyelocytic functionality is also now well understood. IL-10 signals directly through STAT3 (50) thereby inhibiting monocytic differentiation to dendritic cells while promoting macrophage development (51). Blockade of STAT3 signaling has been shown to reverse this inhibition, thereby breaking the dysfunctional immunosuppressive myeloid phenotype (52-54). Other effects of IL-10 on monocytes and macrophages include suppression of pro-inflammatory cytokine production (55;56), upregulation of the secretion of anti-inflammatory molecules (57;58), and decreased antigen presentation by downregulating MHCII and CD86 (55;59;60), hence skewing monomyelocytic cells towards a strong M2 macrophage phenotype. Ironically, our inability to fully deplete B cells actually supports our hypothesis since IL-10 produced from anti-CD20 resistant Breg populations would severely dampen the FcγR-mediated ADCC capability of mononuclear phagocytes (61). In support of our results, this phenomenon has been observed in another study demonstrating B1 cells residing in the peritoneum mediated resistance to Rituximab therapy (42). A follow up study from this group went on to demonstrate these B1 cells were predominantly of a $CD5^+/CD1d^{hi}$ phenotype that encompassed the majority of IL-10 producing B cells and greatly

enhanced A20 lymphoma growth in the midst of α-CD20 treatments (8). Macrophages co-cultured with these B10 cells displayed decreased MHCII and CD86 expression as well LPS stimulated TNF-α and NO production, a strong indication that B10 cells directly induced M2 polarized phenotypes. Although not analyzed, a similar percentage of B1 cells in the peritoneum presumably resisted CD20 depletion as well in our model along with the residual splenic Breg populations (Fig. 3-9), potentially providing an additional IL-10 reservoir for MDSC induction.

The similarities between M2 macrophages and MDSC are well appreciated but both myeloid populations are considered morphologically distinct from one another (62). Both myeloid subsets can express immunosuppressive molecules such as Arg1 in both M2 and MDSCs subsets and iNOS in MDSC. Our putative MDSC population was characterized systemically as $CD11b^{+}/Ly6G^{-}/Ly6C^{hi}$ mononuclear cells that suppress via a mechanism of iNOS but not Arg1 (Fig. 3). This is a classic phenotype of inflammatory monocytes that express high levels of CCR2, distinguished from $CCR2^{lo}/CX3CR1/Ly6C^{lo}$ "patrolling" monocytes (63). Alternatively activated M2 macrophages are hypothesized to differentiate from $Ly6C^{lo}$ monocyte progenitors and dendritic cells from $Ly6C^{hi}$ subsets but the evidence for this is still highly controversial. However, one study provided evidence that $Ly6C^{hi}$ inflammatory monocytes seeded tumors for continuous renewal of all macrophage subtypes and their phenotypes depended on the geographical context of their intratumoral residency (64). We did observe a substantial increase in dermal resident $F4/80^{+}$ cells and upregulation of CCL2 in RAS expressing skin (Fig. 3-2C) and this may indicate a corresponding infiltration of MDSC inflammatory monocytes that under the differentiating roles of IL-10 favor macrophage development. In turn, skin microenvironmental cues may skew these macrophages towards M2 phenotypes. Indeed, greater upregulation of Arg1 transcripts were also observed in RAS expressing skin (Fig. 4-2C)

suggesting increased skin resident M2 phenotypes in these mice but more evidence will be needed to clarify this hypothesis.

Of further interest was the result that the level of RAS oncogene expression directly correlated with reduced cytokine expression and in turn, reduced immunosuppressive behavior in MDSC populations (Fig. 4-5 and 4-6). This would seemingly underscore the hypothesis that MDSC phenotypes are acquired as a result of quantitatively high cytokine environments. This postulate is supported by a cancer vaccine study in mice that defined a threshold dose of GM-CSF adjuvant in tipping the balance from anti-tumor to pro-tumor CD11b^{+} cells (65). As it appeared both the expansion of MDSC populations decreased as well as the functional ability to suppress T cells, it is likely multiple cytokine levels correlate with oncoprotein expression levels. The physiological relevance would be that when the severity of the pathological insult is great enough, self-limiting immunoregulation would be favored to immediately resolve a potentially damaging inflammatory response. This hypothesis has already been proposed for Th2/M2 driven immunity to parasitic infections that can cause significant tissue destruction requiring wound healing (66) that cannot take place until Th1/M1 immunity has effectively resolved (67). The inflammatory balance perverted by progressing malignancies favoring the chronic presence of regulatory leukocytes such as Bregs and MDSCs would fit such a paradigm.

While we show strong evidence that IL-10 producing Bregs are likely responsible for inducing immunosuppressive behavior of Ly6C^{hi} MDSC in our RAS model, we do not yet provide definitive proof for this mode of action. In future studies, we plan to reconstitute DT*Rag1-/-* mice with *IL-10-/-* B cells to test whether IL-10 is required for the recovery of suppressive capabilities observed in wild type B cell repleted DT/*Rag1-/-* mice (Fig. 4-7A). Also, with advanced sorting methods we can purify putative Breg populations (CD5^{+}/CD1d^{hi})

and compare their ability to regenerate MDSC ability to non-Breg populations that we and others have shown are not IL-10 competent (Fig. 4-10). This assay could also be combined with α-IL-10 ligand neutralization or IL-10R blockade for further demonstration of a specific IL-10 mechanism. As it will be important to also demonstrate immunosuppression in mice, our *ex vivo* suppression assay could extrapolated *in vivo* by transfer of CFSE labeled T cells with or without B cells into DT*Rag1-/-* mice. Finally, we will seek to further characterize the phenotype of Ly6C^{hi} monocytes by examining changes to known IL-10 target genes such as: downregulation of CD86, MHCII, and LPS-induced TNF-α and IL-12 expression as well as upregulation of SOCS3 and hyperactivation of STAT3. We will also sort myeloid populations from K14Ras skin to examine the immunosuppressive status of tissue resident inflammatory cells. Whether this phenomenon has an effect on squamous tumor development will be difficult to prove because of our current lack of a B cell or IL-10 deficient FVB/n mouse. Examining skin tumor development in B cell reconstituted DT*Rag1-/-* mice will not support our claims that dampened anti-tumor immunity due to MDSC immunosuppressive activity enhances tumor progression because of the lack of T cell targets to for Ly6C^{hi} cells to suppress in *Rag1-/-* mice. Furthermore, we clearly show that total depletion of B cells is not possible so classic remove and add-back experiments are not presently possible.

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Chapter 5 – Inflammatory responses specific to oncogene expression in basal and post-mitotic epidermal compartments may contribute to malignant potential

5.1 Introduction

One of the more centralized questions in the field of cancer biology is the localization of the initiated cancer cell within the epithelial compartment. The epidermis is organized into a stratified squamous epithelium with a basement layer of basal proliferating keratinocytes beneath progressively differentiating squames of post-mitotic layers that are irreversibly in cell cycle exit culminating in apoptotic cornification or “sloughing off”. This process is tightly regulated by calcium concentration and integrin-mediated cell to cell contacts with the basement membrane that direct apical and basolateral polarity (1;2). Epidermal compartments are not only indentified by spacial localization in relation to one another but also by their unique cytokeratin expression patterns where basal keratinoctyes distinctly express the pairs of type 5/14 (K5/K14) and suprabasal keratinocytes express K1/K10 in addition to the terminal differentiation markers Involucrin, filaggrin and loricrin. This tissue organization extends to hair follicle structures where additional subsets of undifferentiated keratinocytes reside in bulge region niches, biochemically identifiable through co-expression of (K15) and the stem cell marker (CD34). These unique stem cell keratinocytes also expresses the K5/K14 basal markers and are widely thought to be the primary target of malignant epithelial growth because of their pluripotent potential (3).

Benign lesions arising on epithelial skin surfaces during the chemical carcinogenesis tumor protocol have a variable progression to malignant conversion. Standard DMBA/TPA protocols require 1 time DMBA application to initiate H-Ras/A61T transversions followed by once or twice weekly TPA treatments to promote clonal proliferation of mutated keratinocytes.

This method yields numerous papilloma outgrowths by 10-15 weeks where a proportion can spontaneously regress after removal of TPA promotion and another population progresses to SCC around 20 weeks albeit at a relatively low ratio (3-5%) of the total tumor burden. However, an alternative protocol that terminates phorbol ester treatments after 5 weeks produces much less frequent papilloma numbers that do not regress in the absence of TPA and convert to SCC with significantly greater probability (15-25%) (4). The reasons for this discrepancy are still unclear, but these studies suggest that there may be a subset of target cells derived from different epidermal compartments that are genetically inclined for greater potential to cause malignant conversion. DMBA application to the dorsal surface of rodents is ubiquitous where all keratinocytes, regardless of differentiation stage, are exposed to the carcinogen and thus have an equal probability of acquiring an H-Ras mutation. Cancer cell transformation only occurs when multiple mutational events (5 in humans, 2-3 in mice) successively activate proliferative cell cycle entry while simultaneously deactivating tumor suppressor pathways. Ras oncogene activates cellular growth arrest through INK4A/ARF and p53 in primary keratinocytes thereby blocking transformation in vitro (5) and mutation of one or the other is required for RAS to cause progression to SCC in the 2-stage assay (6). As the majority of H-RAS mutations caused by DMBA would no doubt be lost in the process of terminal differentiation, the necessary amount of genetic alterations required to induce tumor formation are more likely to occur in an epidermal resident with inherently long lived and self-renewal properties like interfollicular and/or bulge region keratinocyte stem cells.

Regression of papillomas during low risk protocols likely implies cytotoxic immunosurveillance can ensue following TPA removal. This also suggests that mutant RAS has the ability to intrinsically regulate the promotion of clonal expansion of transformed

keratinocytes in the absence of a potent pro-inflammatory phorbol ester. Based on these results, a number of groups have targeted a RAS oncogene with promoters expressed in various layers of the epithelium to demonstrate the malignant potential of mutations arising from keratinocytes are different stages of differentiation. A K10-Ras transgene produced benign papillomas that never progressed to SCC on areas of the skin associated with grooming and scratching suggesting wounding may promote tumor growth (7). Similarly, an independent study showed K1-Ras expression resulted in almost an identical phenotype (8). MEK1 expression driven by an Involucrin promoter could also spontaneously induce benign papilloma formation that was drastically increased by punch biopsy wounding (9;10). In stark contrast, targeting constitutive or conditionally inducible RAS expression to a K5 or K14 promoter caused SCC conversion on multiple epithelial surfaces; malignant lesions that were refractory to TPA promotion and completely independent of wounding (11-13). These seminal studies indicate that while skin tumors can form from any mutated keratinocyte, SCC development almost certainly arises from a basal/stem cell lineage. While there are likely numerous keratinocyte intrinsic mechanisms (cell cycle status) that can account for an enhanced inclination to progress to malignancy, no one has examined the possibility that cancer cells provoke extrinsic factors unique to mutations in proliferating or differentiating epithelial cell layers that may influence tumor outcome. Of note, Arwert *et al* did demonstrate that the Inv-MEK1 benign tumor formation was dependent on γδT cells and macrophages (9). Interestingly, when characterizing microarray gene expression profile signatures unique to either high or low risk papillomas, low T cell infiltration was a distinguishing factor for high risk skin tumors supporting the potential for immune-derived mechanisms of malignant progression associated with basal/stem cell mutations (14).

