

**REGULATION OF ANTIGEN PROCESSING MACHINERY COMPONENT
EXPRESSION IN HEAD AND NECK CANCER BY SIGNAL TRANSDUCERS AND
ACTIVATORS OF TRANSCRIPTION AND SRC HOMOLOGY-2 DOMAIN-
CONTAINING PHOSPHATASE**

by

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Michael S. Leibowitz, Ph.D.

Squamous cell carcinoma of the head and neck (SCCHN) cells can escape recognition and lysis by tumor antigen (TA)-specific cytotoxic T lymphocytes (CTL) by downregulation of antigen processing machinery (APM) components, such as the transporter associated with antigen processing (TAP)-1/2 heterodimer. APM component upregulation by interferon gamma (IFN- γ) restores SCCHN cell susceptibility to lysis by CTL, but the mechanism underlying TAP1/2 downregulation in SCCHN cells is not known.

Because IFN- γ activates signal transducer and activator of transcription (STAT)-1, we investigated phosphorylated (p)-STAT1 as a mediator of low basal TAP1/2 expression in SCCHN cells. SCCHN cells were found to express basal total STAT1 but low to undetectable levels of pSTAT1. The association of increased pSTAT1 levels and APM components likely reflects a cause-effect relationship, since STAT1 knockdown significantly reduced both IFN- γ -mediated APM component expression and TA-specific CTL recognition of IFN- γ treated SCCHN cells. On the other hand, since oncogenic pSTAT3 is overexpressed in SCCHN cells and was found to heterodimerize with pSTAT1, we also tested whether pSTAT3 and STAT1:STAT3 heterodimers inhibited IFN- γ -induced APM component expression. First, STAT3 activation or depletion did not affect basal or IFN- γ induced expression of pSTAT1 and APM components, or recognition of SCCHN cells by TA-specific CTL. Second,

STAT1:STAT3 heterodimers did not interfere with IFN- γ induced STAT1 binding to the TAP1 promoter or APM component protein expression. These findings demonstrate that APM component downregulation is regulated primarily by an IFN- γ -pSTAT1-mediated signaling pathway, independent of STAT3 in SCCHN cells.

Interestingly, treatment of SCCHN cells with a broad phosphatase inhibitor, sodium orthovanadate, increased pSTAT1 levels, suggesting that a phosphatase might be responsible for maintaining low basal pSTAT1 and APM component levels, as a mechanism for CTL escape by tumor cells. Indeed, immunohistochemical analyses of 14 SCCHN tumors and paired adjacent normal mucosa demonstrated that src homology-2 domain-containing phosphatase (SHP2) was significantly upregulated in the tumor tissue compared to surrounding mucosa. Moreover, SHP2 specific knockdown using siRNA resulted in significant upregulation of pSTAT1, APM components, and HLA class I in SCCHN cells. Furthermore, SHP2 depletion restored the recognition of SCCHN cells by TA-specific CTL, and induced secretion of Regulation upon Activation, Normal T cell Expressed, and presumably Secreted (RANTES) and IFN- γ -inducible protein 10 (IP10). These novel findings identify SHP2 as an important molecular regulator contributing to low basal pSTAT1 levels and APM-mediated immune escape in SCCHN cells, and provide a potential inhibitory strategy for enhancing the clinical activity of T cell-based immunotherapy.

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PREFACE

Science is not easy. It requires mentorship, knowledge, critical thinking, charisma, technical ability, patience, dedication, passion, integrity, humility and a little bit of luck. I am fortunate to have met so many great mentors that have taught me the qualities that make the best scientists.

First, I would like to thank my mentor, Dr. Robert Ferris, for giving me a home to explore the fascinating field of tumor immunology. I am grateful for your support, encouragement, and wisdom you shared. I am also indebted to Dr. Soldano Ferrone for providing APM component antibodies that were critical for my studies. I admire your work ethic, humility, and kindness. I also thank Dr. Richard Steinman for always challenging me to think critically about a hypothesis or experiment, but more importantly, for being a friend and confidant. I also thank my thesis committee, Drs. Storkus, Grandis, Ray and Binder for guidance and helpful suggestions over the past three and a half years.

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1.0 INTRODUCTION

1.1 SQUAMOUS CELL CARCINOMA OF THE HEAD AND NECK

Squamous cell carcinoma of the head and neck (SCCHN) is a malignancy of epithelial origin that primarily affects the mucosa of the oropharynx, oral cavity, hypopharynx, and larynx. Tobacco and alcohol abuse are the two major risk factors for SCCHN development and recently, human papillomavirus (HPV) was identified as a significant risk factor for cancer of the oropharynx (2). SCCHN is the fifth most common cancer worldwide, diagnosed in over half a million people each year. In 2010, approximately 50,000 people in the United States (US) are estimated to develop SCCHN and about 12,000 people are expected to die from the disease (3, 4). The death rate for patients with SCCHN in the US has decreased over the past decade perhaps due to improvements in detection and treatment of this disease as well as increased frequency of HPV⁺ disease which confers a better prognosis (3). The development of novel treatments such as cetuximab, a human monoclonal antibody (mAb) that targets epidermal growth factor receptor (EGFR), has demonstrated immunomodulatory properties and clinical efficacy (5, 6) providing a strong rationale for the continued development of immunotherapy for cancer. Understanding the role of the immune cell recognition, and mechanisms of evasion from immune elimination of tumor cells, is an important effort to enhance the progress for future SCCHN immunotherapy.

1.2 CANCER IMMUNOSURVEILLANCE

The notion that the immune system can recognize and eliminate primary tumors was first proposed by Paul Ehrlich in 1909 (7). At that time, the field of immunology was in its infancy and the methods to test this hypothesis experimentally were not yet available. About 50 years later, Burnet and colleagues theorized that lymphocytes were responsible for eliminating continuously arising cancer cells in the body based on the increasing understanding of the role of lymphocytes in transplantation, and coined the term cancer immunosurveillance (8-10). The development of athymic nude mice in the 1970s enabled researchers to investigate tumor formation in an immunocompromised host. Interestingly, the number of chemically induced tumors in nude mice were not significantly greater than what was found in wild-type control mice (11). Although not known at the time, athymic nude mice were not the ideal immunodeficiency model to test tumor development since components of the adaptive and innate immunity remained in those mice, principally $\alpha\beta$ T cells and natural killer cells. Nevertheless, the concept of cancer immunosurveillance was largely discarded by many in the tumor immunology field.

About a decade later, several studies demonstrated that interferon gamma (IFN- γ) and perforin were important in preventing the growth of transplanted and chemically induced tumors in mice (12-14). These findings provided clear evidence that aspects of the immune system could control tumor development in mice. Since the publication of those papers, the notion that the immune system can identify and eliminate cancerous cells became widely accepted among

tumor immunologists, yet the perplexing question as to how tumors routinely develop in immunocompetent hosts remained a mystery. A relatively new perspective on the well accepted interaction of tumor and immune cells, termed *cancer immunoediting*, attempts to explain this observation through dissecting the complex interactions between the immune system and cancer cells (15, 16).

1.2.1 Cancer immunoediting

Cancer immunoediting is divided into three phases, elimination, equilibrium, and escape (15, 16) (Fig 1). The first phase of cancer immunoediting, the *elimination* phase, is the same process described in cancer immunosurveillance, which is innate and adaptive immunity can recognize transformed cells and destroy them. The elimination phase results in two distinct outcomes, either complete destruction of the tumor cells thereby protecting the host from tumor formation, or incomplete destruction of tumor cells. In the latter case, the remaining tumor cells are characterized by reduced immunogenicity which leads to the second phase of cancer immunoediting, termed *equilibrium*.

The equilibrium phase is characterized by a subclinical malignancy where strong immune pressure causes tumor cell destruction and Darwinian selection of tumor cells that are progressively more resistant to immune attack called *tumor cell variants*. In this way, the immune system facilitates the formation of a heterogeneous bed of tumor cells with genetic and epigenetic mutations that subsequently lead to tumor cell outgrowth. Evidence for the equilibrium phase exists in both humans and mouse models. A recent case report described two individuals who died from a secondary melanoma originating from the kidney they received from the same donor who was treated for and felt to be “cured” of primary melanoma 16 years

prior to transplant (17). This report suggested that the transplant donor's intact immune system maintained the metastatic melanoma in an equilibrium state, but the pharmacologically immunosuppressed transplant recipients could not prevent the transplanted melanoma cells from progressing secondarily and ultimately succumbed to the malignancy. Furthermore, Schreiber and colleagues used 3'-methylcholanthrene (MCA) to induce sarcomas in wild-type (WT) C57/BL/6 mice and selected mice with stable masses at the injection site. Interestingly, T cell depletion and IFN- γ neutralization caused the stable sarcomas to begin growing in 60% of the mice, whereas the sarcomas in control treated mice did not (18). These data, along with the clinical findings provide evidence that tumors can undergo an equilibrium phase that is mediated in part by the adaptive immune response.

The last phase of cancer immunoediting, referred to as *escape*, describes any process employed by tumor cell variants to evade detection and destruction by the immune system which leads to tumor expansion and frank cancer. The immune escape mechanisms that tumor cells utilize are both tumor cell intrinsic, such as downregulation of MHC class I molecules, or tumor cell extrinsic, such as secretion of cytokines to recruit regulatory T cells into the tumor microenvironment to dampen an antitumor immune response.

Evidence that cancer immunoediting occurs comes from MCA induced sarcoma cell lines derived from WT-mice and *Rag2*^{-/-} mice (lack T cells, NKT cells, and B cells). Interestingly, *edited* sarcoma cells derived from WT mice formed tumors in both WT and *Rag2*^{-/-} mice, whereas *unedited* sarcoma cells from *Rag2*^{-/-} mice only grew in 40% of the WT recipients, suggesting that edited sarcoma cells from the WT mice were less immunogenic than the unedited sarcoma cells from the *Rag2*^{-/-} mice (18). The cancer immunoediting hypothesis provides insight as to why tumors occur in immunocompetent hosts. The notion that the immune

system can both protect a host from neoplastic disease and create a more malignant tumor phenotype that is resistant to immune attack represents a major challenge for the development of cancer immunotherapies.

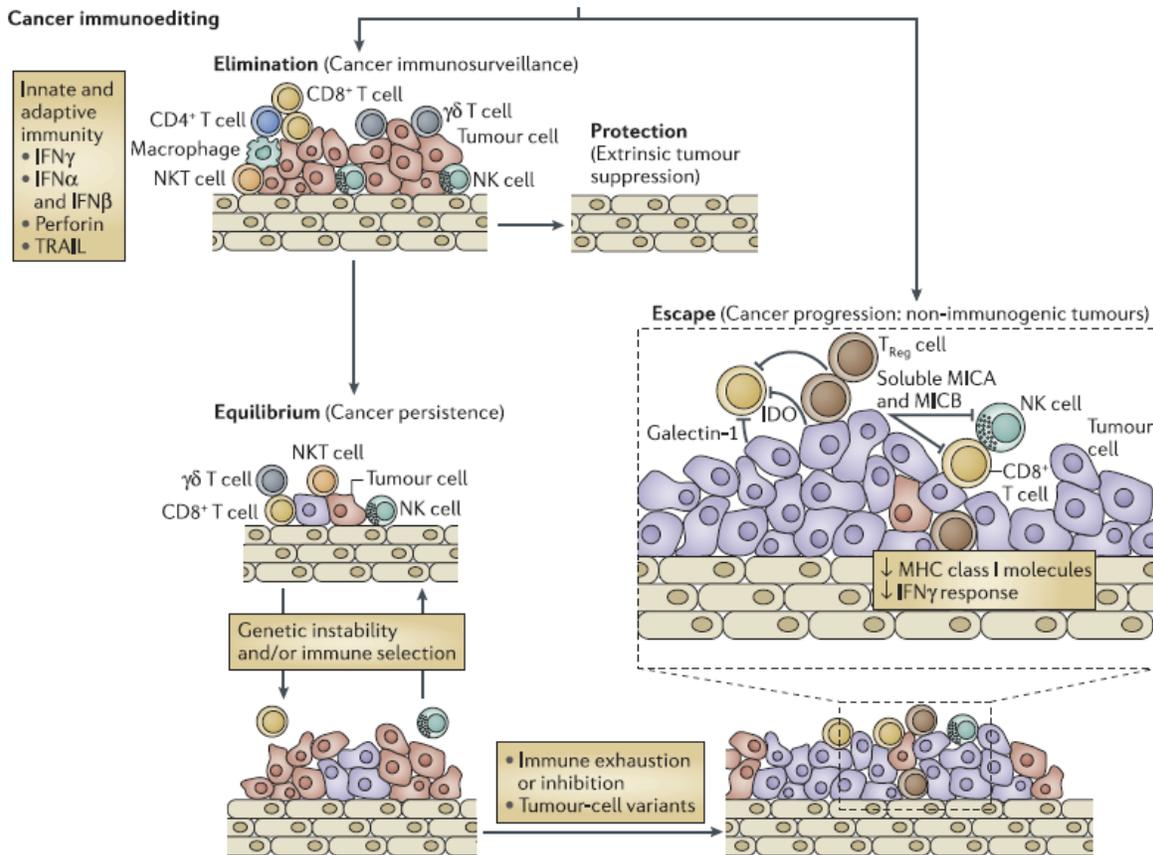


Figure 1. Cancer immunoediting.