To address the hypothesis that immune mediated mechanisms influence the disparate malignant outcome of skin carcinogenesis initiated from pre and post-mitotic oncogene expression, we compared inflammatory responses in InvtTA and K14rTA tetOHRAS^{G12V} mice. The results presented herein demonstrate cytokine signaling patterns unique to RAS expression in each compartment that may explain the variable progression risk between differentiated and stem cell driven skin cancer.

5.2 Results

We have initially compared acute inflammation in both InvRas (7 days off dox) and K14Ras (4 days on dox) mice in order to investigate any immediate differences to RAS expression that would be mainly attributable to keratinocyte autonomous RAS signaling. The most overt difference between these two Ras expression models was the lack of neutrophil microabscesses in K14rTA/tetORAS (K14Ras) mice despite obvious dermal infiltration of leukocytes and systemic expansion of Gr-1/CD11b myeloid cells (Fig. 5-1A). However, closer examination of blood leukocytes revealed Gr-1 expression on the Ly6G^{+} neutrophilic population was markedly reduced from K14Ras mice and there was a greater expansion of Ly6G^{-} cells in proportion to Ly6G^{+} cells (Fig 5-1C). The immature nuclear morphology of sorted Ly6G^{+} cells suggest neutrophils responding to basal RAS expression had a blunted activation phenotype and were more immature than suprabasal RAS neutrophils although this has yet to be validated functionally (Fig 5-1D). Also, in support of previous studies, all tumors arising from InvRas expression were of a benign phenotype but preliminary studies on tumor development in K14Ras mice produced 1 SCC from 2 mice although both mice were sacrificed before a full histopathological analysis could be performed (Fig. 5-1B). These data indicate that cytotoxic

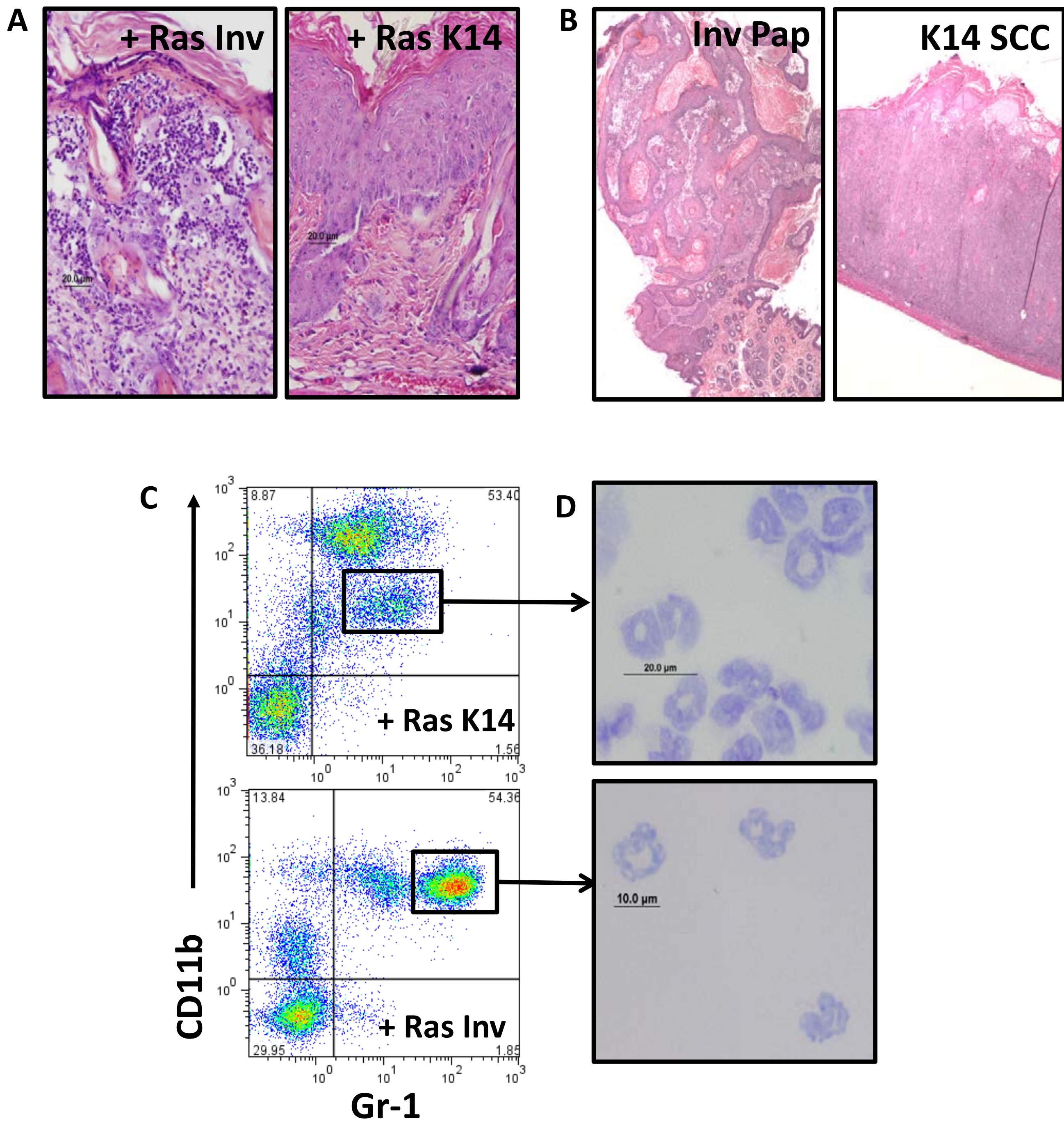


Figure 5-1: Basal RAS expression leads to myeloid expansion devoid of epidermal microabscesses and tumor progression to SCC. A) Representative H&E images of K14rTA/tetORas mice on dox chow for 5 days or InvtTA/tetORas mice off dox water for 7 days displaying lack of microabscesses in K14Ras skin. B) Tumor H&E stains from transgenic mice on low dox dose protocols exhibiting benign (Inv Pap) and malignant (K14 SCC) histology. C) Gr-1/CD11b FACS profiles gated on $CD45^+$ population from peripheral blood collected at the time of necropsy as in A. Boxes indicated $Ly6G^+$ population. D) Cytopsins of sorted $Ly6G^+/CD11b^+$ cells from the groups indicated. Note lack of PMN morphology in $Ly6G^+$ cells from K14Ras mice.

respiratory burst mechanisms were inhibited in neutrophils that expanded systemically in K14Ras mice possibly due to dampened maturation and myeloid inflammation that favored monocytic expansion over neutrophils.

Because of the drastic quantitative difference in peripheral blood myeloid populations between these two epidermal RAS models, we hypothesized there also may be a qualitative functional discrepancy in the monomyelocytic subset. As before, the immunosuppressive potential of $Ly6G^-/CD11b^+$ cells purified from spleens was compared in InvtTA and K14rTA models. K14Ras $Ly6G^-$ cells, again, potently inhibited CD3/CD28 induced proliferation of $CD4^+$ and $CD8^+$ T cell but the same population isolated from InvRas mice exhibited no suppressive capability (Fig. 5-2A). Furthermore, $F4/80^+/CD11b^+$ cells isolated from skin of InvRas mice were also incapable of inhibiting T cell proliferation (Fig. 5-2B). A parallel study of immunosuppressive properties of $F4/80^+$ cells from K14Ras skin has not been done yet but the expression of the key immunosuppressive gene Arg1 was upregulated in skin of K14Ras mice but not InvRas mice. This result suggests that skin resident macrophages in K14Ras mice could have similar suppressive function as that observed in splenic monocytes (Fig. 5-2C).

Based on these data we postulated that cytokine expression originating from the skin in these two models may not be equivalent, thus causing their distinct inflammatory responses. Indeed, relative to InvRas skin, K14Ras skin demonstrated increased mRNA transcripts of S100A9, CCL2 and KC, while InvRas skin had higher expression of GM-CSF, IL-1α, and IL-1β (Fig. 5-3). S100A9 has been shown to be a critical cytokine in inducing MDSC activity by preventing myeloid differentiation and CCL2 is important in monocyte MDSC recruitment supporting the previous results on the immunosuppressive functions of $Ly6G^-/CD11b^+$ cells in each RAS model (15). This indicated to us that oncogenic RAS may have a varying potential to

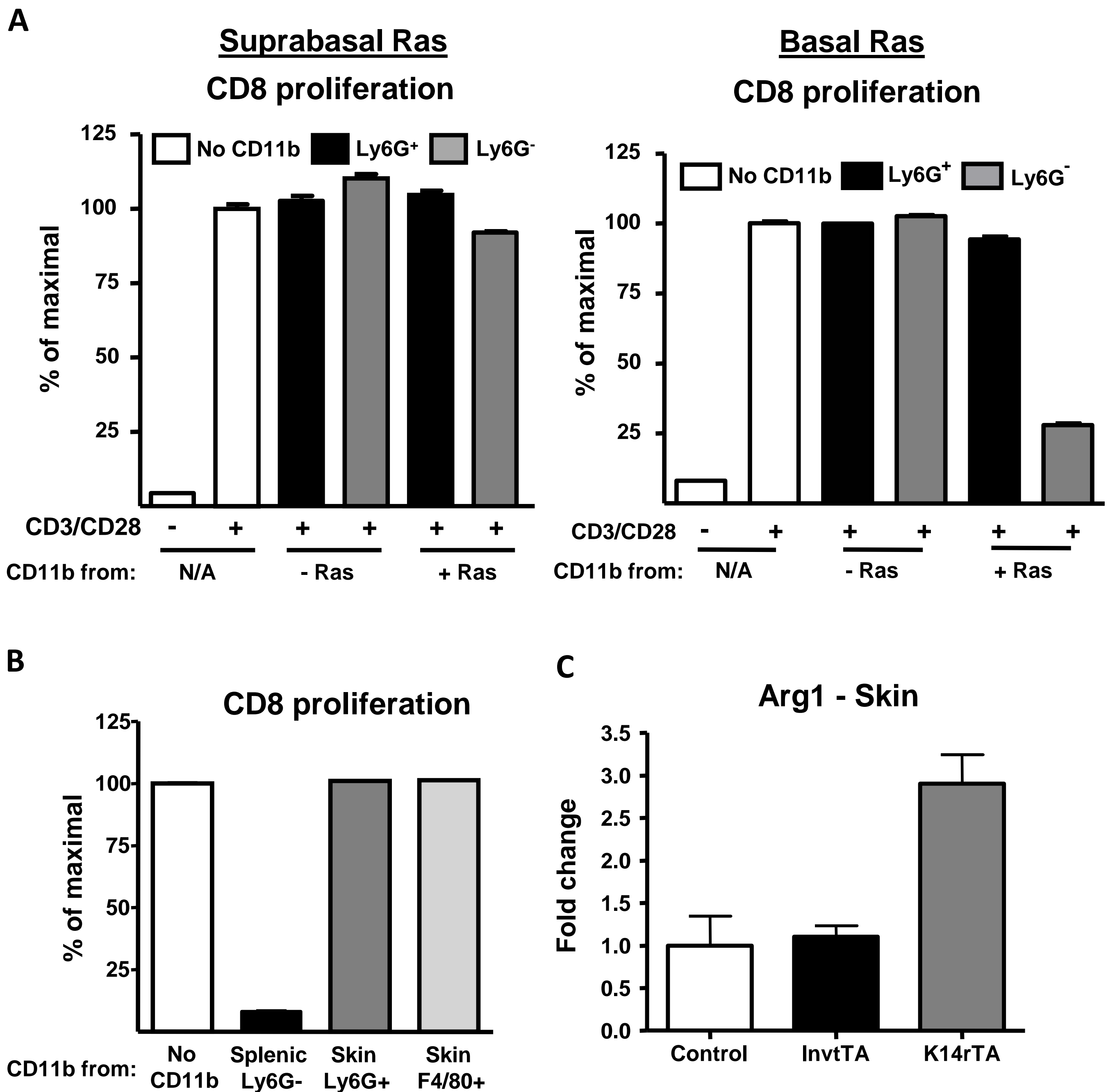


Figure 5-2: Splenic Ly6G⁻/CD11b⁺ and skin F4/80⁺/CD11b⁺ cells from InvRas mice are not suppressive. A) Suppression assay using sorted CD11b⁺ cells from spleens of InvRas (left) or K14Ras (right) mice respectively. B) Skin single suspensions were prepared from InvRas mice and sorted for F4/80⁺ or Ly6G⁺/CD11b⁺ cells and placed in the suppression assay. Ly6G⁻ cells from K14Ras spleens were used as a positive control. C) QRT-PCR for Arg1 transcripts in total skin from the indicated groups.

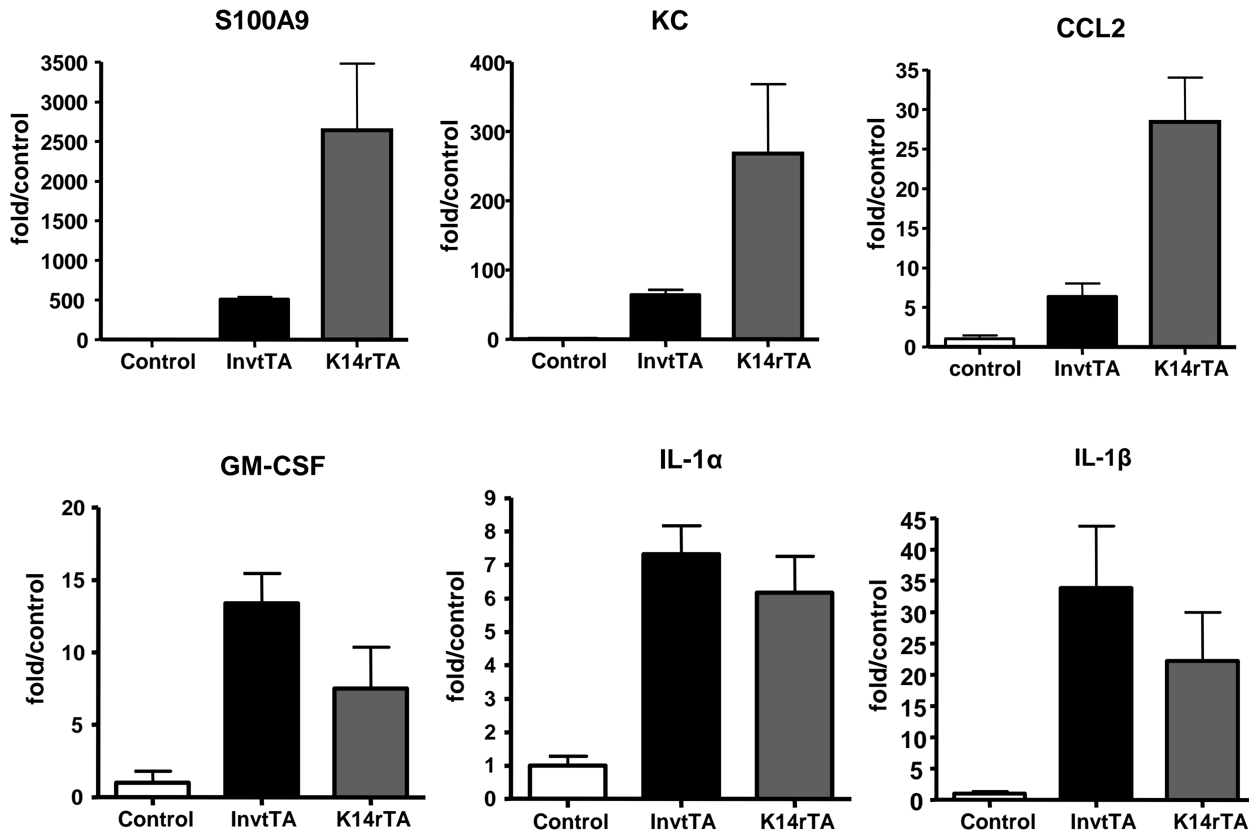


Figure 5-3: Differential cytokine expression patterns in InvRas and K14Ras mice. QPCR amplification of dorsal skin cDNA from non-Ras expressing (control), InvRas, and K14Ras mice for the genes listed. Each group is the mean and standard error of 4-6 mice.

activate downstream signaling dependent on the differentiation status of the cell it is expressed in. Indeed, p-ERK1/2 immunoblotting of total skin lysates from InvRas or K14Ras mice showed that even though total RAS transgene levels were higher in InvRas skin, ERK activation was equivalent or somewhat diminished compared to its basal RAS counterparts (Fig. 5-4A). Supporting this concept, p-ERK1/2 immunohistochemical staining on dorsal skin sections from both models showed ubiquitous staining in K14Ras epidermis compared to sporadic and non-uniform p-ERK1/2 in InvRas skin (Fig. 5-4B).

Next we analyzed primary keratinocyte RAS signaling *in vitro* to eliminate paracrine contributions from stromal cells that could confound any interpretation of the strictly cell autonomous RAS downstream signal transduction in basal and differentiating keratinocytes. We also examined only K14Ras keratinocytes and manipulated their differentiation states by changing extracellular calcium concentrations to avoid kinetic variables between the InvtTA and K14rTA dox inducible systems. When keratinocytes were differentiated with a 0.5mM Ca^{2+} (HiCal) switch prior to dox induction of RAS transgene, ERK1/2 activation was noticeably reduced, confirming that RAS in terminally differentiated keratinocytes is less capable of activating its immediately downstream MAPK pathway (Fig. 5-5A). Importantly, this phenomenon may be specific to ERK1/2 as phosphorylation of AKT was left unaffected (Fig. 5-5A). Correspondingly, cytokine expression induced by RAS was inhibited by both calcium increases and the MEK1/2 inhibitor U0126 (Fig. 5-6A and B). These results suggest a critical role for the involvement of ERK in mediating specific cytokine expression induced by RAS.

As most of these cytokine genes induced by RAS have NFκb consensus elements in their promoters and thus potentially regulated by NFκb transcriptional activity, we tested whether RAS expression directly activated NFκb and if blockade of NFκb signaling could inhibit

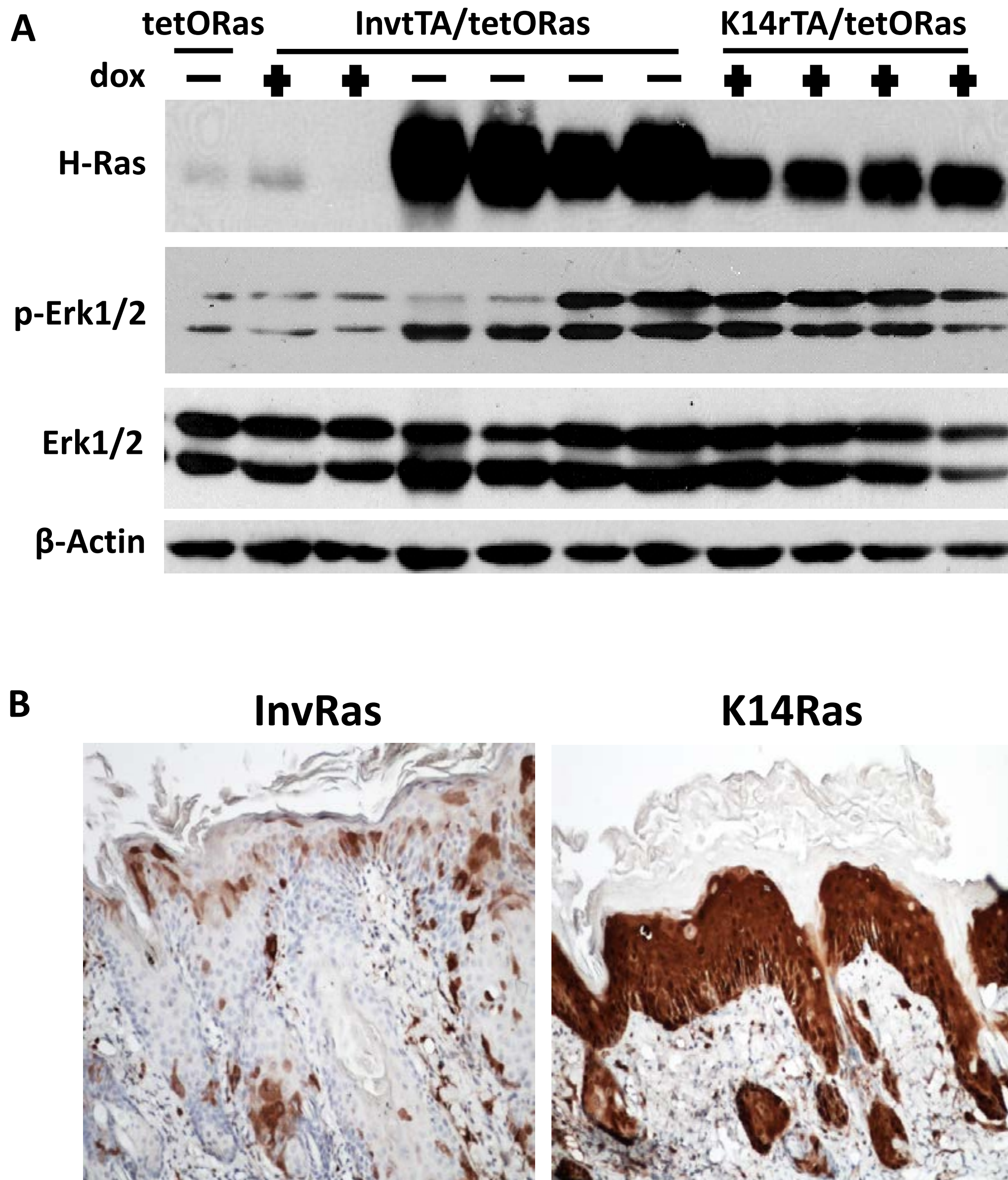


Figure 5-4: InvtTA/tetoRas mice exhibit reduced ERK1/2 activation. A) Immunoblot on total skin lysates from the groups listed stained with antibodies against H-Ras, p-ERK1/2, total ERK1/2, and the loading control β-actin. B) Immunohistochemical staining of dorsal skin sections with α-p-ERK1/2. Control – Ras sections had negligible pERK1/2 staining in the epidermis (not shown).