The three phases of cancer immunoediting are *elimination*, *equilibrium*, and *escape*. The elimination phase is the same process described by cancer immunosurveillance, whereby the immune system recognizes and kills transformed cells, eliciting protection from neoplastic disease. The second phase, equilibrium, occurs when the elimination phase fails to completely destroy all of the tumor cells. As a result, the remaining tumor cells persist subclinically but are prevented from expanding by immune pressure. The last phase of cancer immunoediting, escape, is the outgrowth of tumor cells that can occur due to the emergence of tumor cell variants which demonstrate reduced immunogenicity and suppress antitumor immune responses. (Adapted by permission from Macmillan Publishers Ltd: [Nature Reviews Immunology](#)⁽¹⁶⁾, 2006).

1.3 CANCER IMMUNOTHERAPY

In the late 19th century, a New York surgeon named William B. Coley began treating patients with inoperable head and neck tumors by local injections of heat-killed *Streptococcus pyogenes* and *Serratia marcescens* cultures based on prior evidence that linked acute bacterial infections with the regression of certain malignancies. This bacterial mixture named “Coley’s toxins” had variable clinical outcomes; in some cases complete regression of the tumor was observed, while in others patients, severe morbidity and mortality occurred. Further trials by Dr. Coley revealed that injection of the toxins at remote anatomic locations could produce robust anti-tumor effects, and that the severity of infection correlated with tumor regression (19-21). Dr. Coley’s work demonstrated that eliciting an immune response, albeit a non-cancer-specific response was sufficient to induce tumor regression in a fraction of his patients. This work provided the foundation of modern day cancer immunotherapy.

1.3.1 T cell-based immunotherapy

CTL are felt to be critical mediators of tumor immunity, and several strategies have been developed to enhance therapeutic TA-specific CTL in patients with cancer. One promising approach is adoptive T cell therapy, which is the transfusion of autologous or allogeneic T cells into tumor-bearing hosts. In the 1960’s, Southam and colleagues subcutaneously injected a suspension containing autologous human cancer cells and autologous peripheral blood lymphocytes (PBL) into patients with nonresectable cancers and demonstrated that tumor growth was inhibited in about half of the patients (22). This suggested that patients with advanced

cancer contained lymphocytes that could inhibit the growth of cancer cells. About thirty years later, Schreiber and colleagues demonstrated that mice containing T cells that were unable to produce interferon gamma (IFN- γ) and secrete perforin had a greater incidence of primary tumor development (23). Taken together, these findings suggested that T cells could be a viable effector population for adoptive immunotherapy for cancer.

Current approaches for adoptive T cell therapy (ACT) involve *ex vivo* polyclonal or antigen specific expansion of tumor infiltrating lymphocytes (TIL) or PBL followed by infusion into patients (24, 25) (Fig 2). Significant barriers to the success of ACT involve generating a sufficient quantity and quality of T cells for infusion. Dendritic cells (DC) would be an ideal candidate to expand T cells *ex vivo*, but the difficulty in obtaining large numbers of autologous DCs limits the utility of these cells. June and colleagues have overcome these issues by developing an *in vitro* cell proliferation system using artificial antigen presenting cells (aAPC) (26). The aAPC is an erythromyeloid K562 cell that does not express major histocompatibility complex (MHC) class I or class II proteins, and therefore does not induce allogeneic T cell proliferation. Furthermore, these cells have been lentivirally transduced to express several proteins important for T cell expansion such as human leukocyte antigen (HLA)-A2 and 4-1BBL, which can present specific tumor antigens (TA) and engage the T cell receptor providing signal 1, and engage costimulatory molecules and provide signal 2, respectively. This system has been able to achieve greater than 1×10^9 fold expansion of CTL (26).

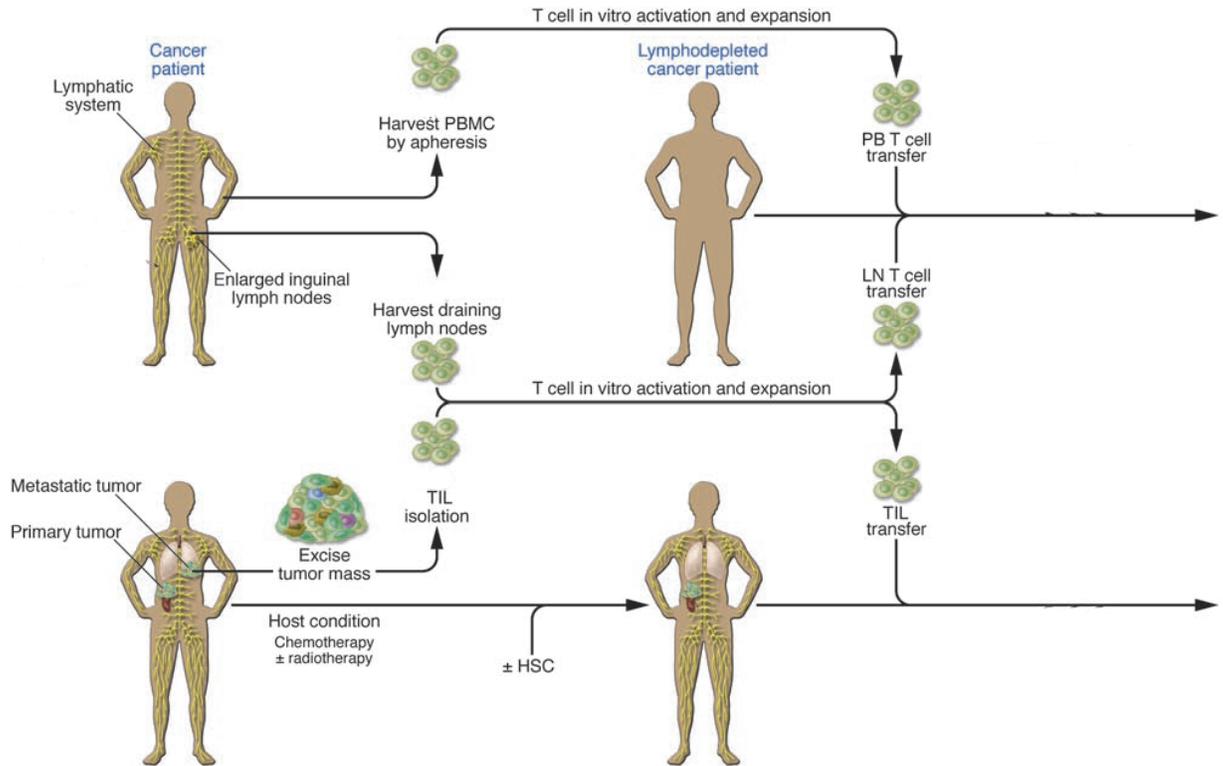


Figure 2. Approaches for adoptive transfer of T lymphocytes.

Lymphocytes are isolated from the peripheral blood (PB), draining lymph nodes (LN) or tumors, expanded *in vitro*, and infused into the patient's PB or LN. (Reproduced with permission from the American Society for Clinical Investigation⁽²⁴⁾)

Despite advances in T cell culture technology, ACT has been met with mixed clinical success. A phase I study using autologous MART-1 and gp100-specific cytotoxic T lymphocytes (CTL) to treat patients with refractory metastatic melanoma resulted in 8 of 10 patients having minor, mixed or stable responses (27). Another phase I study used autologous Melan-A-specific CTL to treat patients with metastatic melanoma which showed antitumor responses in only 3 of 11 patients (28). Interestingly in both studies, TA-specific CTL eliminated the tumor cells expressing the targeted TA, resulting in an outgrowth of tumor cells that lacked expression of the TA (27, 28), analogous to the immune pressure leading to the escape phase described in the cancer immunoeediting hypothesis. These findings highlight a

major limitation in ACT, which is the heterogeneous expression of TAs, resulting in the inability of CTL to recognize and destroy its tumor cell target. To overcome this barrier, one could transfer CTL that target multiple TA, but it is unlikely that all of the tumor cells would be eliminated. Alternatively, one could focus on strategies to increase the immunogenicity of the tumor. Thus far, the tumor immunology field has chosen the former, with an emphasis on the identification of so called “universal tumor antigens”, which are antigens that are broadly expressed by a variety of cancers (29). Although promising in name, this approach has been less successful in practice (30). The latter approach would bypass the need to identify universal TA, and augment ACT and the endogenous antitumor immune responses in patients.

1.4 CLASSICAL MHC CLASS I PROCESSING AND PRESENTATION

CTL recognize antigenic peptides bound to major histocompatibility complex (MHC) class I molecules expressed on the surface of nucleated cells. They consist of the glycoprotein heavy chain (human leukocyte antigen (HLA)-A,-B,-C) which is encoded by polymorphic and polygenic MHC genes, a non-covalently bound light chain, β_2 microglobulin (β_2m), and a short 8-10 amino acid long peptide derived from proteins degraded principally in the cytosol by the proteasome. The peptide binding cleft is formed by the distal domains of the class I heavy chain which consists of two antiparallel α helices above several strands of antiparallel β pleated sheets (31). Only distinct amino acid residues of the MHC groove (called antigen-binding pockets) and the antigenic peptide (called anchor residues) are able to form stable high-affinity interactions. Thus, the highly polygenic and polymorphic nature of the MHC class I gene is able to create a variety of antigen-binding pockets to bind different peptides. T cell receptor (TCR) contacts

both the antigen and the α helices flanking the peptide binding groove providing antigen specificity and MHC restriction.

1.4.1 Antigen processing

Rock and colleagues demonstrated that the ubiquitin-proteasome pathway mediates the majority of endogenous cytoplasmic protein degradation (32). They used a peptide aldehyde protease inhibitor to block the proteolytic degradation of ovalbumin peptide which inhibited the formation of MHC-ova peptide complexes (32). These peptide aldehyde inhibitors were not proteasome specific and also inhibited cysteine proteases, calpain and cathepsin B (32). Later studies used a more specific proteasome inhibitor, lactacystin (33), which confirmed the critical role of the proteasome in cytoplasmic peptide degradation and subsequent loading onto MHC class I molecules.

The proteasome is a hollow cylindrical multicatalytic protease composed of four rings containing either 7α or 7β subunits for a total of 28 subunits. The 20S core particle is lined by the active sites of the proteolytic β subunits and degrade proteins in an ATP-dependent fashion (34). The 20S core is surrounded by additional complexes forming the 26S particle. IFN- γ induces the replacement of several 20S subunits including MB1, δ , and Z with low molecular mass polypeptide (LMP)-2, LMP7 and LMP10 respectively forming the immunoproteasome (34, 35). The immunoproteasome increases the cleavage of peptides after hydrophobic and basic residues and inhibits cleavage after acidic residues (36), thereby creating peptides with carboxy-terminal residues that form anchor residues which can interact with most antigenic binding sites in the MHC cleft. As a result, the immunoproteasome creates more peptides capable of forming

stable MHC-peptide complexes. IFN- γ also upregulates proteasome activator (PA)-28 α and PA28 β which consists of six subunits that form a ring that can bind to and open up both ends of 20S proteasomes. Consequently, the rate at which peptides are released from the proteasome increases significantly (34). Taken together, IFN- γ increases the rate, repertoire and quality of peptides generated by the proteasome (Fig 3).

1.4.2 Antigen presentation

The majority of peptides generated by the proteasome are degraded by aminopeptidases and utilized by the cell to maintain homeostasis (37). Those peptides destined for the endoplasmic reticulum (ER) must first bind to the transporters associated with antigen processing (TAP)-1/2 heterodimer (Fig 3). TAP1/2 is a member of the ATP-binding cassette (ABC) family of transporters and consists of two hydrophobic transmembrane domains and two hydrophilic nucleotide binding domains located in the cytoplasm and are involved in ATP hydrolysis (1). TAP1/2 functions to translocate peptides from the cytoplasm to the ER lumen in an ATP dependent fashion. Expression of both TAP1 and TAP2 is required for efficient peptide binding (34). Peptides between 8-12 amino-acids long with carboxy-terminal residues, which are produced by the immunoproteasome, bind best to TAP1/2 heterodimers (1). Generated peptides are stabilized in the cytosol by chaperone proteins called heat shock proteins (HSP) (38). Furthermore, HSP70 and gp96 can bind to and stabilize exogenous proteins and facilitate cross-presentation through engagement of HSP receptors such as CD91 (38, 39). The exact mechanism of TAP1/2 peptide translocation is still unclear, but the purported mechanism is that peptide binding causes TAP1/2 to undergo a conformational change inducing ATP hydrolysis and peptide transport. Within the ER, peptides may undergo further trimming to the appropriate

length for binding to MHC class I molecules by the ER aminopeptidase associated with antigen presentation (ERAAP) enzyme (40). Additionally, insulin-regulated aminopeptidase (IRAP) is a recently identified trimming enzyme localized within endocytic vesicles that assist in the cross-presentation of peptides from endosomes (41).