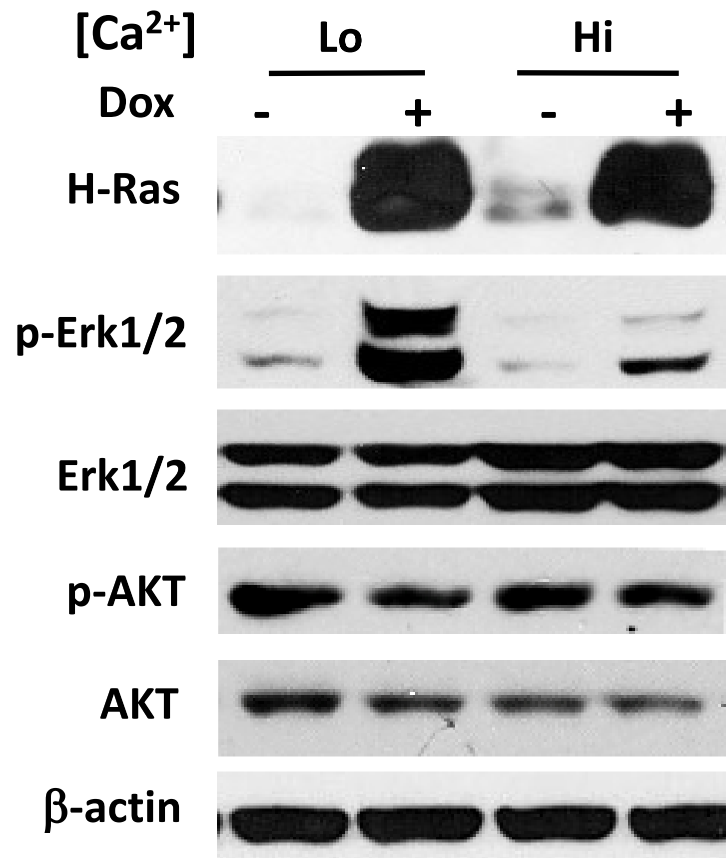


Figure 5-5: Differentiating primary keratinocytes blocks ERK1/2 activation by RAS. Primary keratinocytes isolated from K14rTA/tetoRas mice were cultured in LoCal medium (0.05mM) for 2 days and a group was switched to HiCal (0.5mM) for 24 hours to induce differentiation. Dox (1ug/ml) was then added to + dox groups and protein lysates harvested 24 hours later. SDS-PAGE and western blots were subsequently run and stained with the antibodies indicated. This experiment was repeated 2 other times independently

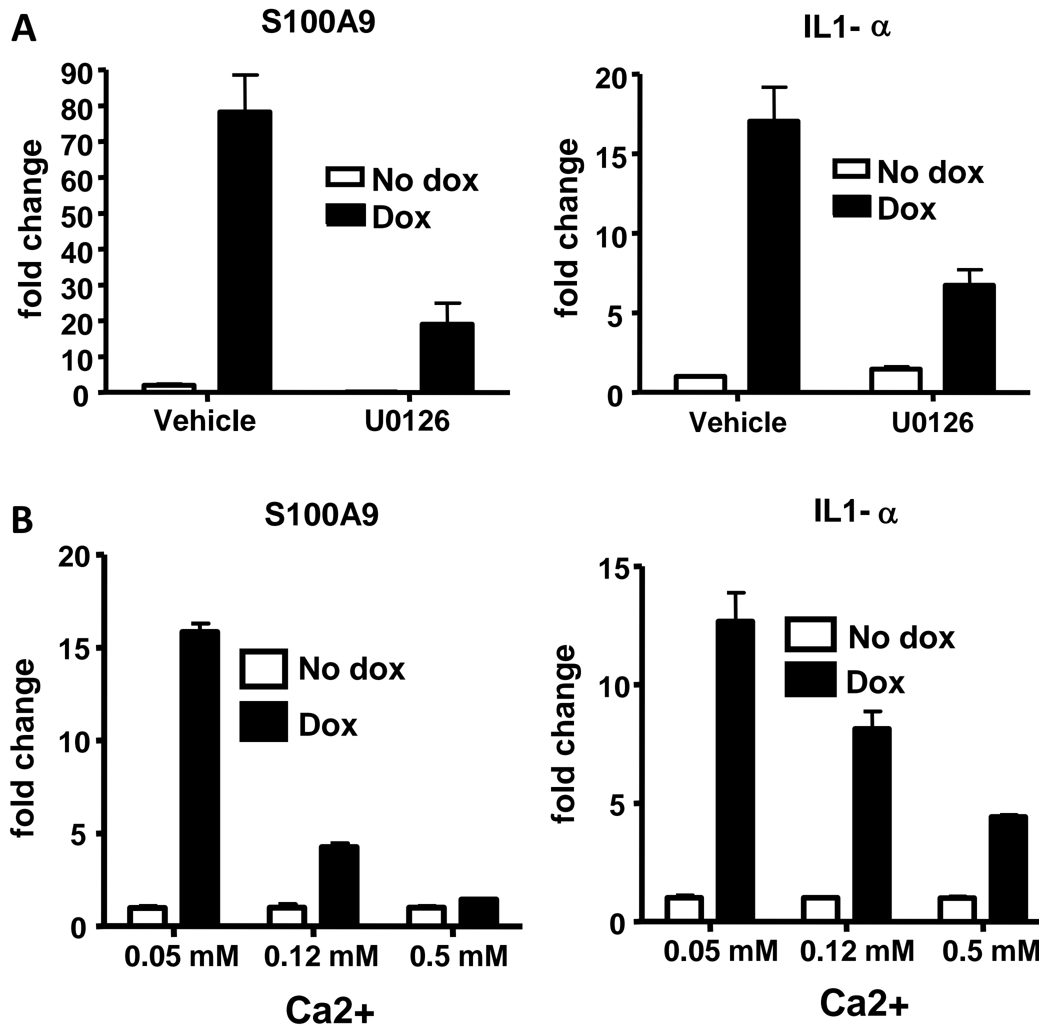


Figure 5-6: MEK1/2 inhibition and Ca2+ differentiation attenuates RAS induced cytokine upregulation. A) QPCR for S100A9 and IL-1α was performed on cDNA from K14Ras keratinocytes +/- dox for 48 hours followed by 8 hour treatments with either DMSO vehicle or the MEK1/2 inhibitor U0126 (10μM). B) As in “A”, S100A9 and IL-1a QPCR analysis was carried out on cDNA from K14Ras keratinocytes treated with the $Ca2^{+}$ concentrations indicated followed by +/- dox (1ug/ml) treatments for 36 hours. These experiments were repeated in 2 separate keratinocyte preps.

cytokine transcriptional activity. Upon RAS activation in cells maintained in LoCal media, both p50 and p65 subunits increased their nuclear localization (Fig. 5-7A). Introduction of the dominant negative IκBα super repressor (IκBsr) by adenoviral transduction prior to dox treatment blocked RAS's ability to upregulate numerous, but not all cytokine genes, confirming the requirement for NFκb nuclear translocation in RAS induced pro-inflammatory gene transcription (Fig. 5-7B). When bitransgenic keratinocytes were infected with an adenovirus expressing A-FOS, a modified Fos gene which blocks all DNA binding by AP-1 transcription factors, expression of virtually every cytokine gene tested was enhanced, indicating in the context of oncogenic RAS expression, AP-1 was transcriptionally repressive for these genes. Conversely, AP-1 or NFκb blockade in HiCal keratinocytes was ineffectual in preventing or synergistically activating RAS induced GM-CSF expression which suggests differentiated keratinocytes do not require either transcription factor for RAS mediated cytokine transcription (Fig. 5-7C). However, as both of these proteins are known to play a direct role in the process of terminal differentiation blocking their activity after inducing differentiation may be after the key transcriptional events have already taken place. Further studies will be required to elucidate this discrepancy and if differentiated keratinocytes upregulate a specific set of cytokines in discordance to basal keratinocytes.

Since InvRas mice exhibited increased pro-inflammatory cytokine expression in vivo and displayed an obviously inflamed skin tissue phenotype, we next wanted to see if RAS expressed in terminally differentiated cells preferably activated a distinct signaling pathway downstream from RAS that could be linked to specific cytokine expression patterns. Previous studies have shown that blocking EGFR or ERK signaling during TNF-α, TGF-α or IFN-γ treatments of primary human keratinocytes switched downstream signaling cascades toward p38 and JNK

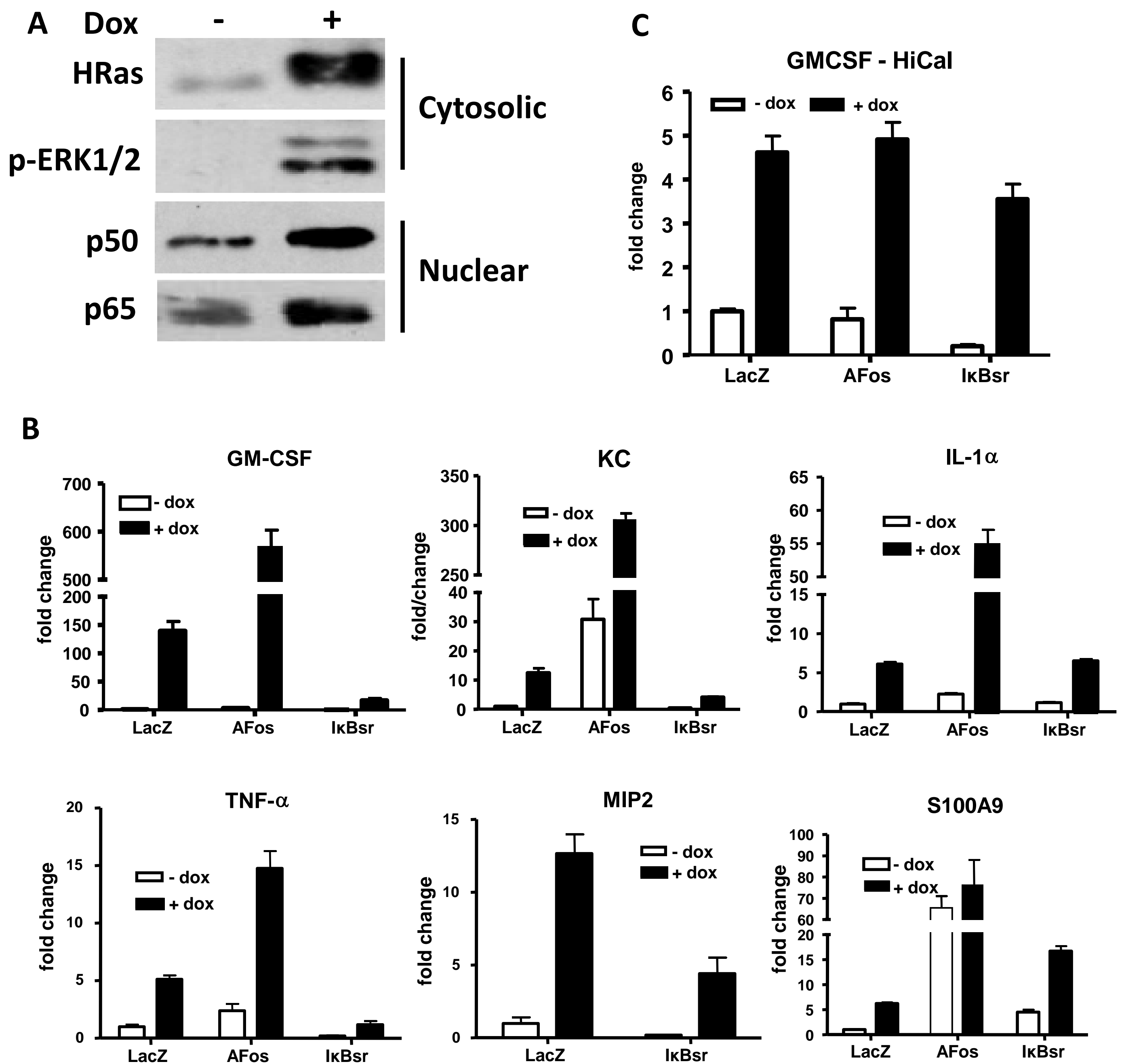
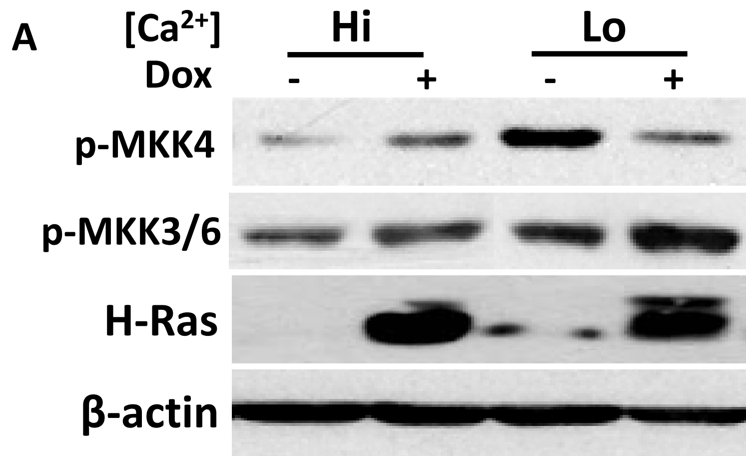


Figure 5-7: RAS activated NFκb is required for cytokine upregulation while AP-1 is repressive in basal keratinocytes. A) Western blots on fractionated cytosolic and nuclear protein lysates isolated from K14Ras LoCal keratinocytes 3 hours after dox treatments for the NFκb subunits p50 and p65. B) Adenoviral vectors carrying LacZ control or the AP-1 and NFκb dominant negative genes A-FOS and IκBsr were infected into keratinocytes 24 hours prior to dox treatments in LoCal media. RNA was harvested 24 hours later, made into cDNA and analyzed by QPCR of specific mRNA transcripts. C) As in B, except keratinocytes were cultured in HiCal media for 24 hours prior to adenoviral transduction and maintained in HiCal media for the duration of the experiment.



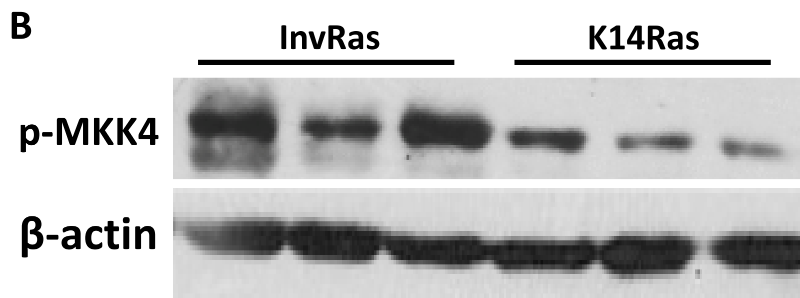


Figure 5-8: RAS expression in differentiated cells may selectively activate the JNK pathway over p38 or ERK1/2. A) Western blotting for the activated forms of the JNK kinase MKK4 and the p38 kinase MKK3/6 on protein lysates from Lo/HiCal keratinocytes +/- dox for 24 hours. B) As in “A”, immunoblotting for p-MKK4 and loading control on InvRas or K14Ras total skin lysates. Non-ras expressing control skin had negligible MKK4 activation.

mediated upregulation of CCL2, CCL5, and CXCL10 and suppression of CXCL8 (16;17). Indeed, blotting for the kinases directly upstream from JNK and p38 revealed that expression of RAS in basal/proliferating keratinocytes blocked MKK4 expression while expression of transgene in differentiating keratinocytes enhanced MKK4 phosphorylation (Fig 5-8A). Increasing $Ca2^{+}$ concentrations also blocked RAS activation of MKK3/6 indicating multiple MAPK pathways were non-responsive to downstream Ras signaling in differentiated cells (Fig. 5-8A). We also observed reduced levels of p-MKK4 in K14Ras total skin lysates compared to InvRas skin, supporting the concept of differential pathway activation dependent on differentiation status (Fig. 5-8B). However, as total MKK4 and MKK3/6 protein levels have not yet been determined, we cannot exclude the possibility that increases in phosphorylated levels may represent upregulation of these genes in InvRas skin and differentiated keratinocytes. Since the culmination of JNK signaling is c-Jun transcriptional activity these results suggest oncogenic RAS signal transduction preferentially activates this MAPK cascade in differentiated cells over the RAF-MEK-ERK pathway that basal RAS expression selects. In turn, this difference in RAS signaling may be one reason why InvRas mice provoke a cytotoxic neutrophil inflammatory response and have a benign tumor phenotype and K14Ras expression induces an immunosuppressive monocyte response that contributes to a greater risk of SCC development.

5.3 Discussion

In this chapter, we show preliminary evidence for specific inflammatory responses unique to oncogenic RAS expression in basal and post-mitotic epithelial layers. These data, although intriguing, require greater experimental proof that can definitively link a particular myeloid inflammatory response to a greater risk of malignant progression. It is likely that

suprabasal RAS expression has paracrine effects on basal keratinocytes in the InvRas model. Indeed, proliferation data in chapter 2 showed that most of the BRDU positive cells in InvRas dorsal skin are constrained to the basal layer. The study on Inv-MEK1 mice demonstrated that epidermal proliferation increased when transgenic mice were crossed with a K14-IL-1R1 mouse where the IL-1 receptor is overexpressed in the basal layer (18). Furthermore, Inv-MEK1 wound induced tumor onset was decreased by application with an IL-1R antagonist supporting the concept that paracrine activation of basal cells by suprabasal derived cytokines is a necessary component to stimulating proliferation in the non-oncogenic layer (9). Conversely, targeting a MYC oncogene to the Involucrin promoter causes loss of differentiation and proliferation to the suprabasal keratinocytes MYC is activated in (19). Whether InvRas expressing keratinocytes are de-differentiating over time with RAS expression is an important concept that has yet to be determined in our model.

Subunits of AP-1 dimers have variable expression patterns in the epidermis depending on the differentiation status and their role in mediating keratinocyte terminal differentiation has been well studied (20). Interestingly, in murine epidermis Fos and Fra-1 are both expressed in the basal layer and whereas only Fra-1 is predominantly expressed in the suprabasal layer (21). Furthermore, whereas Fos is primarily a positive regulator of gene transcription, Fra-1 has been shown to be largely repressive (22;23). Both subunits are activated upon v-ras transduction in keratinocytes indicating both play roles in epidermal transformation (24). Our data shows that in basal keratinocytes, NFκb is activated in response to *H-RAS*^{G12V} expression and mediates RAS induced cytokine gene upregulation but AP-1 blockade has the reverse effect. This likely implies that although RAS activates both transcription factors, they have opposite functions on inflammatory gene transcription. Cataisson *et al* demonstrated that the IκBsr could block KC

and MIP2 expression in v-ras infected basal keratinocytes (25). This same group also showed an A-FOS transgene could not block the inflammatory skin phenotype in TPA treated K5-PKCα mice and enhanced pro-inflammatory gene expression *in vitro* in response to TPA (26). Introduction of the IκBsr in a subsequent study did reverse the effects of PKCα hyperactivity (27). Since Ras activates PKCα in keratinocytes (28) these results are directly applicable with ours. PKCα is also a well known upstream activator of NFκb which would link RAS to NFκb activity in our model (29). Because AP-1 is indeed repressive to cytokine transcription, it would make sense that suprabasal epidermal layers would have a decreased potential to increase gene expression because Fra-1 would be expressed and JNK signaling is preferably activated in differentiated keratinocytes. MKK4 activation was also decreased in basal RAS expressing keratinocytes in our model but in another study conditional K14-Cre mediated epidermal ablation of MKK4 resulted in reduced tumor kinetics in the 2-stage CC assay suggesting greater complexity during tumorigenesis linked to this MAPK pathway (30).

Regulation of cytokine expression by ERK1/2 in keratinocytes has been exquisitely demonstrated in human keratinocytes where ERK1/2 activation by TGF-α treatment coordinated high CXCL8 expression but suppressed CCL2, CCL5, and CXCL10 (16). The reverse effect occurred when EGFR signaling was blocked in TNF-α treated cells through a mechanism of increased mRNA stability of the latter 3 chemokines that ultimately lead to qualitatively distinct inflammatory responses. This novel study indicates that in the context of inflammation, constitutive ERK1/2 activity can serve as an inflammatory rheostat by regulating keratinocyte cytokine/chemokine output. The potential role of EGFR signaling in this study and ours cannot be ignored. RAS is the immediate downstream signaling effector of EGFR stimulation and EGFR and its ligands are potently upregulated during skin carcinogenesis, wound healing and

psoriasis (31;32). RAS itself induces EGFR ligand synthesis formulating a critical growth factor autocrine loop that likely effects cytokine expression as well (33). Supportively, GM-CSF expression by TNF-α or IFN-γ treatment of human keratinocytes was potentiated by TGF-α co-treatment (34). Tumor growth from grafts of v-ras infected EGFR null primary keratinocytes was markedly reduced, evidence for the critical role of *in vivo* EGFR signaling during squamous tumor progression (35).

Taken together, our data establishes an important extrinsic contribution to malignant progression in squamous tumors where oncogenic cytokine signaling is determined by the differentiation status of the transformed epithelial cell. Chronic suppression of adaptive immunity by MDSC in basal/stem cell driven epidermal cancer would remove the immunosurveillant checkpoints that would be intact in tumors with a suprabasal mutation thus providing a fertile environment for progression to SCC. Future studies will be critical to confirm if specific intracellular signaling pathways in cancer cells leading to MDSC formation do exist in order to selectively antagonize them to enhance anti-tumor immunity and reduce risks of malignant conversion. Current therapeutic attempts at mitigating RAS signaling in the treatment of malignant disease include targeting B-Raf kinase in melanoma (36), RAS post-translation farnesylation in hematologic cancers (37), EGFR blocking mAb and tyrosine kinase inhibitors in various solid tumors (38), MEK1/2 (39), and PI3K (40). Our findings could have broad implications for the diagnosis and treatment of multiple cancer types arising in multilayered stratified epithelium where stem cells are thought to contain the greatest malignant potential.

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Chapter 6: Conclusion and Future Direction

6.1 Discussion

The data presented herein provides strong evidence for the putative tumor promoting roles of lymphocytes in oncogenic RAS initiated skin carcinogenesis. Although similar studies have been carried out before, the genetic nature of the RAS transgenic tumor models and direct examination of primary immune cell interactions *in vivo* indicate that lymphocytes exert tumor-promoting actions in physiologically relevant tumor settings during the onset of inflammation in pre-malignant stages. These contextual differences are worth noting because solid human tumors are not solely composed of genetically monoclonal cancer cells that autonomously regulate sustained proliferation, evasion of apoptotic and senescent pathways, and metastatic dissemination. Instead, malignant tissue masses arise utilizing proximal contributions from normal somatic cells that include resident and infiltrating leukocytes, stromal fibroblasts, endothelial cells and pericytes. Together, these cells form an accessory relationship with transformed cells that is needed to achieve the defined hallmarks of cancer (1). It should be reiterated that similar biology to that demonstrated in these studies has been observed in analogous skin tumors models where $CD8^+$ T cells and B cells (independently) were required for 2-stage chemical carcinogenesis and B cells during K14-HPV16 driven squamous progression (2-5). The fact we have supported these claims using our RAS transgenic models leads us to believe that there may be tissue specificity in determining standardized immune responses to pathological events rather than disease specific mechanisms.