MHC class I heavy chain is a glycoprotein that is translated into the ER and inserted into the membrane. Several chaperone proteins such as calnexin and the immunoglobulin-binding protein (BiP) facilitate proper folding of the heavy chain after translation. BiP recognizes misfolded heavy chains and calnexin targets those proteins for degradation. When β_2m binds to the heavy chain, calnexin is replaced by calreticulin. Additional ER-resident chaperone proteins such as ERp57 and tapasin bind to the heavy chain. ERp57 is recruited to heavy chain through interactions with calnexin and calreticulin and mediates disulfide bond formation in the folding glycoprotein. Tapasin functions to link the MHC class I molecule in association with the TAP1/2 heterodimer complex and facilitates loading of peptide onto MHC class I molecules. Upon peptide binding, this trimeric complex, MHC class I heavy chain, β_2m light chain, and peptide are translocated via the Golgi apparatus to the cell surface where it can interact with CTL (1) (Fig 3).

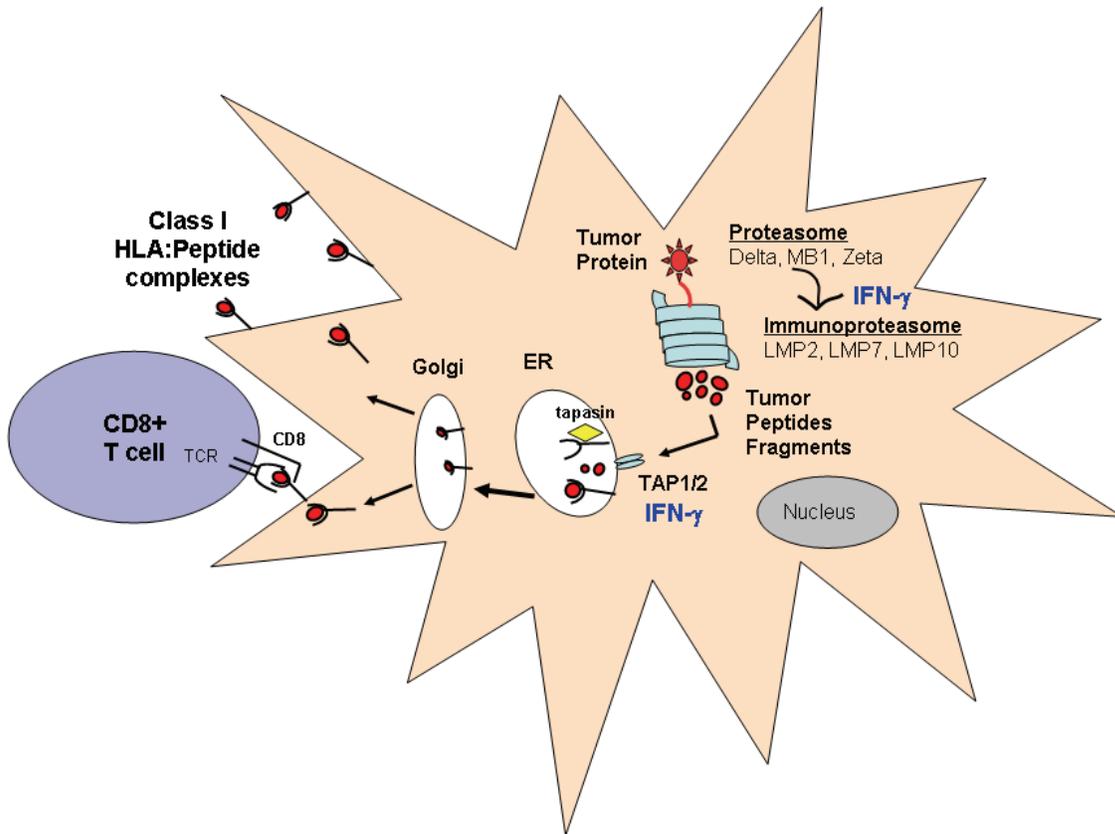


Figure 3. Classical MHC class I antigen processing and presentation.

With kind permission from Springer Science+ Business Media: Current Oncology Reports, Head and Neck Cancer Immunotherapy: Clinical Evaluation 10(2), 2008, page 163, Michael S. Leibowitz, Jayakar V Nayak, and Robert L. Ferris, Figure 1⁽⁴²⁾.

1.5 SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION (STAT)-1 AND STAT3

1.5.1 STAT1 signal transduction

Signal transducer and activator of transcription (STAT)-1 can be phosphorylated and activated by several cytokines and growth factors (43) such as type I (IFN-α) (44) and type II (IFN-γ) (45)

interferons. IFN- α is secreted at low levels by hematopoietic cells (46), and activates STAT1 and STAT2. IFN- α activated STAT1 can form homodimers or heterodimers with STAT2. STAT1:STAT2 heterodimers can bind to interferon regulatory factor (IRF)-9 forming the interferon-stimulated gene factor (ISGF)-3 heterotrimer complex that binds to interferon- α stimulated response elements (ISRE) (47). IFN- γ is produced by activated T cells, NK cells, NKT cells, B cells and professional antigen presenting cells (46). IFN- γ activates STAT1 inducing homodimers that bind to gamma activated sequences (GAS) in the promoters of its target genes (48).

Despite their shared ability to activate STAT1, IFN- α and IFN- γ are structurally unrelated and they bind to different receptors. There are few reports of IFN- α upregulating APM components (49), which is consistent with the observation that IFN- α and IFN- γ -mediated STAT1 homodimers bind to some but not all of the same target gene promoters (47). IFN- γ -mediated STAT1 signal transduction will be discussed further as a model system.

The IFN- γ receptor (IFNGR) is composed of two ligand binding chains (IFNGR1) and two signal transducing chains (IFNGR2). The intracellular domain of the IFNGR1 contains binding sites for the Janus activated kinase (Jak)-1 at residues 266-269 and STAT1 at residues 440-441 (46, 50). IFNGR2 contains binding sites for Jak2 at residues 263-267 and 270-274 which participates in signal transduction (46). Ligand binding to IFNGR1 induces Jak2 autophosphorylation and transphosphorylation of Jak1 (46, 51). Activated Jak1 phosphorylates tyrosine 440 on the cytoplasmic domain of both IFNGR1 chains, providing docking sites for the src homology 2 (SH2) domains of STAT1. Upon STAT1 binding, it is phosphorylated in the C-terminus on tyrosine residue 701 likely by Jak2 (52, 53) and dissociates from the receptor. STAT1 is also serine phosphorylated at residue 727 by an unknown serine kinase in response to

IFN- γ which is thought to provide target specificity and induce maximal transcriptional activity (54-56). Activated STAT1 forms homodimers or heterodimers by binding to the tyrosine phosphorylated SH2 domain of another STAT (57). STAT1 homodimers translocate to the nucleus where it binds to GAS elements (TTCN(2-4)GAA) in the promoters of its target genes (48). Interferon regulatory factor (IRF)-1 is an important IFN- γ responsive gene activated by STAT1 homodimers (51). STAT1 and IRF1 act as transcriptional activators of several interferon responsive genes (54) (Fig 4).

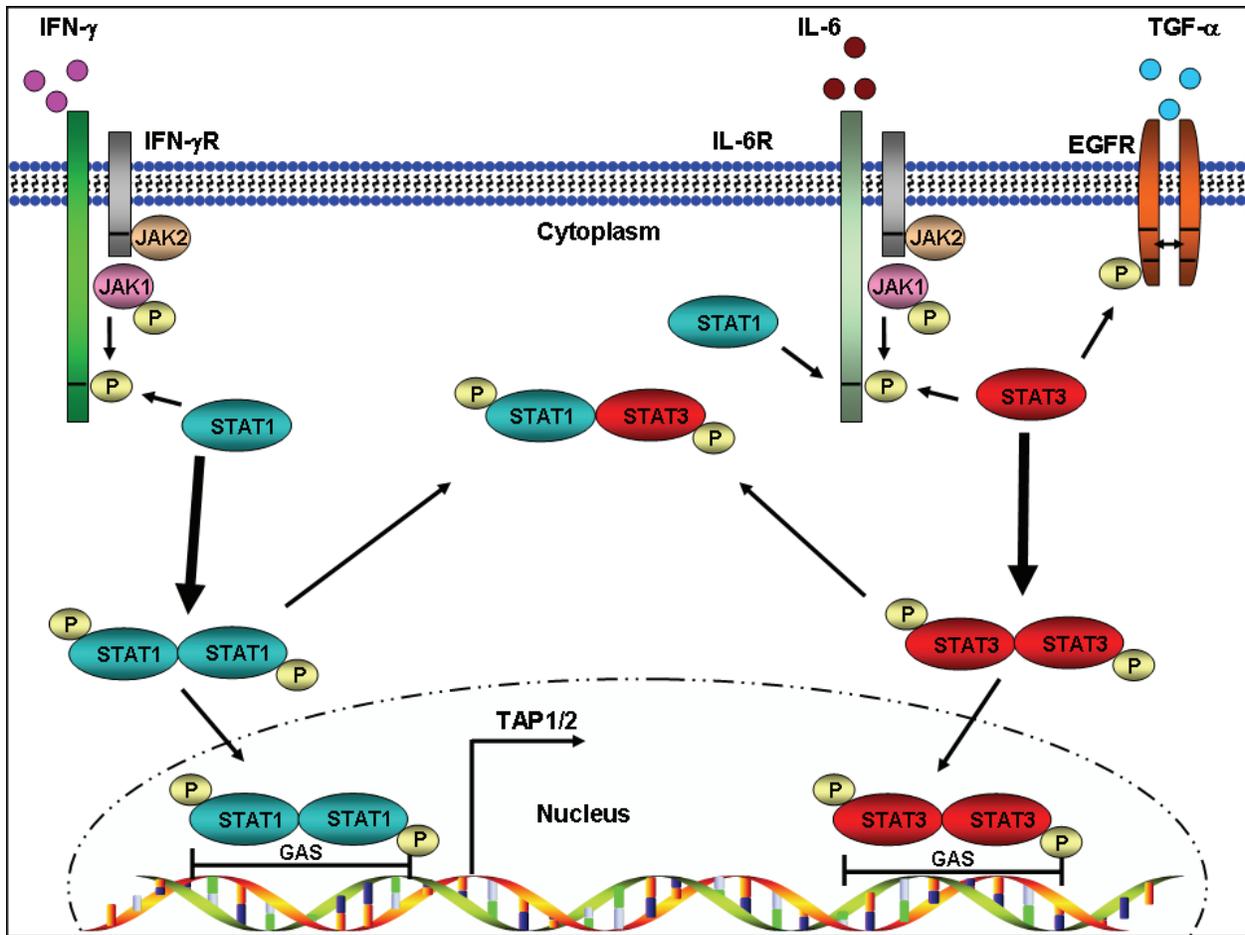


Figure 4. Simplified STAT1 and STAT3 signal transduction.

IFN- γ induces Jak aggregation and transphosphorylation. Activated Jaks phosphorylate the cytoplasmic domain of the IFN- γ R where STAT1 binds. STAT1 is tyrosine phosphorylated at residue 701 by Jak and homodimerizes or heterodimerizes with STAT3. STAT1 homodimers bind to GAS sites in the promoters of target genes such as TAP1 and TAP2. IL-6 induces both STAT1 and STAT3 phosphorylation in a similar manner to IFN- γ activation of STAT1. TGF- α induces reciprocal transphosphorylation of EGFR providing a docking site for STAT3. STAT3 binds to EGFR and is tyrosine phosphorylated at residue 705. Activated STAT3 can form a homodimer and bind to GAS sites or heterodimerize with activated STAT1. The function of STAT1:STAT3 heterodimers is not known.

1.5.2 STAT3 signal transduction

STAT3 activation in SCCHN can occur through stimulation of cytokine receptors such as interleukin (IL)-6R (58) and IL-10R (59, 60), receptor tyrosine kinases such as epidermal growth

factor receptor (EGFR) (61), and non-receptor tyrosine kinases such as Src (62, 63). Interestingly, there are numerous studies that have shown that SCCHN cells secrete IL-6 (58, 64-66) and a recent report demonstrated that activation of STAT3 in these cells occurs by autocrine/paracrine stimulation of the IL-6R (58). Moreover, serum IL-6 levels were found to be significantly increased in patients with SCCHN compared to healthy controls (67-72). These findings prompted investigation of serum IL-6 as a prognostic marker for SCCHN. Several large controlled studies determined that pretreatment serum IL-6 could serve as a biomarker for predicting recurrence and overall survival in patients with SCCHN (73-75). Higher levels of IL-6 were associated with increased recurrence and decreased survival (73-75). From an immunologic and clinical perspective, IL-6 activation of STAT3 is the most relevant and will be discussed further.

IL-6 is a pleiotropic cytokine that has profound effects on multiple biological systems including bone and cartilage formation, immune regulation, inflammation and oncogenesis (76). IL-6 activates STAT proteins by binding to an 80 kDa receptor which exists in both a membrane bound form and a soluble form. The receptor is composed of a non-signaling ligand binding α -chain, and the signal transducing 130 kDa polypeptide chain called gp130 (77). gp130 is ubiquitously expressed and utilized by several other cytokine receptor systems, but the expression of the α receptor is more restricted and tightly regulated limiting the number of cells that respond to IL-6 (76, 78, 79). Both the α and gp130 chains exist in a soluble form and are present in human serum. It is believed that the soluble form of the receptor functions to antagonize and downmodulate systemic responses to IL-6 (80).

IL-6 signal transduction occurs when one IL-6R complex interacts with a second complex forming two IL-6R- α proteins, and two gp130 proteins. Dimerized gp130 proteins

cause Jak1 proteins to bind to hydrophobic residues on its membrane-proximal region (81). gp130 activates Jak1 proteins by phosphorylation (81) inducing STAT activation in a similar process described in section 1.5.1, except that both STAT1 and STAT3 are tyrosine phosphorylated, and STAT3 tyrosine phosphorylation by Jak1 occurs on residue 705 (Fig 4). STAT3 is also serine phosphorylated on residue 727 to induce maximal transcriptional activity (54). Phosphorylated STAT1 and STAT3 can form homodimers or heterodimers, and both bind to GAS sites in the promoters of target genes. It is unclear how STAT1 and STAT3 differentially activate their target genes. It has been proposed that differential binding affinities, the recruitment of co-activators, the number of sequential GAS sites, and the spacing of palindromic half sites may be responsible for selective gene activation (57, 82-84). Furthermore, the function of STAT1/3 heterodimers has not been elucidated.

1.5.3 STAT1 as a tumor suppressor protein

STAT1 is widely classified as a tumor suppressor protein due to the observation that STAT1-knockout mice develop chemically induced tumors more rapidly and with greater frequency compared to wild-type mice (13). The increased rate in tumor formation can be attributed in part to impaired innate immunosurveillance (85). Subsequent *in vitro* analyses have elucidated the role of STAT1 in preventing tumorigenesis. Human U3A fibroblast cells that lack STAT1 protein expression were not growth inhibited by IFN- γ treatment, whereas the parental cells that express STAT1, 2fTGH cells, and U3A cells reconstituted with STAT1 displayed significant growth impairment after IFN- γ treatment (86). The mechanisms for the observed growth arrest can be attributed to the ability of STAT1 to prevent cell cycle progression through inhibition of *c-myc* (87) and downregulation of anti-apoptotic genes such as Bcl-2 and Bcl-X_L (88).

Furthermore, STAT1 can induce apoptosis by activation of caspases. IFN- γ treatment of U3A cells expressing WT STAT1 induced cleavage of caspases 2, 3, and 7 and which was partially blocked by a caspase inhibitor (89). U3A cells lacking STAT1 did not induce caspase cleavage after IFN- γ treatment (89). Interestingly, a common chemotherapeutic agent, doxorubicin, enhances IFN- γ -mediated STAT1 activation and apoptosis of breast cancer cells via caspases (90). These data demonstrate that caspases are involved in STAT1 mediated apoptosis.

STAT1 target genes have been shown to inhibit angiogenesis and metastasis of tumor cells. Huang and colleagues demonstrated that syngeneic subcutaneous STAT1 knockout murine fibrosarcoma cells produced progressively growing tumors *in vivo* with numerous lung metastases (91). In contrast, STAT1 reconstituted fibrosarcoma cells produced much smaller tumors with significantly fewer lung metastases (91). Moreover, IHC analyses of the tumors from STAT1 knockout cells demonstrated increased expression of proangiogenic proteins such as vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMP)-2 and MMP9 which correlated with a significant increase in mean vessel diameter compared to the tumors from STAT1-expressing fibrosarcoma cells (91). These data demonstrate that STAT1 mediates numerous important antitumor effects, and its absence or lack of activation may facilitate tumorigenesis (Table 1).

Interestingly, analysis of serum cytokines from patients with SCCHN revealed decreased levels of IFN- γ compared to age-matched controls (70, 71), likely reflecting the status in the tumor microenvironment. As a result, IFN- γ dependent tumor immunesurveillance, antigen processing and presentation, and STAT1 tumor suppressor activity could be significantly impaired, thereby providing SCCHN cells with an escape mechanism from immune destruction.

1.5.4 STAT3 as an oncogene

In normal cells, STAT activation is transient, lasting minutes to hours, however in tumor cells, STAT activation, particularly phosphorylated (p)-STAT3, is constitutive. Constitutive pSTAT3 has been detected in a variety of solid and hematologic tumors including but not limited to SCCHN, breast cancer, melanoma, lung cancer, ovarian cancer, pancreatic cancer, prostate cancer, and several leukemias and lymphomas (82). One of the earliest insights into STAT3's contribution to oncogenesis was the finding that STAT3 was activated in src transformed fibroblasts, and that expression of a dominant negative STAT3 protein (STAT3 β) blocked the transformation of these cells (92). Subsequent studies demonstrated that a constitutively activate form of STAT3 (STAT3C) could transform fibroblasts and produce tumors in mice, providing direct evidence for the role of STAT3 in tumorigenesis (93).

STAT3's role in oncogenesis is multifaceted and highly complex. STAT3 has been shown to inhibit apoptosis, induce cell cycle progression, and promote angiogenesis, invasion and metastases. STAT3C transfection of murine fibroblasts induced protection from apoptosis induced by UV light or serum withdrawal (94). Others have shown that human SCCHN xenografts treated with a STAT3 antisense oligonucleotide (ASO) expressed less of the anti-apoptotic protein, Bcl-X_L, compared to control treated mice (95). STAT3 has also been shown to increase proliferation and cell cycle progression of SCCHN cells *in vitro* and *in vivo*. Transfection of SCCHN cells that express relatively low levels of pSTAT3 with STAT3C significantly increased the growth rate of tumor cells compared to cells transfected with a control vector (96). *In vivo* studies demonstrated that STAT3C transfected SCCHN cells grew subcutaneous tumors more rapidly in nude mice compared to control cells (96). The observed

increased in proliferation by STAT3C transfected cells correlated with an upregulation of Bcl-X_L and cyclin D1, which inhibit apoptosis and induce cell cycle progression through the G₁/S cell check point respectively (96). STAT3 has also been shown to regulate other cell cycle proteins such as MYC (97) and pro-survival genes such as Bcl-2 (98) and survivin (99). Several studies have implicated STAT3 in promoting tumor angiogenesis directly by activating the vascular endothelial growth factor (VEGF) gene and indirectly through activating hypoxia inducible factor-1 α (100-103). STAT3 also been shown to regulate tumor invasion and metastases through activation of matrix metalloproteinase (MMP)-2 (104) (Table 1).

STAT3 signaling activates several important oncogenes within tumor cells, but it also suppresses antitumor immunity thereby contributing to tumor immune escape. B16 murine melanoma cells transiently transfected with STAT3 β or STAT3 ASO caused increase transcription of proinflammatory cytokines, TNF- α , IL-6 and IFN- β , and chemokines, RANTES and IP10 (105). A recent report found similar results in SCCHN cells (106). STAT3 small interfering RNA (siRNA) transfection resulted in a significant increase in the secretion of IL-6, IL-8 and IP10 compared to control treated cells (106). These data demonstrate that STAT3 can inhibit tumor cell expression of proinflammatory cytokines and chemokines.

STAT3 signaling has also been shown to both directly and indirectly suppress innate and adaptive immunity. Electroinjection of STAT3 β into established B16 tumors in C57BL/6 mice increased infiltration of the tumors with macrophages and neutrophils (105) and suppressed the growth of the tumor (107). The mechanism of growth arrest may be in part due to increased antitumor activity of the tumor infiltrating macrophages and neutrophils since supernatants derived from STAT3 β treated B16 cells caused strong induction of nitric oxide and RANTES

expression in macrophages and TNF- α production from neutrophils (105). There are also numerous studies demonstrating the negative regulatory role of STAT3 signaling in DC maturation and function. Supernatants derived from murine tumor cells induced phosphorylation of Jak2 and STAT3 in myeloid cells and prevented their differentiation into mature DC by LPS compared to conditioned medium from control fibroblast cells (108). Interestingly, viral transduction of myeloid cells with a STAT3 dominant negative protein restored the ability of these cells to differentiate into DC by LPS in the presence of tumor cell condition medium (108). Subsequent studies demonstrated that pharmacologic inhibition of the Jak2/STAT3 pathway with JSI-124 could induce differentiation of myeloid cells into DC in the presence of tumor cell conditioned medium (109). Furthermore, DC isolated from JSI-124 treated mice had significantly greater allostimulatory activity of CD4⁺ T cells compared to DC from control treated mice (110). Moreover, exposure of DCs to conditioned medium from B16 tumor cells expressing STAT3 β (105) or disruption STAT3 signaling in DCs (111) resulted in potent activation of naïve and tolerant T cells to proliferate and produce IL-2 and IFN- γ respectively. In another study, STAT3 ablated splenic DC isolated from tumor bearing mice induced greater antigen specific T cell proliferation *ex vivo* compared to STAT3 expressing splenic DC isolated from tumor bearing mice (112).

Inhibition of STAT3 in B16 tumor cells *in vivo* by STAT3 β electroinjection led to an increase in TILs and activation of TA-specific CTLs compared to control treated mice (105). The observed increase in TIL and TA-specific CTL is probably related to higher quality and quantity of DC generated in the tumor microenvironment from inhibiting STAT3 in the tumor cells. Indeed, VEGF, which is regulated by STAT3, when neutralized by antibodies in B16 supernatants could suppress the inhibitory effects on DC maturation, but not supernatants from

other tumor cells (105). A recent report demonstrated that DCs from STAT3 knockout were resistant to IL-10 mediated inhibition of LPS induced MHC class II and CD86 upregulation and cytokine secretion (113). These data demonstrate that multiple tumor derived factors downstream of STAT3, including VEGF and IL-10, inhibit DC maturation and function.

A recent report evaluated the role of STAT3 in innate and adaptive antitumor immune responses, independent of STAT3 activity within tumor cells, by developing a mouse model where STAT3 was deleted from hematopoietic cells by an inducible *Mxl-Cre* recombinase system (112). Interestingly, neutrophils and NK cells from STAT3 ablated mice demonstrated increased cytolytic activity compared to their WT counterparts with intact STAT3 signaling (112). Furthermore, T cells from STAT3^{-/-} mice challenged with B16 tumors showed increased tumor infiltration with a reduced regulatory T cell phenotype and stronger responses against tumor antigens *ex vivo* compared to T cells from STAT3^{+/+} mice (112). Moreover, ablation of STAT3 in hematopoietic cells before and after established B16 tumors led to significant growth inhibition, and complete eradication of MB49 bladder carcinoma cells (112). The observed antitumor responses were T cell dependent since injection of CD4⁺ and CD8⁺ depleting antibodies caused similar tumor growth kinetics in STAT3^{+/+} mice (112).

Table 1. Opposing roles of STAT1 and STAT3 in oncogenesis.

	STAT1	STAT3
	-STAT1 KO mice develop tumors faster with greater frequency than WT mice	-Constitutive expression of STAT3 transforms cells and forms tumors in mice
Cell cycle	-Inhibits c-myc	-Upregulates myc and cyclin D1
Apoptosis	-Downregulates Bcl-2 and Bcl-X _L -Induces cleavage of caspases 2,3,7	-Upregulates Bcl-X _L , Bcl-2, and survivin
Angiogenesis	-Downregulates VEGF	-Upregulates VEGF and HIF-1 α
Metastases	-Downregulates MMP-2/9	-Upregulates MMP-2

1.5.5 STAT1 in antigen processing and presentation

There are conflicting reports in the literature as to the mechanism of STAT1 and other cofactors that induce APM component expression. Min and colleagues found that STAT1, and not IRF-1, mediates TAP1 transcription by IFN- γ in Hela cells (114). However, another study utilized a bidirectional reporter system to analyze the transcriptional activity of *TAP1* and *LMP2* genes (which share a 593 bp bidirectional promoter (115)) and found that mutation of the IRF-1 binding site in the reporter construct completely inhibited IFN- γ induction of TAP1 and LMP2 in Hela cells (116). A report in melanoma cells found that binding of IRF-1 or STAT1 was sufficient for TAP1 transcription, while binding of both factors were required for LMP2 transcription (117). Others have found that IRF-2 and not IRF-1 cooperates with STAT1 to induce TAP1 transcription (118). Mouse embryonic fibroblasts from IRF-1/2 knockout mice and 293T cells that lack IRF-1/2 binding activity were transfected with an IRF-2 expression vector and a TAP1 promoter reporter construct (118). Both cells showed increased TAP1 promoter

activity demonstrating that IRF-2 was sufficient to induce TAP1 gene expression (118). In contrast, IFN- γ mediated TAP1 and LMP2 expression in murine macrophages were found to be regulated by STAT1 and IRF-1 (119). Furthermore, macrophages from STAT1 knockout mice failed to upregulate TAP1 and LMP2 protein after IFN- γ treatment, demonstrating that STAT1 is required for IFN- γ induced expression of these genes (119). Despite the lack of consensus regarding which transcription factors regulate APM component expression, STAT1 has been consistently found to be necessary and important in regulating APM gene transcription.