When closely examining the kinetics of our squamous tumor study, the removal of $CD8^+$ T cells caused a greater latency of tumor onset only during the early stages of development. Conversely, during later timepoints, CD8-depleted DT/*Rag1*+/+ mice demonstrated a rapid

recovery of total skin tumor burdens. Additionally, CD8 repletion of DT/*Rag1-/-* mice initially stimulated tumor outgrowth similar to DT/*Rag1+/+* mice, albeit for a short duration, thus confirming the requirement for additional lymphocyte subsets to sustain papillomatous outgrowth. Intra-tumoral neutrophil infiltration in these groups also mirrored that of DT/*Rag1+/+* cohorts implying that the acute inflammatory skin biology is extrapolated to malignant stages. This suggests that the pro-inflammatory effects of $CD8^+$ T cells promote early pre-malignant proliferation of cancerous keratinocytes requisite for the nascent appearance of benign lesions but potentially transition to an inhibitory phenotype later on. Alternatively, the type of myeloid cell (neutrophil) that $CD8^+$ T cells specifically affect may be distinct from that of other lymphocyte lineages whereby neutrophils only serve purpose for inducing acute epidermal proliferation but do not greatly contribute to other tumor tissue alterations like stromal remodeling and angiogenesis; events required predominantly in later stages of malignancy. As we observed similar cytokine profiles in $CD8^+$ T cells infiltrating acute inflamed skin as well as those isolated from endpoint tumors, the latter hypothesis seems more plausible. Girardi and colleagues did provide experimental evidence for the separation of two $CD8^+$ T cell phenotypes showing co-expression of IFN-γ, IL-17, and IL-10 simultaneously occurred with downregulation of perforin during 2-stage chemical carcinogenesis; a required phenomenon for the promotion of tumor volume and malignant conversion (2;3). Similarly, $CD8^+$ T cell cells conditioned in strong Tc17 polarizing conditions (TGFβ, IL-6, IL-23, IFN-γ, IL-1β, α-IL4) potently expressed IL-17 and TNF-α but not granzyme B, perforin and FAS ligand (6). It should be noted, however, that a separate *in vitro* conditioning study using only TGFβ and IL-6 to polarize $CD8^+$ T cells into Tc17 phenotypes did result in cytotoxic Tc17 effectors with comparable granzyme B expression to Tc1 polarized cells (7). We did not, however, examine expression of cytolytic

effector molecules such as granzyme or perforin so comparing the pro-inflammatory versus cytotoxic potential of these cells cannot presently be concluded. Other further interest was the result that only high dose but not low dose TPA treatment during chemical carcinogenesis was able to provoke disparate papilloma volumes and malignant conversion ratios in *CD8-/-* and *TCRβ-/-* vs. WT FVB/n mice (2;3;8). This may suggest that $CD8^+$ T cell functionality and phenotypes may depend on the intensity of the inflammatory microenvironment. High dose phorbol ester application and RAS overexpression per our model would likely qualify for such environments. If there indeed is a distinct functional difference between pro-inflammatory Tc17 and cytotoxic Tc1 cells, it will be imperative to categorize these differences so targeted therapies aimed at phenotypic favoritism could be developed depending on disease context.

In support of the hypothesis that skin tumor progression requires multiple lymphocyte lineages activating different myeloid sets, the studies in the K14-HPV16 model clearly linked IgG production by B cells with recruiting and activating angiogenic pathways in mast cells and macrophages that led to the enhancement of dysplastic cutaneous architecture (5;9). Meanwhile, B cell reconstitution of *Rag2-/-* mice only partially recovered DMBA/TPA squamous tumors (4) again supporting the claim that B cells *and* $CD8^+$ T cells are required for maximal squamous tumor formation. $CD8^+$ T cells can express a wealth of genes that directly affect neutrophil egress and respiratory burst including IFN-γ, TNF-α, GM-CSF, G-CSF and IL-3 providing a direct link between the neutrophilia and cytotoxic activities of skin infiltrating neutrophils controlled by RAS induced pro-inflammatory $CD8^+$ T cells. Since CD8 reconstitution only recovered neutrophilic and not mast cell inflammation in DT/*Rag1-/-* acute hyperproliferative skin and papillomas, our data also fits with this dual lymphocyte hypothesis. The study implicating $CD8^+$ "T-pro" cells did not compare 2-stage CC tumor development in *TCRβ-/-* or

CD8-/- mice to that in total lymphocyte deficient *Rag-/-* groups preventing a direct comparison between these two studies. In the future, it will be necessary to reconstitute DT*Rag1-/-* mice with purified $CD8^+$ T and B lymphocytes to experimentally validate this postulate.

Only two human cancer studies to date have implicated a causal role for pro-inflammatory Tc17 cells. Kuang *et al* observed a high proportion of Tc17 cells in the invading edges of hepatocellular carcinomas where these cells also produced IFN-γ, TNF-α and were again largely deficient in perforin and granzyme B (10). Ji *et al* also FACS analyzed 21 nasopharyngeal carcinomas and discovered increased percentages of TNF-α/IFN-γ secreting Tc17 cells (11). The idea that the pro-inflammatory activities of $CD8^+$ T cells can lead to augmentation of pathological disease states is a relatively new concept in the field of tumor immunology. Because of their obvious significance in directly mediating cytotoxic effector mechanisms, these cells were likely overlooked for their potential to cause opposable functions in tumors. We argue, however, that $CD8^+$ T cells inherently contain all of the same capabilities and effector mechanisms observed during any given inflammatory cascade regardless of disease classification. In this light, the histological and immunological similarities between psoriasis and our InvtTA/tetORas mouse model should not be ignored. A hyperproliferative basal epidermal layer, acanthosis, neutrophil "Munro" microabcesses, activated T cell infiltration, IL-17/IL-23 production, and residency of diverse myeloid cell subpopulations are all key features shared between our model and chronic inflammatory diseases of the skin (12). Indeed, the etiology of the autoimmune condition, psoriasis, is widely believed to be driven by the chronic infiltration of IFN-γ and/or IL-17 secreting Th and $CD8^+$ T cells that drive abnormal tissue accumulation of activated neutrophils. Both CD3 depletion and experimentation on a *Rag2-/-* background greatly attenuated the disease scoring during imiquimod induction of a psoriasis-like phenotype in mice

(13). IL-17^{+}/CD8^{+} cells but not Th17 cells were overrepresented in human psoriatic plaques (14) supported by a similar study that showed these cells could also produce TNF-α and IFN-γ while exhibiting cytotoxic behavior *ex vivo* (15). Th1 and IFN-γ^{+} Tc cells are increased in the lesional epidermis and blood of psoriasis patients (16;17). And perhaps the most compelling evidence linking the immunobiology of psoriasis and skin cancer is that α-TNF-α (Etanercept) therapy is highly effective at alleviating psoriasis plaque pathology (18) and *TNF-α-/-* mice are resistant to chemical carcinogenesis (19). Yet, with all of the data linking chronic inflammation to the risk of cancer development, psoriasis persists as one of the lone examples of a negative corollary between pre-existing inflammation and malignant onset (20). We propose the reasons for this are two-fold: 1) Cells expressing a mutationally activated oncogene or deactivated tumor suppressor disparately respond to cytokines and immune derived factors than those of the same cell type without a mutation; 2) Epithelial cells of different tissue origin inherently contain a predetermined cytokine/chemokine expression potential that may depend on constitutive PAMP signals from symbiotic microfluora specific to that microenvironment.

There is experimental evidence to support these hypotheses. TNF-α and IFN-γ treatment of normal primary keratinocytes leads to irreversible activation of growth arrest pathways that involves upregulation of p16 and p21 (21). But in the context of chronically inhibited growth arrest, as would be the case with E6/E7 transgenic blockade of p53 and Rb in the K14-HPV16 model, cytokine signaling may lead to very different cellular responses. For instance, NFκb is commonly regarded as a master regulator of cytokine/chemokine gene transcription but its paradoxical roles in regulating keratinocyte cell cycling, cutaneous inflammation and skin homeostasis make it unique when examining biology specific to the skin. In normal basal keratinocytes, NFκb is activated in response to UV stress (22), cytoplasmic in proliferating basal

keratinocytes but nuclear in differentiated suprabasal layers (23) and prevents proliferation by inducing p21 dependent growth arrest in numerous studies in vitro and in vivo (24-26). RAS transformation of NIH-3T3 fibroblasts is dependent on NFκb transcriptional activation (27) by suppressing oncogenic RAS activated apoptotic pathways independent of p53 (28). Transgenic overexpression of a dominant negative form of IkBα (IkBαSR) targeted to the basal layer of the epidermis leads to super repression of NFκb nuclear translocation causing spontaneous SCC development and dichotomously, increased epidermal apoptosis (29). Furthermore, this forced prevention of NFκb translocation by degradation resistant IκBα transgenic expression or genetic ablation of epidermal IKKβ surprisingly results in significant TNFR-dependent skin inflammation despite the absence of this major pathway to cytokine transcriptional activity (30;31). Intriguingly, both models also required macrophages to initiate and sustain this inflammation (32). Because of NFκB's pro-survival roles in epithelial cells, it has been proposed that the increased apoptotic cell death may be the trigger for the influx of inflammatory phagocytes. In direct opposition to the studies, K5Cre-IκBα$^{fl/fl}$ mice yielded almost identical skin inflammatory phenotypes suggesting that both persistent inhibition *and* activation of NFκb signaling in keratinocytes redundantly provokes cutaneous inflammation; although the mechanisms for this contradiction may be distinct (33). Finally, retroviral transduction of H-Ras^{G12V} concomitantly with dominant negative IκBα in human keratinocytes overcame Ras activated p15/p16/p21/Rb mediated growth arrest that introduction of oncogenic Ras could not achieve alone, demonstrating that blockade of NFκb senescent signaling was required for SCC development driven by a Ras oncogene (34). Taken together, these studies indicate that in skin tumors that have inactivated tumor suppressor pathways, persistent NFκb activation by the presence of immune derived pro-inflammatory cytokines could lead to positive reinforcement of

intrinsic cytokine/chemokine expression without the ability to simultaneously activate growth arrest in cancer cells. Therefore, cytokine signaling in keratinocytes during the steady state may result in very different biological outcomes depending on the mutational status of key cell cycle checkpoints.

Regarding involvement of commensal microorganisms and the probability for tissue specificity, chronic TLR signaling on colonic epithelia has become a recognized regulatory mechanism for gut homeostasis (35). Similar to skin keratinocytes, IKKγ/NEMO genetic ablation targeted to intestinal epithelial cells ($NEMO^{IEC-KO}$) results in TNFR1-dependent colitis (36). However, crossing $NEMO^{IEC-KO}$ mice onto a MYD88 deficient background greatly attenuated inflammatory disease progression demonstrating a requirement for TLR signaling in causing this inflammation (37). Intriguingly, MYD88 knockout mice are also more resistant to 2-stage chemical carcinogenesis skin tumorigenesis, strong evidence for the direct role TLR signaling can play in promoting skin tumor growth (38). In a similar manner deactivation of NFκb signaling in liver parenchymal cells (LPC-hepatocytes and bile duct epithelial cells) by IkBαSR expression or genetic knockout of IKKβ/IKKα, NEMO, or p65 leads to increased sensitization to TNF-α or LPS induced acute liver damage (39-41). LPC-targeted *NEMO-/-* mice also spontaneously develop chronic hepatitis as a prerequisite to HCC onset (42). Conversely, deploying similar methods of inhibiting NFκb signaling in CNS tissue (43), muscle (44), and pancreas (45) resulted in ameliorated inflammatory conditions in those tissues. The most logical theory for these tissue specific differences is that the liver, skin, and intestine are organs exposed to environmental stimuli such as toxins and microbial products whereas muscle, brain, and pancreas are relatively protected. As symbiotic microorganisms express a variety of different PAMPs (CpG, LPS, ss/dsRNA, bacterial flaggelin, fungal zymosan) each activating a specific

TLR subtype, it is plausible that inherent epithelial TLR signaling activated by tissue specific commensal (or pathogenic) microbiota may affect the outcomes of carcinogenic inflammatory signaling unique to certain microenvironments.