1.6 IMMUNE ESCAPE IN SCCHN

A greater understanding of SCCHN immune escape is necessary to develop therapeutic strategies to increase the immunogenicity of this malignancy and augment the efficacy of ACT. Two of the most studied immune evasion mechanisms in SCCHN include induction of T cell apoptosis and defective antigen processing machinery (APM) component expression.

1.6.1 Apoptosis

CTL derived from patients with SCCHN have been shown to be more sensitive to apoptosis (120). Interestingly, expression of Fas ligand has been identified on SCCHN cells and tumor biopsies (121). Fas-Fas ligand are part of the tumor necrosis factor (TNF) family and can induce apoptosis of activated T cells as a homeostatic mechanism to maintain appropriate T cell numbers (122). The apoptotic activity of Fas ligand expression on SCCHN cells was confirmed by inhibiting the apoptosis of activated T cells co-cultured with SCCHN cells in the presence of

an anti-Fas ligand antibody (121). Furthermore, analysis of Fas expression on CD3⁺ cells from 37 patients with SCCHN was significantly higher compared to normal controls (123). The expression of Fas on CD3⁺ cells and Fas ligand on SCCHN tumors may be responsible for the significant reduction in absolute numbers of CD3⁺, CD4⁺, and CD8⁺ T cells observed from the peripheral blood of 146 patients with SCCHN compared to normal controls (124). These findings provide strong evidence that SCCHN cells evade T cell lysis by exploiting the Fas-mediated apoptosis pathway to eliminate antitumor effector T cells.

1.6.2 Antigen processing machinery component defects

Abnormal expression of the MHC class I antigen processing machinery (APM) components, such as LMP2, LMP7, TAP1, TAP2 and tapasin represent a major mechanism of immune escape in SCCHN (125-131), and a variety of other cancers including but not limited to renal cell carcinoma (132), colorectal carcinoma (133), small cell lung carcinoma (134) and melanoma (135). Of important clinical note, APM component downregulation in SCCHN lesions correlated with poor prognosis (125, 127, 129, 130), which likely reflect the inability of the tumor cell to efficiently process and presentation antigen to CTL. Interestingly, it has been recently demonstrated that treatment of SCCHN tumor cells with IFN- γ upregulates several APM components including LMP2, LMP7, LMP10, TAP1, TAP2, and tapasin, resulting in TA-specific CTL lysis (126). SCCHN cells transected with TAP1 cDNA restored their recognition and lysis by TA-specific CTL, suggesting that TAP1 is a critical APM component that is defective in these cells (126).

The molecular mechanisms responsible for APM component defects are not well understood. In a melanoma cell line, a single nucleotide deletion at position 1489 of the *TAP1*

gene resulted in unstable TAP1 mRNA and deficient protein expression (136). In another study, a non-functional TAP1 allele was discovered in a human small cell lung cancer cell line causing a TAP1 deficiency phenotype because only the defective allele was transcribed (137). Others have found an epigenetic mechanism for APM component downregulation in mouse melanoma cells (138, 139). Treatment of the tumor cells with a histone deacetylase inhibitor (HDACi) upregulated TAP1, TAP2, LMP2 and tapasin (138, 139). Furthermore, subcutaneous tumors in C57BL/6 syngeneic mice treated with intraperitoneal injections of an HDACi significantly decreased tumor growth which correlated with an increase in TAP1 promoter activity (139). The current understanding of the molecular players underlying defective APM component expression in human tumors is insufficient. Interestingly, inactivating mutations, defective alleles, and epigenetic repression appear to be quite rare (140), and probably do not represent the major mechanism responsible for defective APM component expression in cancer, rather pointing to regulatory mechanisms playing a crucial role.

1.6.2.1 Impaired IFN- γ signaling

IFN- γ “unresponsiveness”, measured by an inability to form STAT1 DNA-binding complexes by electrophoretic mobility shift assay, is frequently found in human cancer (13). Approximately 33% of 33 melanoma and 17 non-adenocarcinoma lung tumor cell lines exhibited a quantitative reduction in IFN- γ sensitivity, while 4 out of 17 lung adenocarcinoma cell lines were totally unresponsive to IFN- γ (13). The mechanism for the observed IFN- γ insensitivity was variable. Some cells lacked expression of IFN- γ R α chain and Jak1, while others produced abnormally phosphorylated or inactivated Jak2 proteins (13). In another study, 2 out of 57 melanoma cell lines were unresponsive to the IFN- γ treatment determined by the inability to upregulate MHC

class I molecules (141). The phenotype for the observed IFN- γ resistance included failure to induce tyrosine phosphorylation of STAT1 and silencing of IRF-1 by methylation (141). Similar findings were reported in a human renal cell carcinoma cell line that failed to induce TAP1 and LMP2 expression after IFN- γ treatment (142). In these cells, IFN- γ treatment did not induce STAT1 phosphorylation or IRF-1 expression (142). The inability of IFN- γ to induce TAP1 and LMP2 in human uterine leiomyosarcoma cells was due to a point mutation in the ATP binding region of the Jak1 tyrosine kinase domain resulting in a lack of STAT1 phosphorylation and subsequent IRF1 expression (143).

1.6.2.2 STAT3 directly inhibiting APM component expression

The role of STAT3 in APM component expression is also controversial. IL-10 activates Jak1 inducing STAT3 phosphorylation (59, 60), which is essential for all known functions of IL-10 (59). IL-10 is an anti-inflammatory immunosuppressive cytokine secreted significantly by T regulatory cells (59) and has been found to be elevated in the serum of patients with SCCHN (144). IL-10 has been implicated in the downregulation of MHC class I heavy chain and APM components in melanoma. Treatment of human melanoma cells with IL-10 induced resistance to lysis by autologous TILs *ex vivo* and downregulated MHC class I in a dose-dependent fashion of up to 50% (145). Subsequent studies revealed that a synthetic peptide homologous to the functional domain of IL-10 could downregulate IFN- γ induced TAP1/2 heterodimer expression in melanoma cells (146). Murine tumor cells treated with IL-10 or transfected to express IL-10 also demonstrated downregulation of TAP1 and TAP2 expression (147, 148). In another study, B cells infected with EBV secrete IL-10 and mediate the downregulation of TAP1 and LMP2

transcription and protein (149). Taken together, these studies suggest that activation of STAT3 by IL-10 could mediate APM component downregulation in SCCHN cells.

1.6.2.3 STAT1 and STAT3 cross regulation

STAT1 and STAT3 have opposing roles in tumorigenesis yet are activated by the same Jak proteins and are simultaneously activated by IL-6. Several studies have been performed to dissect the mechanisms by which STAT1 and STAT3 interact and cross regulate each other. IL-10 predominantly phosphorylates STAT3 and suppresses LPS-induced TNF- α production in monocytes. However monocytes pretreated with IFN- γ were no longer inhibited from secreting TNF- α by IL-10 (150). Interestingly, IFN- γ treated monocytes caused IL-10 to activate both STAT3 and STAT1 (150). In STAT3 knock out MEFs, IL-6 mediated prolonged STAT1 activation and induced the expression of numerous IFN- γ inducible genes such as LMP7 (151). Another report found similar results in T cell leukemia and lymphoma cells. STAT3 knockdown in those cells caused IL-6 to induce prolonged STAT1 activation and switch to an IFN- γ -like response by upregulating MHC class I and inducing apoptosis and growth arrest (152). These data demonstrate that in normal cells, IL-6 predominantly activates STAT3 which functions to inhibit an IFN- γ -STAT1-like response. In SCCHN, it is unclear whether inhibition of STAT3 modulates the antitumor effect of STAT1. One report demonstrated that STAT1 siRNA did not alter the cytotoxicity of an oligonucleotide that binds to STAT3 and prevents its physical binding to DNA (153), however another report found that the growth inhibitory effect of a Jak-STAT3 inhibitor required STAT1 expression (154).

STAT1 and STAT3 target gene expression can be altered by activation of the opposing transcription factor, however the mechanisms for these observations are still unclear. One

hypothesis is that activation of STAT3 suppresses phosphorylation of STAT1 and vice-versa. Human monocytes pretreated with IL-10 inhibited IFN- γ and IFN- α mediated IP10 and ICAM-1 transcription (155). Interestingly, IL-10 inhibited both interferons from inducing tyrosine phosphorylation of STAT1 (155). In a prostate cancer cell line, IFN- γ treatment decreased tyrosine phosphorylation of STAT3 (156). This observation appeared to be dependent on intact mTOR signaling because treatment of the cells with rapamycin significantly inhibited IFN- γ mediated STAT3 dephosphorylation (156). Surprisingly, STAT1 knockdown did not inhibit IFN- γ mediated STAT3 dephosphorylation in these cells (156). A recent clinical report demonstrated that tumor cells from patients with melanoma treated with IFN α 2b upregulated pSTAT1 and TAP2, but downregulated pSTAT3 (157).

Another hypothesis as to how STAT1 and STAT3 negatively regulate each other's target gene expression is through formation of STAT1:STAT3 heterodimers, thereby decreasing STAT1 and STAT3 homodimers. In human monocytic cells, STAT3 reduced IFN- α mediated transcription of STAT1, IRF-1, and chemokines MIG and IP10, however not through inhibiting STAT1 tyrosine phosphorylation or nuclear translocation (158). Interestingly, IFN- α treated monocytes transduced to express high levels of STAT3 contained predominantly STAT1:STAT3 heterodimers and significantly diminished STAT1 homodimers compared to control cells (158). These data suggest that cells with increased STAT3 expression may sequester STAT1 in STAT1:STAT3 heterodimers, and inhibit STAT1 homodimerization and target gene expression. Similar findings were reported in two myeloma cells in which IFN- α treatment decreased STAT3 homodimerization and STAT3 mediated expression of *Bcl-X_L*, *MCL-1*, and *survivin*, thereby inducing apoptosis (159). IFN- α caused a shift from predominantly STAT3 homodimers to STAT1 homodimers and STAT1:STAT3 heterodimer formation (159). Interestingly,

transfection of the myeloma cells with STAT3C protected the cells from IFN- α induced apoptosis by maintaining STAT3 homodimers and *Bcl-X_L*, *MCL-1*, and *survivin* expression (159).

1.6.2.4 Impaired STAT1 phosphorylation by src homology-2 domain-containing phosphatase (SHP)-2

SHP2 is a non-receptor protein tyrosine phosphatase (PTP) that is ubiquitously expressed in humans (160). SHP2 is composed of two tandem N-terminal SH2 domains, a PTP domain, and a C-terminal tail with two important tyrosine phosphorylation sites (Tyr542 and Tyr580) (161). The SH2 domain mediates binding of SHP2 to phosphorylated tyrosine residues on other molecules (162). Under basal conditions, SHP2 is inactivated by the N-SH2 domain wedged into the PTP domain forming a “backside loop” thereby preventing substrate access (163) (Fig 5). Upon growth factor (EGF) or cytokine stimulation such as IL-6 or IFN- γ , SHP2 binds (via its SH2 domains) to phosphorylated tyrosine residues on various receptors causing a conformational change in N-SH2 domains and release of the PTP domain from its auto-inhibitory state (160, 161, 163). The phosphorylation of Tyr542 and Tyr580 may also contribute to the activation of SHP2 through intramolecular interactions with N-terminal SH2 domain and C-terminal PTP domain (164).

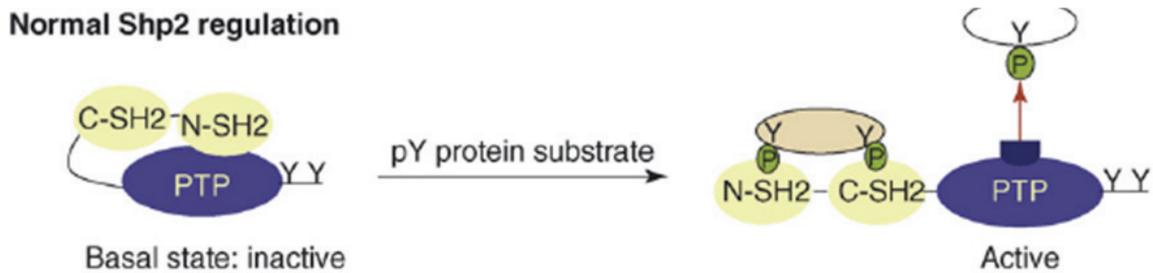


Figure 5. Basal auto-inhibition of SHP2.