Furthermore, we are not excluding the demonstrable effects of $CD4^+$ Th cells, more specifically Th17 subsets. Depletion of CD4 cells proved virtually ineffectual in regards to acute epidermal hyperproliferation, mast cell residency and neutrophil inflammation, contrasting with CD8-depletion (Fig. 3-7). However, as we did not assess transgenic tumor development in CD4-depleted DT mice, their fundamental contributions during RAS driven skin carcinogenesis cannot fully be analyzed at this juncture. Since skewing of $CD4^+$ helper T cell lineages has a more established paradigm of functional diversity, depleting all CD4 cells may yield ambiguous results. Both lymph node Tregs and skin resident Th17 cells significantly increased upon transgenic RAS activation and these two cell types have counteracting and opposable functions. Interestingly, skin carcinogenesis on FVB/n *CD4-/-* mice using a low dose TPA promotion protocol indicated $CD4^+$ T cells were actually inhibiting squamous tumor formation (2), conflicting with results gathered from the same experiment performed on C3H/HeN strains (46). We also observed that depleting total CD4 cells resulted in enhanced CD8 activation suggesting that anti-inflammatory dampening by Tregs may be responsible for this. The study by Denardo *et al* showed that $CD4^+$ T cells were dispensable for primary tumor growth in MMTV transgenic mice and only affected metastatic disease progression (47), further supporting a tumor stage specific hypothesis on the pro-tumorigenic effects of $CD4^+$ T cells. In addition, Th17 cells may play an important role in enhancing CTL tumor immunity. Removal of $CD8^+$ T cells in our model diminished Th17 differentiation which implies that some of the decreased tumor promotion in those mice may be due to a lack of Th17 activity. Their adoptive transfer into B16

bearing mice was proportionally more effective than Th1 cell transfer at provoking tumor specific CTL activity (48). In a Pan02 pancreatic mouse model, Th17 skewing led to delayed tumor growth (49). In humans, Th17 intratumoral levels in ovarian cancer patients correlated with increased survival by inducing CXCL9/10 recruitment of NK and $CD8^+$ T cells (50) and slower disease progression has also been linked in prostate (51), lung adenocarcinoma, and SCC (52). In sum, depleting total CD4 cells in our skin tumor model may not lead to the systematic mechanistic dissection required to elucidate a putative immunological role for $CD4^+$ T cell subsets in our model. A better method would be reconstitution of *CD4* or *Rag1-/-* mice with purified $CD4^+$ T cells genetically deficient in Th polarizing and effector genes such as *Foxp3* (Treg), *RORγT* and IL-17 (Th17), IL-4 (Th2), and IFN-γ (Th1).

Healthy and effective immune systems are able to deactivate immune responses just as quickly as they mount them. In addition to Tregs and Bregs, expansion and activation of immunosuppressive myeloid populations seems to be a common self-regulatory mechanism employed by the immune system during periods of potentially harmful inflammation. Their evolutionary purpose seems logical as the chronic hyperactivation of T lymphocytes would certainly lead to life threatening allergy and autoimmunity. Cancers, in their unique and seemingly intuitive ability to hijack homeostatic immunological processes, take advantage of MDSC biology by chronically suppressing immunosurveillant lymphocytes that seek to limit tumor cell proliferation and malignant progression. Tumor microenvironments are endless and abundant sources of chemokines such as CXCL1/2, CCL2, CCL7, CCL5, CSF-1/2/3, S100A8/9 that ensure the constant hematopoietic expansion and infiltration of myeloid cells. However, since dendritic cells, macrophages, and neutrophils are important innate immune effectors in shaping and activating cytotoxic adaptive responses to cancer cells, attempting to completely

shut down these pathways to prevent further tumor growth seems counterproductive. Furthermore, MDSC are by definition only a functional description of their phenotype, not a separate myeloid lineage and are likely immature myelocytes stuck in various stages of differentiation due to the cytokine environments they are exposed to. A more rational approach would be to target immunosuppressive pathways or effectors thereby not only inhibiting T cell non-responsiveness but also potentially reversing the grip on myeloid cell differentiation. Indeed, clinical research trials have aimed at reversing the immature and suppressive phenotypes of MDSC. Vitamin D3 treatments on HNSCC bearing patients effectively caused the differentiation of $CD34^+$ iMC, thereby improving $CD8^+$ T cell responses, although clinical tumor responses were not tested (53). In a study on renal cell carcinoma patients, all-trans-retinoic acid (Vitamin A) produced similar observations (54) and can directly cause in vitro MDSC differentiation into macrophages and dendritic cells (55). Treatment with sildenafil-citrate, a PDE5 inhibitor, resulted in downregulated immunosuppressive markers Arg1, iNOS, and IL4Rα in MDSC of tumor bearing mice which lead to greater CD8-mediated inhibition of tumor growth. When HNSCC and myeloma bearing patients were treated with sildenafil in the same study, proliferation of *ex vivo* stimulated CD4/CD8 T cells significantly increased (56). Use of a nitro-aspirin adjuvant increased cancer vaccine elicited CTL responses by directly inhibiting iNOS and Arg1 enzymatic activity in MDSC (57). Utilizing the inducibility of our RAS transgenic system we provide proof that the immunosuppressive capability of MDSC may be manipulated without affecting other facets of myelocytic inflammation. Whether this effects tumor development or not in our model will need to be tested in the future.

The broad supportive role B cells may play, not only in skin carcinogenesis but also for the development of other solid tumors, should draw wide therapeutic interest since anti-CD20

therapy is relatively safe and B cell depletion is an effective method of alleviating autoimmune disease progress. Our studies, however, suggest that a specific CD20-resistant Breg population may significantly contribute to the pro-tumorigenic properties of monomyelocytic cells thus ruling out Rituximab as an adjuvant therapy when seeking to decrease the immunosuppressive affects of MDSC. Based on other reports in conjunction with our findings, it is tantalizing to speculate that B cells are intimately involved in the progression of a wide variety of cancers and not just those limited to the skin. The majority of human cancer patients mount specific autoantibody responses raised against their tumors (58). Since autoantibody production is attributed to the $CD5^+$ B-1 subset of B lymphocytes, it seems plausible to hypothesize that most of the pro-tumorigenic properties of B cells are restricted to regulatory populations. The aforementioned studies on the K14-HPV16 and 2-stage CC skin tumor models support this idea (4;9). Malignant xenografts on B cell deficient mice in two different studies produced decreased tumor growth and increased CTL anti-tumor immunity (59;60), pro-tumoral M2 macrophage phenotypes were influenced directly by Bregs in two other studies (60-62), and the anti-inflammatory polarizing effects of IL-10 on monomyelocytic leukocytes are well understood (63). Perhaps this represents B cell biology unique to conditions of "sterile" inflammation where an immune system would have no imperative to eliminate a pathogenic microorganism and instead would favor resolution of acute inflammation to avoid harmful, chronic immune activation. IL-10 expressing Bregs are significantly expanded in numerous autoimmune models and dampen contact hypersensitivity reactions (64) and EAE disease progression (65). IL10 null mice spontaneously develop inflammatory bowel disease (66) a disease where increased numbers of tissue resident $IL10^+/CD1d^{hi}$ B cells have been found and suppress disease progression through modulating IL-1 and STAT3 signaling (67). Transfer of anti-CD40 and

collagen activated B cells from IL-10 WT but not KO mice prevented CIA development in mice, a T cell-dependent model of rheumatoid arthritis (68). Finally, the transfer of B cells into NOD mice conferred protection from type 1 diabetes, a phenotype that again IL10-/- B cells could not recapitulate (69). This is in stark contrast to the overwhelming amount of evidence suggesting B cells are critical components of initiating and perpetuating autoimmune diseases (70). Excessive production of self-reactive immunoglobulins are no doubt a recognized pathogenic mechanism of exacerbating autoimmunity and CD20 depletion is used to abrogate symptoms stemming from systemic lupus erythematosus (SLE), arthritis, and other autoimmune pathologies (71). However, as plasma cells and other mature B2 subsets are the predominant producers of immune stimulating antibodies, removing these populations while letting Breg subsets persist may account for Rituximab's therapeutic efficacy.

The capability of Bregs to administer their activity in vivo is extraordinary when calculating how many IL-10^+ B cells we transferred into DT/*Rag1-/-* mice while still achieving the acquisition of immunosuppressive functionality in Ly6C^{hi} monocytes. We transferred 5 million total naïve B cells of which only ~ 3% (150,000) were theoretically IL-10^+. The other study confirming these data also needed to transfer relatively small numbers of IL-10 expressing $CD5^+$/CD1d^{hi} Bregs (200,000) to promote lymphoma growth where the TLR stimulators LPS, CpG, and PolyI:C effectively reversed the IL-10 induced anti-inflammatory M2 phenotype. These data demonstrate the impressive potency of IL-10 on mononuclear phagocytes. As Rituximab therapy requires FcγR-dependent ADCC of B cells by macrophages/monocytes and not NK cells (72) and IL-10 upregulates expression of inhibitory FcγRIIB (with an ITIM domain) on these cells (73) while downregulating pro-inflammatory properties, it makes sense that by observing the inability of mononuclear cells to deplete α-CD20 bound Bregs that they

would then also be primed for immunosuppressive functionality when acute inflammation was invoked by RAS expression. IL-10R signaling primarily activates STAT3 (74) and this transcription factor is a vital player in mediating MDSC biology. The prevention of iMC differentiation into dendritic cells by tumor cell conditioned media was shown to be mediated through a STAT3 mechanism (75). The same group subsequently showed that pharmacological inhibition of STAT3 *in vivo* could enhance anti-tumor immunity and slow tumor growth (76). Moreover, exosomes derived from tumors induced immunosuppressive pathways in MDSC via activation of STAT3 and siRNA knockdown of STAT3 in MDSC abrogated their *in vitro* suppressive ability (77). The most definitive study implicating STAT3 and MDSC biology came from Kortylewski *et al* where conditional genetic ablation of STAT3 targeted to bone marrow cells allowed greater IL-12 production and antigen presenting functions in dendritic cells, increased neutrophil cytotoxicity, decreased intratumoral Treg infiltration, enhanced antigen specific CD8 responses, and ultimately reduced T cell dependent tumor growth in mice with *STAT3-/-* bone marrow (78). We note that the direct influence of Bregs on monocytes/macrophages is not specific to RAS expressing mice as CD20 depletion in ST littermates resulted in persistence of a similar proportion of CD20-resistant B cells. However, Ly6C^{hi}/Ly6G^{-} monocytes isolated from normal mice do not inhibit T cell proliferation in vitro suggesting that IL-10 priming events are necessary but not sufficient for the RAS induced appearance of MDSC populations. We propose that a series of coordinated events leads to the genesis of MDSC where hematopoietic expansion, prevention of differentiation, upregulation of immunosuppressive effector genes, and tissue chemotaxis are governed by independent processes and paracrine mediators. The data in this thesis provides evidence for just one of those steps. It will be necessary in future studies to explore the mechanism of how B cells activate

immunosuppressive Ly6C^{hi} monocytes through the use of IL-10 deficient B cell transfer and performing a molecular characterization of the putative MDSC population for distinct gene expression and signaling alterations dependent on B cell derived factors.