Under normal conditions, SHP2 is maintained in an inhibited state in the absence of growth factor or cytokine stimulation (Reprinted from *Current Opinion in Genetics & Development*, 17, M Golam Mohi and Benjamin G Neel, The Role of SHP2 (PTPN11) in cancer, 23-30, 2007, with permission from Elsevier⁽¹⁶¹⁾).

SHP2 has been implicated as a negative regulator of the IFN-Jak-STAT1 signal transduction pathway (165). In SHP2^{-/-} MEFs, IFN- γ and IFN- α treatment resulted in increased cytotoxicity and enhanced STAT1 DNA binding activity compared to WT MEFs (165). Expression of WT SHP2 in SHP2^{-/-} MEFs decreased the growth inhibitory effects of the interferons (165). Indeed, IFN- γ treatment of SHP2^{-/-} MEFs enhanced STAT1 tyrosine and serine phosphorylation compared to control cells (166). Furthermore, Her-2/neu and SHP2 overexpressing bladder cancer cells exhibited resistance to IFN- γ -mediated STAT1 phosphorylation and growth inhibition (167). Treatment with a HER2-neu-specific inhibitor decreased SHP2 levels, upregulated pSTAT1, and restored the ability of IFN- γ to induce growth arrest in these cells (167). Others have shown glycogen synthase kinase-3 β (GSK-3 β) could inhibit SHP2 activity in murine macrophages and human epithelial lung cells (168). Treatment of these cells with GSK-3 β inhibitors or siRNA decreased STAT1 phosphorylation and increased SHP2 Tyr542 phosphorylation (168). The observed effects from the GSK-3 β inhibitors were reversed in the presence of a SHP2 inhibitor (168). Moreover, human embryonic fibroblasts

infected with CMV upregulated SHP2 and exhibit reduced STAT1 tyrosine phosphorylation in response to IFN- γ treatment (169). SHP2-specific siRNA transfection restored IFN- γ induced STAT1 tyrosine phosphorylation in these cells (169).

SHP2 has been recently identified as an oncogene (161). SHP2 activating mutations account for approximately 35% of sporadic juvenile myelomonocytic leukemia, and various childhood leukemias including acute myeloid leukemia (4%), myelodysplastic syndromes (10%) and acute lymphoid leukemia (7%) (170). In a recent study, 44 adult patients with leukemia were screened for SHP2 mutations (171). Interestingly, no gain of function mutations were detected in the leukemic cells which was consistent with reports describing SHP2 mutations as uncommon in adult leukemia (172). Instead, they found SHP2 expression to be elevated in primary leukemia cells compared to normal progenitors which correlated with the hyperproliferative phenotype in the patients (171). Furthermore, SHP2 ASO induced apoptosis in K562 leukemia cells, which express high levels of SHP2 (171). Although rare in solid cancers, SHP2 expression was significantly elevated in infiltrating ductal carcinoma of the breast tissue compared to normal breast tissue (173).

1.7 SPECIFIC AIMS

1.7.1 Specific Aim 1 (Chapter 2): To investigate the role of STAT3 and STAT1:STAT3 heterodimers in APM-mediated immune escape in SCCHN cells

APM component downregulation is a major mechanism of immune escape in SCCHN however the predominant mechanism responsible for this phenotype remains unknown. In other

malignancies, STAT3 activation has been shown to downregulate APM component expression. Recent studies revealed that activated STAT3 could sequester and inhibit STAT1 target genes through formation of STAT1:STAT3 heterodimers. Therefore, we hypothesized that overexpressed STAT3 sequesters STAT1 into STAT1:STAT3 heterodimers and inhibits STAT1 homodimerization and APM component gene activation. Indeed, STAT1 depletion in SCCHN cells significantly reduced APM expression and TA-specific CTL recognition, demonstrating the critical role for STAT1 in APM component expression. However, STAT3 depletion did not enhance basal or IFN- γ induced APM component expression or TA-specific CTL recognition of SCCHN cells. Furthermore, induction of STAT1:STAT3 heterodimers in SCCHN cells did not alter STAT1 binding to the TAP1 promoter, APM transcription, APM protein or TA-specific CTL recognition. These data demonstrate that APM component downregulation is regulated primarily by an IFN- γ -pSTAT1-mediated signaling pathway, independent of oncogenic STAT3 in SCCHN cells.

1.7.2 Specific Aim 2 (Chapter 3): To examine the role of SHP2 in APM-mediated immune escape in SCCHN cells

SCCHN cells were found to express abundant levels of total unphosphorylated STAT1, but low to undetectable levels of pSTAT1. Given the critical role of pSTAT1 in APM component expression demonstrated in specific aim 1, we investigated whether a dysregulated phosphatase might be responsible for low basal pSTAT1 and APM component downregulation in SCCHN cells. Interestingly, treatment of SCCHN cells with a pan phosphatase inhibitor, sodium orthovanadate, upregulated pSTAT1. Immunohistochemical analysis revealed that SHP2 was expressed significantly higher in tumor tissue compared to normal adjacent mucosa. Moreover,

SHP2 depletion in SCCHN cells significantly upregulated pSTAT1, APM protein expression, TA-specific CTL recognition, and induced the secretion of RANTES and IP10. These data demonstrate that SCCHN cells utilize SHP2 to suppress STAT1 phosphorylation and subsequent APM component expression as an antitumor immune evasion mechanism.

2.0 DEFICIENCY OF ACTIVATED STAT1 IN HEAD AND NECK CANCER CELLS MEDIATES TAP1-DEPENDENT ESCAPE FROM CTL RECOGNITION

2.1 INTRODUCTION

Cytotoxic T lymphocytes (CTL) recognize antigenic peptides bound to human leukocyte antigen (HLA) class I molecules on the membrane of target cells. These peptides are mostly, but not exclusively derived from endogenous proteins that are degraded by the proteasome and by the immunoproteasome to 8-10 amino acid long peptides. The generated peptides are then transported into the endoplasmic reticulum by the transporters associated with antigen processing, (TAP)1/2 complex. In the ER, with the help of antigen processing machinery (APM) components, peptides are loaded on β 2 microglobulin-associated HLA class I heavy chains; these trimolecular complexes are then transported to the cell surface for recognition by cognate CTL (174-177).

APM components are frequently abnormal in their expression and/or function in malignant cells, causing impaired expression of HLA class I tumor antigen (TA)-derived peptide complexes on tumor cells (178, 179). The resulting defective interactions with the host's immune system provide tumor cells with a mechanism to escape recognition and lysis by CTL.

Squamous cell carcinoma of the head and neck (SCCHN) cells can evade detection and lysis by CTL through downregulation of APM components such as TAP1 resulting in poor TA

processing and presentation (126-128, 130). These abnormalities have clinical significance since they correlate with poor prognosis in patients with SCCHN (125, 129, 180). Furthermore abnormalities in APM component expression and/or function are likely to have a negative impact on the clinical response to T cell-based immunotherapy. These findings highlight the need to characterize the molecular basis of APM component defects in SCCHN cells, since this information could improve the clinical course of the disease by developing targeted therapies to correct these defects and augment T cell-based immunotherapies.

Incubation of SCCHN cells with interferon gamma (IFN- γ) upregulates APM components, such as TAP1/2, and restores recognition and lysis of SCCHN cells by HLA class I restricted, TA-specific CTL (126). IFN- γ induces activation of signal transducer and activator of transcription (STAT1)-1, which forms homodimers and binds to gamma activated sequences (GAS) in the promoters of APM component genes, such as TAP1 (114, 117-119, 181, 182). We have investigated the role of STAT1 and STAT3 because both of these transcription factors can modulate the signaling activity of interferons by forming STAT1:STAT3 heterodimers and inhibiting their respective gene activation functions (158, 159). Furthermore, IL-10, a potent STAT3 agonist (59), has been shown to inhibit IFN- γ -mediated STAT1 phosphorylation (155) and APM component expression in tumor cells (146-148, 183). Thus, we investigated whether the low level of phosphorylated pSTAT1 is responsible for poor basal APM expression, and whether the beneficial effect of IFN- γ is mediated by the activation of STAT1 and/or by reduction of STAT3 levels and STAT1:STAT3 heterodimers.

2.2 MATERIALS AND METHODS

2.2.1 Cell lines

The HLA-A*0201⁺ SCCHN cell lines, PCI-13 and SCC90 were characterized and described previously (184). Both tumor cell lines were cultured in DMEM (Sigma-Aldrich Inc, St. Louis, MO) supplemented with 10% FBS (Mediatech, Herndon, VA), 2% L-glutamine, and 1% penicillin/streptomycin (Invitrogen Corp, Carlsbad, CA).

2.2.2 Cytokines

Recombinant human IL-6 was purchased from R&D systems (Minneapolis, MN), and IFN- γ was purchased from InterMune (Brisbane, CA).

2.2.3 Antibodies

IFN- γ R α chain monoclonal antibody (mAb) used in intracellular flow was purchased from BD Pharmingen (San Jose, CA) while the IFN- γ R polyclonal antibody (pAb) used in immunohistochemistry was purchased from Abcam (Cambridge, MA). Anti-HLA-A,B,C mAb (w6/32) (Ebiosciences, San Diego, CA) and anti-HLA-DR (L243) mAb (Biolegend, San Diego, Ca) were used in ELISPOT assays. LMP2-specific mAb SY-1 (185), TAP1-specific mAb NOB-1, TAP2-specific mAb NOB-2, and calreticulin-specific mAb TO-11, were developed and characterized as described (185, 186). FITC conjugated IgG anti-mouse mAb was used as a secondary antibody for APM and IFN- γ R α chain staining for flow cytometry and purchased

from Invitrogen Corp. The intracellular pSTAT staining was performed using PE conjugated irrelevant IgG2a mAb isotype control, PE conjugated phosphorylated tyrosine 701 STAT1 mAb (pSTAT1 Tyr701) and PE conjugated pSTAT3 Tyr705 mAb purchased from BD Biosciences (San Jose, CA). Western blotting antibodies include anti-pSTAT1 Tyr701 mAb, anti-pSTAT3 Tyr705 mAb (Cell Signaling Tech, Danvers, MA), anti-STAT1 (C-24) pAb (Santa Cruz Biotech, Santa Cruz, CA), anti-STAT3 (C-20) pAb (Santa Cruz Biotech), anti- β -actin mAb (Sigma-Aldrich Inc), anti-rabbit IgG-HRP (Promega, Madison, WI), anti-mouse IgG-HRP (Biorad, Hercules, CA).

2.2.4 Intracellular flow cytometry for APM component and pSTAT1 expression

Intracellular flow cytometry was performed as described (187). Briefly, the cells were fixed using 1.5% paraformaldehyde for 10 minutes at room temperature (RT) and permeabilized with 100% methanol for at least 24 hours at -20°C. Cells were then washed in 2% FCS/PBS (FACS buffer), and stained with either a PE conjugated primary STAT-specific mAb or sequentially by incubation with an unconjugated primary APM component-specific mAb and then with a FITC conjugated anti-mouse secondary mAb. Both incubations were for 30 minutes at RT. FACS analysis was performed on the same day as staining. Isotype control antibody staining was set at a mean fluorescence intensity (MFI) of 5 on an EPICS XL cytometer (Beckman Coulter, Brea, CA) for each condition and cell line tested. A minimum of 10,000 cells were collected per test. Analyses were performed using EXPO32 ADC software (Beckman Coulter). pSTAT1, pSTAT3 and APM component expression was determined based on MFI and was expressed as a mean \pm SE of the results obtained in at least three independent experiments.

2.2.5 Immunoblot analysis

Cells were lysed in 10 mM Tris HCl, 5 mM EDTA, 50 mM NaCl, 30 mM Na₂P₂O₇, 50 mM NaF, 1 mM NaVO₄, 1% Triton X-100, 1 mM PMSF and vortexed for at least 1 hour at 4°C, sonicated for 6 seconds at 20% of the maximum potency (Cole Parmer Instrument, Verona Hills, IL) and centrifuged at 4°C, 16,100 g for 15 minutes. The supernatant protein was normalized and 40-60 µg of protein were size fractionated through a 4-12% SDS-PAGE gel (Lonza, Rockand, ME), transferred to a PVDF membrane (Millipore, Billerica, MA) and immunoblotted with the indicated antibodies. Densitometry was performed on a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA) according to the manufacturer's instructions.

2.2.6 Small interfering RNA (siRNA)

SCCHN cell lines were transfected at 30-40% confluence with STAT1 targeting siRNA, STAT3 targeting siRNA or a non-targeting siRNA control (Ambion, Austin, Tx), Lipofectamine RNAi max (Invitrogen Corp), and Optimem I (Invitrogen Corp) according to the Lipofectamine RNAi max instructions. Eighteen to twenty four hours after the transfection, cells were washed with PBS and incubated with or without IFN-γ (40-100 U/ml) for 15 minutes or 48 hours at 37°C. Then cells were collected and analyzed by flow cytometry, immunoblot, or ELISPOT analyses.

STAT1: 5'-CCUACGAACAUGACCCUAUTT-3' (s) and

5'-AUAGGGUCAUGUUCGUAGGTG-3' (as)

STAT3: 5'-GCCUCAAGAUUGACCUAGATT-3' (s) and

5'-UCUAGGUCAAUCUUGAGGCCT-3' (as)

Non-targeting control: 5'-AGUACAGCAAACGAUACGGtt-3' (s) and
5'-CCGUAUCGUUUGCUGUACUtt-3' (as)

2.2.7 Immunohistochemistry

Paraffin embedded PCI-13 cells were first deparaffinized and hydrated by successive washes in xylene (15 min), 100% ethanol (3 min), 90% ethanol (3 min), 80% ethanol (3 min), 70% ethanol (3 min). The slides were rinsed with water (5 min) and epitope retrieval was performed by heating the slides in citrate pH 6.0 for 20 min, then cooling for 20 min at room temperature. The slides were incubated in 3% hydrogen peroxide (5 min, room temperature (RT)) to block endogenous peroxidases. The slides were blocked for 1 h at RT with 1% bovine serum albumin (BSA)/5% normal horse serum/T-TBS, then incubated overnight at 4°C with a normal rabbit serum control (1:1000) or IFN- γ R pAb (1:25). The next day, the slides were washed in T-TBS for 25 min and incubated with anti-rabbit-HRP secondary antibody (45 min, RT), washed for 25 min in T-TBS, then DAB chromogen (Dako) was added per the manufacturer's instructions. The slides were then rinsed in water, counterstained in Lillie-Meyer's Hematoxylin solution, and rinsed again in water for 10 min. The slides were dehydrated as follows: 95% ethanol 2 min, absolute alcohol (2 min), absolute alcohol (2 min), absolute alcohol (2 min), xylene (1 min), xylene (1 min).

2.2.8 ELISPOT assay

ELISPOT assays were performed as described (188). Briefly, multiscreen_{HTS}-HA filter plates (Millipore) were coated with anti-human IFN- γ mAb 1-D1K (Mabtech, Mariemont, OH) (10

$\mu\text{g}/\text{mL}$ in PBS) overnight at 4°C . Unbound mAb was removed by four successive washings with PBS. Plates were then blocked for 1 h at 37°C with DMEM supplemented with 10% human serum. p53₆₅₋₇₃ (189) or HPV₇₋₁₅ (190)-specific CTL were added to wells in triplicate (5×10^4) and then SCCHN cells (5×10^4). Following an 18-24 hour incubation at 37°C , plates were washed with PBS/0.05% Tween 20 (PBS-T), and incubated with biotinylated anti-IFN- γ mAb (Mabtech) ($2 \mu\text{g}/\text{ml}$) for 4 hours at 37°C . Plates were washed with PBS-T avidin-peroxidase complex (Vector laboratories, Burlingame, CA) (1 hr at RT). Unbound complex was removed by 5 successive washings with PBS-T followed by PBS. Peroxidase staining was performed with 3,3,5'-tetramethylbenzidine (Vector Laboratories) for 4 minutes and stopped by rinsing the plates under running tap water. Spots were enumerated in triplicate wells as a mean \pm standard error using computer-assisted video image analysis software (Cellular Technology Ltd., Shaker Heights, OH). The HLA class I restriction of the recognition of target cells by the tested CTL was assessed by performing the assay in the presence of an anti-HLA class I specific mAb W6/32 ($10 \mu\text{g}/\text{ml}$); the specificity of the inhibition was assessed using an anti-HLA-DR specific mAb L243 ($10 \mu\text{g}/\text{ml}$).

2.2.9 Co-immunoprecipitation

Cell lysates were prepared as described in the immunoblot analysis section and pre-cleared with protein A agarose beads (Millipore, Temecula, CA) at 4°C for 1 hour. Cell lysates ($750 \mu\text{g}$ of protein/ml) were incubated on a rotator overnight at 4°C with $5 \mu\text{g}$ of anti-STAT1 mAb, anti-STAT3 mAb or control IgG mAb. Protein A agarose beads were then added to the lysates and the incubation was continued for 1 additional hour at 4°C on a rotator. Beads were then washed

with lysis buffer three times, size fractionated through a 4-12% SDS-PAGE gel, transferred to a PVDF membrane, and immunoblotted with the indicated antibodies.

2.2.10 Chromatin immunoprecipitation (ChIP) assay

Cells were serum-starved for 48 hours at 37°C in AIM V (Invitrogen Corp, Carlsbad, CA) prior to incubation with IL-6 (50 ng/ml) for 60 minutes at 37°C, IFN- γ (1000 U/ml) for 30 minutes at 37°C, or sequentially with IL-6 (50 ng/ml) for 30 minutes at 37°C and with IFN- γ (1000 U/ml) for 30 minutes at 37°C in the presence of IL-6. At the end of the incubation, cells were fixed with formaldehyde (1% final concentration) (Sigma-Aldrich Inc.) for 10 minutes at RT. Cells were then quenched with glycine (0.125M final concentration) (Sigma-Aldrich Inc.) for 5 min, washed twice with ice-cold PBS and harvested. After centrifugation at 16,100 g for 12 minutes at 4°C, cells were lysed in SDS lysis buffer (Millipore) containing protease inhibitors. Chromatin was sheared by sonication for 12 seconds at 20% of the maximum potency (Cole Parmer Instrument) to generate fragmented DNA with an average length between 200 and 1000 base pairs. STAT1, STAT3, and IgG control mAbs were used to immunoprecipitate STAT1 and STAT3-bound chromatin (5 μ g of antibody) rotating overnight at 4°C. Protein A agarose beads were added to each IP (60 μ l) and incubated for 1 hr rotating at 4°C. The subsequent washes and elution steps were performed using the Ez-ChIPTM kit (Millipore) and according to the manufacturer's instructions. Protein-DNA crosslinks were reversed at 65°C overnight. After RNase (10 μ g, 30 minutes at 37°C) (Sigma-Aldrich Inc.) and sequential proteinase K (10 μ g, 2 hr at 45°C) (Sigma-Aldrich Inc.) digestion, DNA was purified using the QIAquick PCR purification kit (Qiagen). Purified DNA was used in each PCR (at 94°C for 5 minutes, and 35 cycles at 94°C for 30 seconds, at 55°C for 30 seconds, at 68°C for 1 minute) using the following primers:

TAP1: 5'-AAGTAGGCGTTATCTAGTGAGCAGGC 3' (s) and
5'- ACACCTAGAGCTAGCCATTGGCAC' (as).

2.2.11 Quantitative RT-PCR

RNA was extracted from cell lines using Trizol (Invitrogen Corp, Carlsbad, CA) and purified using an RNeasy Midi Kit (Qiagen, Valencia, CA) as described by the manufacturer. DNA was removed from RNA using a Turbo DNA-free kit (Ambion, Austin, TX) and resuspended in RNA secure solution and quantified. Reverse transcription was performed at 25°C for 10 minutes, 48°C for 30 minutes, and 95°C for 5 minutes using random hexamer primers and MultiScribe (Applied Biosystems, Foster City, CA) reverse transcriptase. Quantitative RT-PCR (qRT-PCR) using validated human taqman gene expression assays (Applied Biosystems, Foster City, CA) for LMP2, LMP7, TAP1, TAP2 and calreticulin was performed on the Applied Biosystems 7700 Sequence Detection Instrument and carried out at 95°C for 10 minutes, and 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Expression of the target genes (LMP2, LMP7, TAP1, TAP2 and calreticulin) were calculated relative to β -glucuronidase (GUS; and endogenous control gene) using the ΔC_T (cycle time) method described previously: relative expression = $2^{-\Delta CT}$, where $\Delta CT = C_{T(APM\ gene)} - C_{T(GUS)}$ (191).

2.2.12 Statistical analysis

Data are expressed as a mean \pm standard error of the results obtained in at least three independent experiments. A two-tailed t-test was used to calculate whether observed differences were statistically significant. $P < 0.05$ was considered significant.

2.3 RESULTS

2.3.1 SCCHN cells express low basal pSTAT1 and high basal pSTAT3

At basal untreated conditions in SCCHN cells, PCI-13 and SCC90, high pSTAT3 (Tyrosine 705) was associated with low to undetectable levels of pSTAT1 (Tyrosine 701) (Fig 6) and APM component expression (125). This low level of pSTAT1 does not reflect a deficiency of STAT1, since these cells express significant levels of total STAT1 (Fig 6b). Treatment of SCCHN cells, with IFN- γ (100 U/ml, 15 min) upregulated pSTAT1 but not pSTAT3 (Fig 6). In contrast, IL-6 (50 ng/ml, 15 min) treatment, which is secreted by SCCHN cells in an autocrine/paracrine fashion (58, 105), strongly upregulated pSTAT3 (Fig 6).

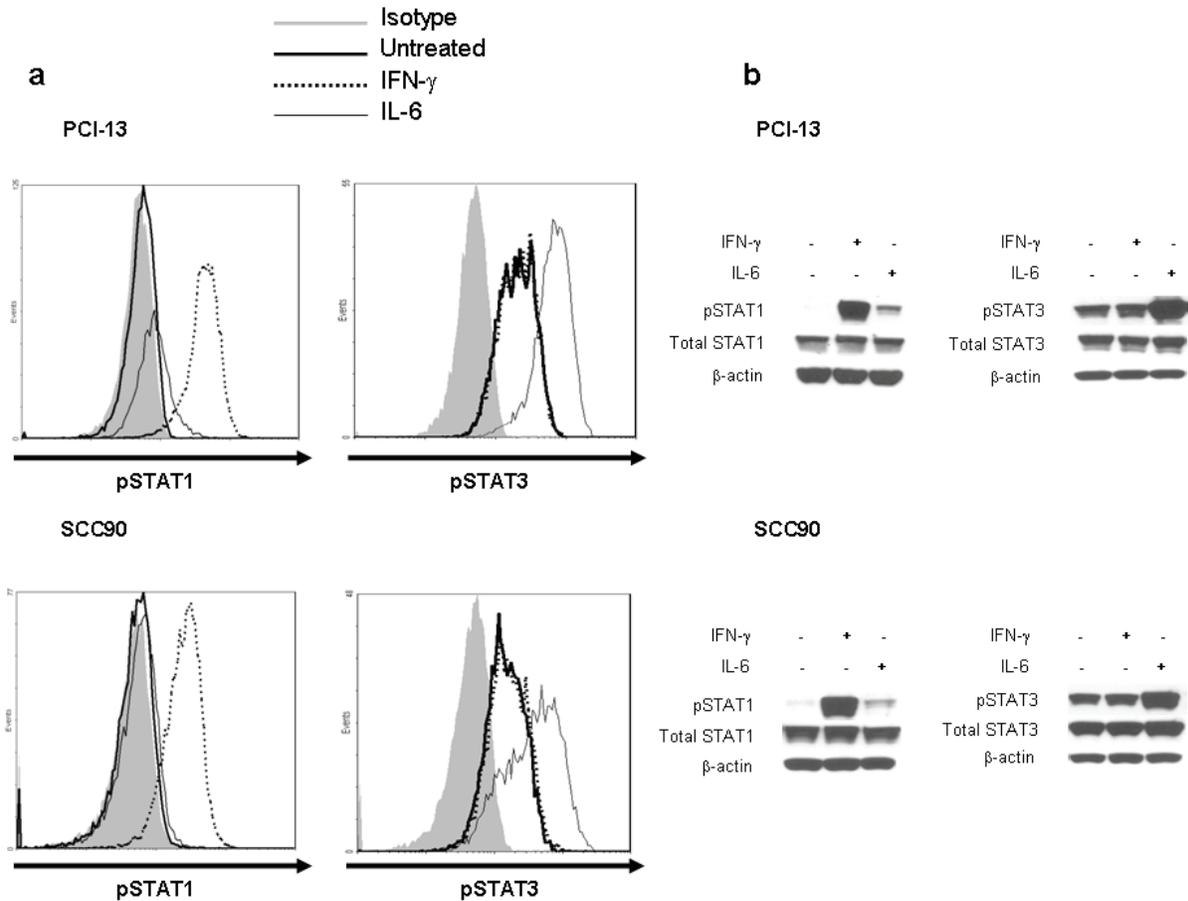


Figure 6. SCCHN cells express low basal pSTAT1 and high basal pSTAT3 that are inducible by treatment with IFN- γ and IL-6.