The logical implication that leukocytes play a causal role in promoting or impeding solid tumor progression is ostensibly realized when surveying the myriad of immune targeted therapies currently approved or being explored for the treatment of malignant diseases (79). Indeed, induction of anti-tumor immunity has long been considered the gold standard for cancer therapy because of the adaptive and self-sustaining nature and establishment of sentinel memory cells that would continually provide cytotoxic elimination of cancer cells. Cancer vaccines would theoretically be the most rational approach to achieving this goal as permanent antigenic education would result from the boosting of specific tumor antigens exposed only during the onset of tumors even when the antigen is technically of "self" origin. A multitude of T cell recognized tumor antigens have been discovered although the therapeutic viability varies greatly (80). Prostatic acid phosphatase administered to prostate cancer patients improved the median survival by 4 months compared to groups with placebo treatments (81;82). Antigen loaded dendritic cells injected intratumorally provoked tumor specific immune responses in ~50% of sarcoma bearing patients (83). However, a recent comprehensive analysis of all stage 3 vaccine clinical trials since 2004 catalogued CR/PR responses in only 3.6% of 984 patients, sobering results by any measure (84). The reasons for these failures can likely be attributed to the chronic presence of immunosuppressive cells that would counteract any acute increases in CD8 reactivity. Without also reversing the cytokine induced stranglehold tumors have on innate immune phenotypes that are notoriously Th2/M2/Treg biased, therapy targeted solely at enhancing CTL immunoreactivity and tumor antigenicity will likely be futile. In light of these

circumstances, depleting Tregs in melanoma patients with an IL-2/diphtheria toxin conjugate (85) or anti-CTLA antibody (86) has yielded encouraging results when combined with vaccine delivery in two different trials. Furthermore, Kao and colleagues used the tyrosine kinase inhibitor Sunitinib to reverse the effects of MDSC in patients with oligometastasis and achieved responses in 59% of treated patients (87). Alternatively, to circumvent the pitfalls of the immunosuppressive environments tumors create on cytotoxic $CD8^+$ lymphocytes, researchers have employed adoptive transfer of in vitro conditioned, autologous $CD8^+$ CTLs transduced with chimeric antigen receptors (CAR) with stronger antigenic specificity. Most of the success has come from CD19 targeting for the treatment of CLL (88;89). However, despite the potency in killing systemic leukemic B cells and reducing bone marrow tumor seeds, the major side effect has been autologous autoimmune reactions resembling graft versus host disease (GVHD). This subsequently requires immunosuppressive treatments that in turn allow for re-establishment of the B cell neoplasm (90). This method of immunotherapy seems promising, but the threat of dangerous autoimmune reactions requiring a lifetime of pharmacological dependence to continually boost and dampen CTL activity runs counter to the goal of generating self-sustaining adaptive memory and immuno-regulation.

The combination of immune targeted adjuvant therapy with the cytotoxic protocols of chemotherapy and radiation is proving synergistically effective in reversing immunosuppression in tumors (91). CT and RT are extremely adept at activating cell death pathways, especially in rapidly dividing cancer cells, and in turn provoke the immune system to clean up the dead tissue. However, normal immune programming instructs macrophages to formulate an M2 phenotype to heal the new “wound”, thus preventing effective anti-tumor immunity to persist and protect cancer patients from malignant recurrence. In support of this, a novel breast cancer study

demonstrated that inhibition of CSF1R signaling in conjunction with paclitaxel chemotherapy synergistically reduced tumor burdens in MMTV mice (92). The mechanism behind this was inhibiting TAM infiltration and macrophage reprogramming from M2 to M1 phenotypes, allowing for greater CD8 tumor reactivity. Additionally, a CD40 agonist/gemcitabine dual regimen led to M1 mediated tumor cytotoxicity and partial tumor regression (93). Immune reprogramming therapies therefore would especially be useful in overcoming a permanent pathological immune status when cytotoxic chemo and/or radio-therapy leaves necrotic tumor tissue capable of recalling "tissue healing" immunological responses.

In sum (Fig. 6-1), the data presented in this dissertation elucidates novel immune mediated mechanisms for the promotion of skin carcinogenesis during complex pre-malignant inflammatory processes. Through the paracrine actions of IFN-γ, pro-inflammatory $CD8^+$ T cells stimulate neutrophilic cutaneous inflammation leading to enhanced keratinocyte proliferation and IL-10 producing Bregs activate specific immunosuppressive pathways in $Ly6C^{hi}$ monocytes that dampen T cell activation in mouse models of epidermal $H\text{-}Ras^{G12V}$ expression. Additionally, the unique inflammatory responses to proliferating and non-differentiating keratinocyte driven RAS expression may contribute to malignant progression where chronic inhibition of T cell activation induced by the basal/stem cell compartments correlates with a higher risk for SCC development. We do not believe these observations of $CD8^+$ T cell and B cell biology are entirely limited to the context of epidermal oncogene expression but likely have broader implications for understanding regulatory immunocellular relationships during any given acute and chronic inflammatory cascade.

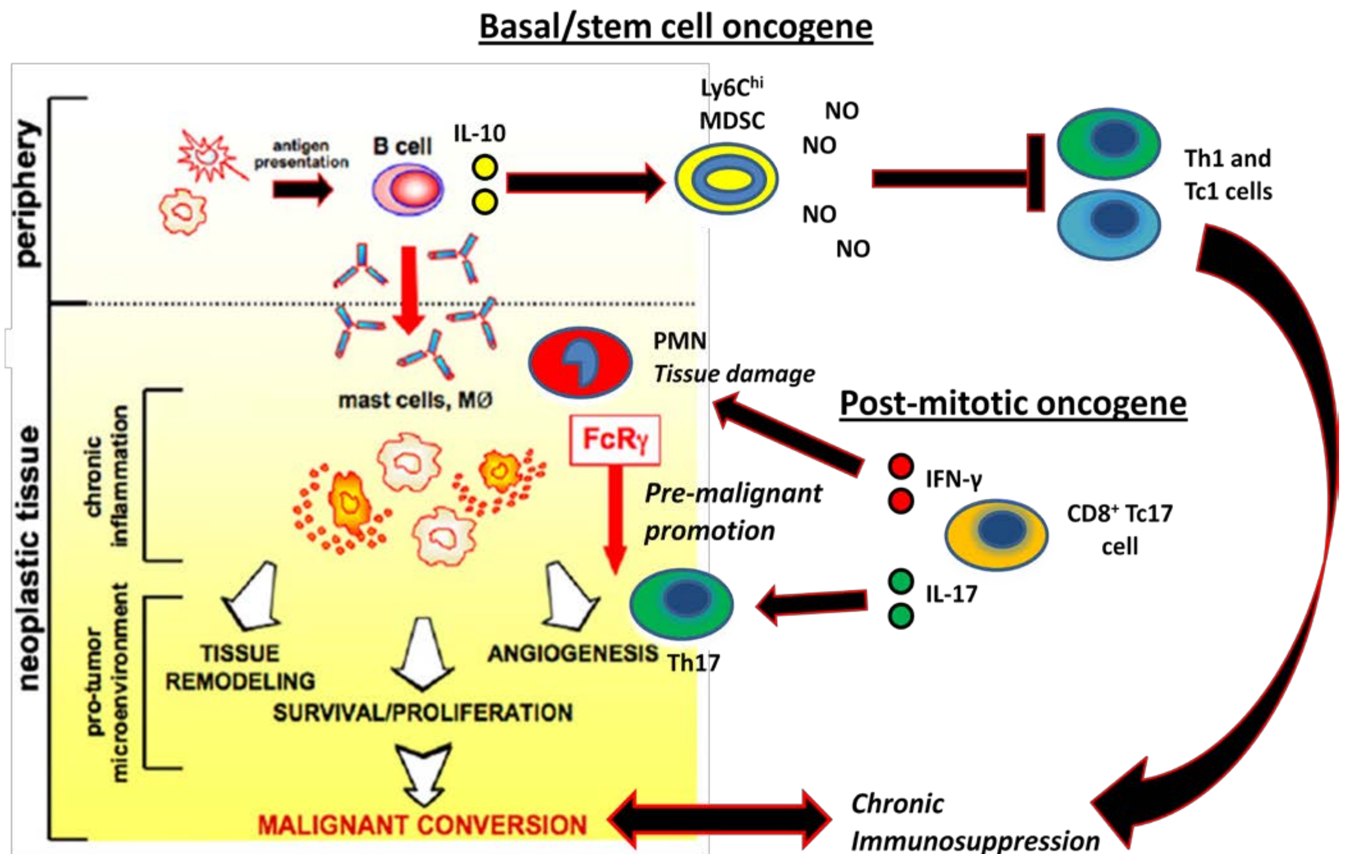

Figure 6-1: Model for inflammatory skin tumor promotion. Keratinocytes expressing a RAS oncogene in differentiated compartments of the epidermis (Post-mitotic oncogene) will stimulate $CD8^+$ T cells to express IL-17 and IFN-γ that in turn drive early inflammatory responses necessary for maximal tumor cell proliferation. These Tc17 cells are sufficient to activate and recruit cytotoxic neutrophils through IFN-γ during acute inflammation but cannot by themselves sustain papilloma outgrowth likely because of a requirement for additional lymphocyte subsets such as B cells. Alternatively, RAS expression driven by a basal proliferating/stem cell keratinocyte, provokes expansion of $Ly6C^{hi}/Ly6G^-/CD11b^+$ immunosuppressive monocytes that seek to dampen $CD4^+$ and $CD8^+$ anti-tumor immunity by nitric oxide production (NO). B cells are necessary and sufficient to mediate the acquisition of this suppressive phenotype, likely through the activities of IL-10 production by regulatory B cell subsets. This chronically immunosuppressed microenvironment could be one mechanism skin cancers initiated in undifferentiated cells contain a higher risk for malignant conversion. *Reprinted and modified from Cancer Cell, Vol. 17, Andreau et al., FcRγ Activation Regulates Inflammation-Associated Squamous Carcinogenesis, pp. 121-134, © 2010, with permission from Elsevier and Lancet.*

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Vita

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Presentations
Poster abstract: Andrew Gunderson, Javed Mohammed, and Adam Glick. Epidermal expression of oncogenic *ras* subverts cytotoxic inflammation by skewing infiltrating myeloid cells towards an immunosuppressive phenotype. American Association of Immunology, 2010 and International Skin Carcinogenesis meeting, 2010.

Poster abstract: Andrew J. Gunderson, Javed Mohammed, Frank Horvath, Cherie Anderson and Adam. Epidermal expression of oncogenic H-RAS provokes tumor promoting inflammation dependent on $CD8^+$ T cells. NCI conference Cancer Immunology and Immunotherapy: Building on success, 2011.

Oral presentation: Andrew J. Gunderson, Javed Mohammed, and Adam B. Glick. B cells are required for immunosuppressive activity in $Ly6C^{hi}$ monocytes during inflammation initiated by epidermally restricted *H-RAS* expression. American Association of Cancer Researchers special conference: Tumor Microenvironment Complexity: Emerging Roles in Cancer Therapy, 2011.

Publications
Javed Mohammed, Andrew Ryscavage, Rolando Perez-Lorenzo, Andrew Gunderson, Nicholas Blazanin, and Adam Glick. TGFβ1 Modulation of Inflammation in Premalignant Squamous Lesions is IL-17-dependent and Independent. Journal of Investigative Dermatology, 2010.

Andrew J. Gunderson, Javed Mohammed, Frank Horvath, Cherie Anderson, Michael Podolsky, and Adam B. Glick. Proinflammatory $CD8^+$ T cells promote *RAS* oncogene-induced cutaneous inflammation and squamous tumor formation. Journal of Immunology, 2012. *In review.*