Cells were treated with IFN- γ (100 U/ml, 15 min) or IL-6 (50 ng/ml, 15 min) and assayed for pSTAT1 (Tyr 701) or pSTAT3 (Tyr 705) by (a) intracellular flow cytometry or (b) immunoblot analyses. Data represent at least three independent experiments.

2.3.2 Induction of IFN- γ -mediated pSTAT1, APM expression, and CTL recognition in STAT3 depleted SCCHN cells

Others have shown that IL-10, a potent STAT3 agonist, can inhibit IFN- γ -mediated STAT1 phosphorylation (155) and TAP1/2 expression in tumor cells (146-148, 183). These studies suggest that pSTAT3 might suppress STAT1 phosphorylation and inhibit APM expression in

SCCHN cells. Since pSTAT3 is expressed at very high levels in SCCHN cells (Fig 6), we investigated whether depleting STAT3 altered the IFN- γ -pSTAT1-APM pathway. Using STAT3 siRNA, target specific reduction of greater than 90% was achieved in PCI-13 and SCC90 cells (Figs 7a-b). Additionally, STAT3 knockdown decreased pSTAT3 in SCCHN cells (Supp Fig 16).

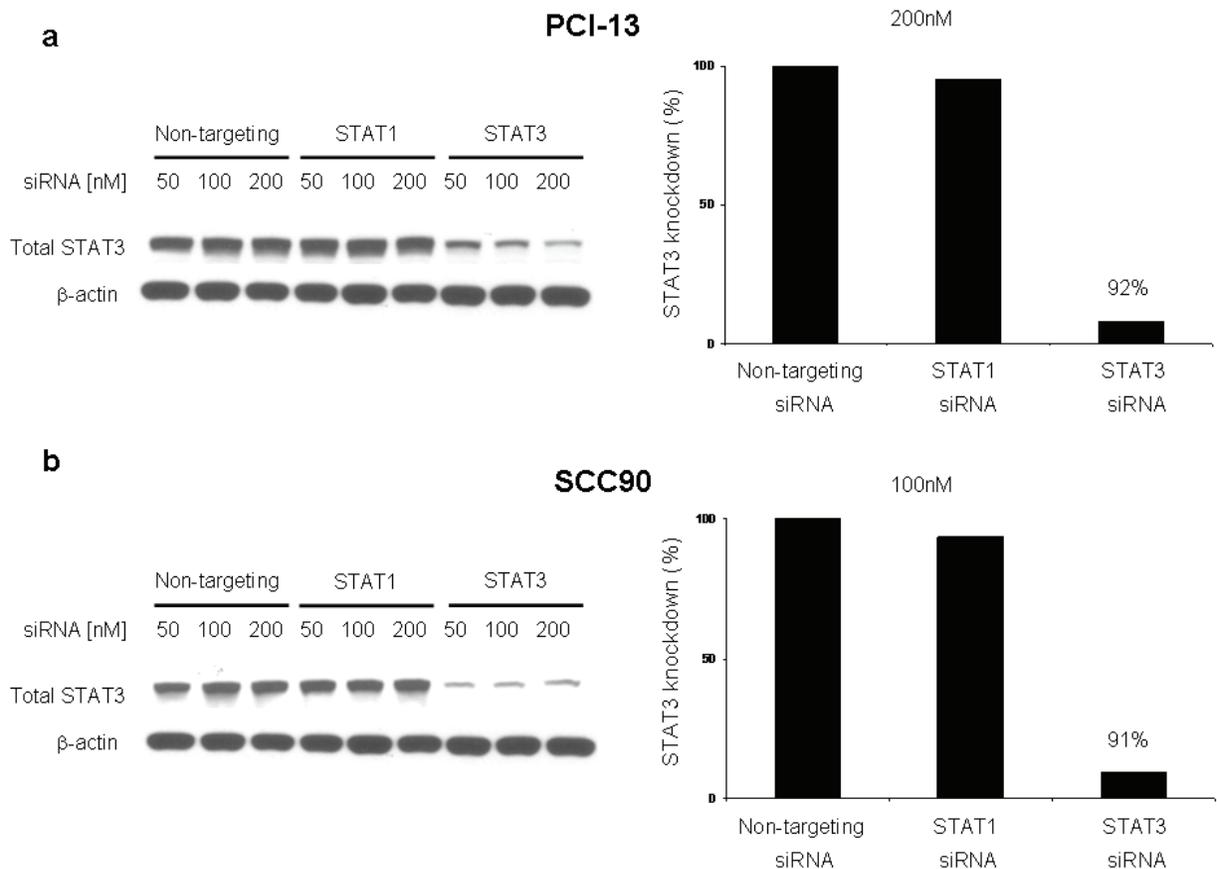


Figure 7. STAT3 siRNA.

(a) PCI-13 cells and (b) SCC90 cells were transfected with various doses of siRNA and probed for total STAT3 protein and β -actin 48 h later. Densitometry was performed.

Interestingly, pSTAT1 and APM component expression levels were equivalent between STAT3 and non-targeting siRNA transfections alone or in combination with IFN- γ treatment

(Figs 8a-d, $p = \text{not significant (NS)}$, two-tailed t-test). Calreticulin, a non-IFN- γ inducible APM component was used as a negative control.

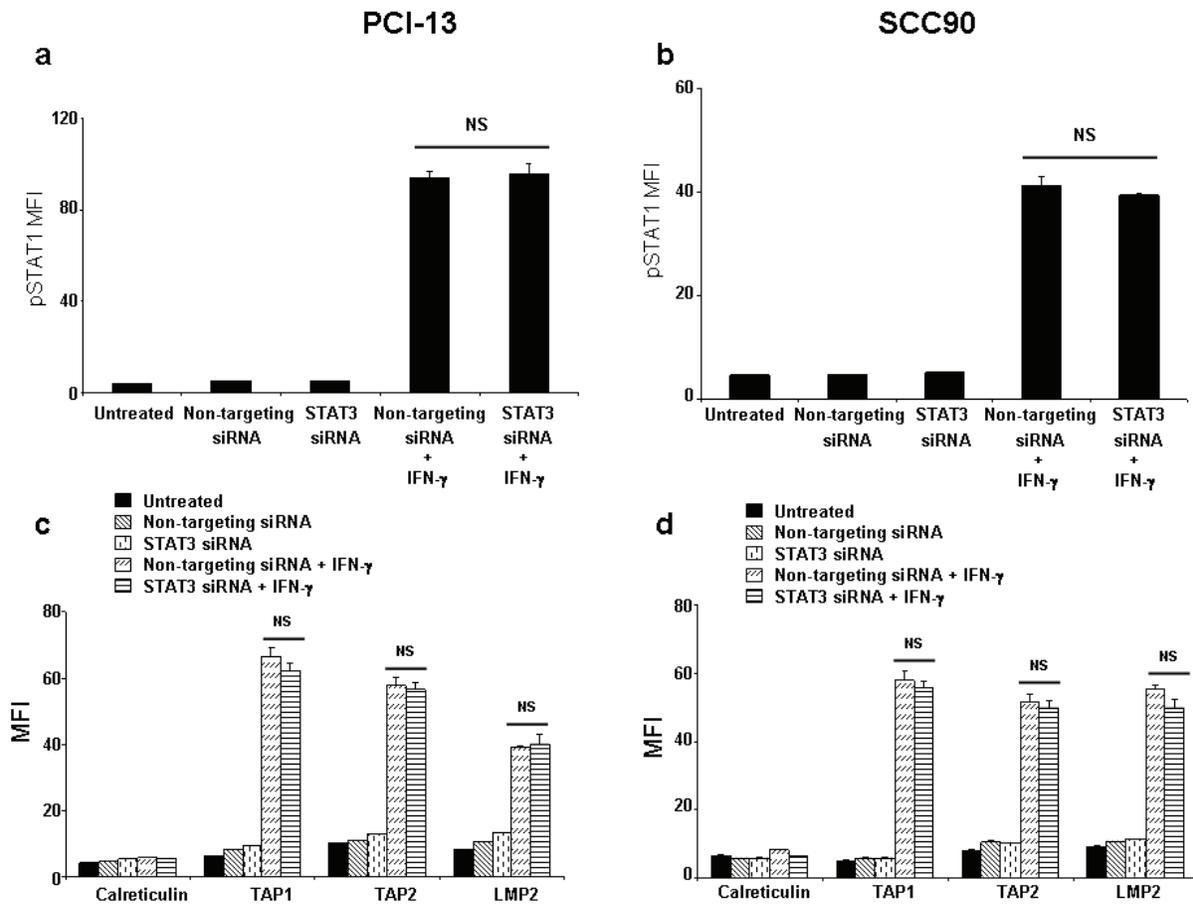


Figure 8. IFN- γ -mediated pSTAT1 and APM expression in STAT3 depleted SCCHN cells.

(a) PCI-13 cells and (b) SCC90 cells were transfected with (200 nM or 100 nM respectively) with non-targeting or STAT3 siRNA alone or in combination with IFN- γ (100U/ml, 15 min), added 48 hours after transfection. Intracellular flow cytometry for pSTAT1 was performed. Data represent the mean fluorescence intensity MFI of at least three independent experiments. ($p = \text{NS}$, two-tailed t-test). Error bars indicate standard error. PCI-13 (c) and SCC90 (d) cells were transfected as described above, except IFN- γ (100U/ml, 48hr) was added 24 hours after transfection. Intracellular flow cytometry for TAP1, TAP2, LMP2 and calreticulin was performed. Data represent the (MFI) of at least three independent experiments. ($p = \text{NS}$, two-tailed t-test). Error bars indicate standard error.

To document the functional impact of the above findings on TA-specific CTL recognition of SCC90 cells, IFN- γ ELISPOT was performed. The beneficial effect of IFN- γ in activating

STAT1 and restoring SCCHN cell recognition by p53₆₅₋₇₃ (189) or HPV₇₋₁₅-specific (190) CTL was unaffected by STAT3 depletion (Figs 9a-b, p=NS, two-tailed t-test). CTL recognition was abrogated using an anti HLA-A,B,C specific mAb demonstrating that CTL were HLA class I restricted. Similar results were obtained with PCI-13 cells. These data illustrate that the APM expression in SCCHN cells is strongly dependent on IFN- γ induced pSTAT1, and STAT3 does not functionally alter the activity of this pathway.

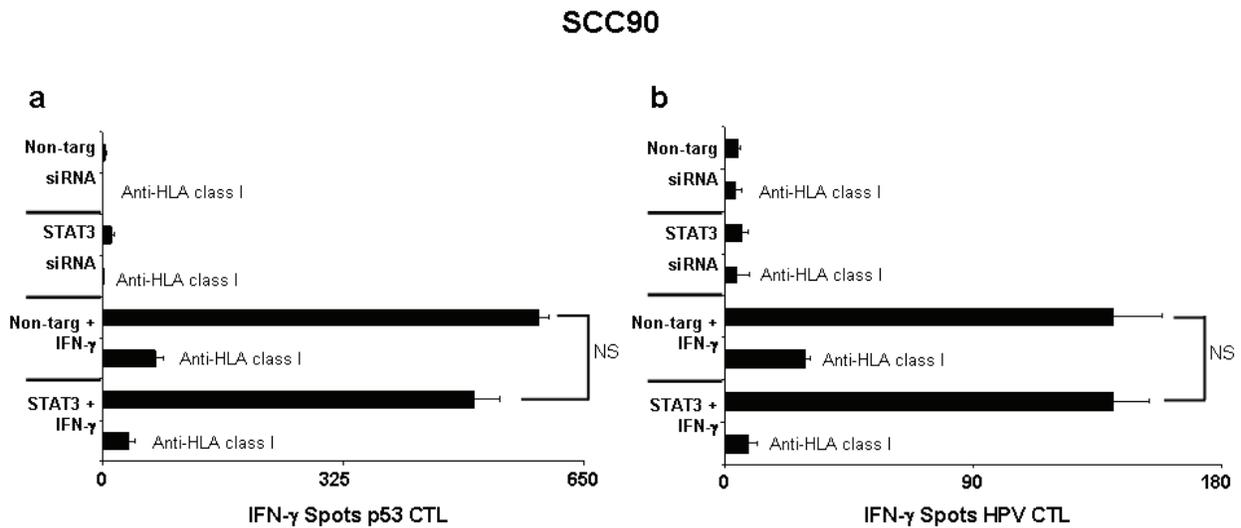


Figure 9. IFN- γ -mediated CTL recognition in STAT3 depleted SCCHN cells.

IFN- γ ELISPOT assay were performed using (a) p53₆₅₋₇₃ or (b) HPV₇₋₁₅ specific cytotoxic T lymphocytes as effector cells and SCC90 cells as targets that have been transfected and treated with IFN- γ as described in Figures 8c-d. An anti-HLA-A,B,C mAb (w6/32) was used to demonstrate that CTL was HLA class I restricted. Error bars indicate standard error (p=NS, two-tailed t-test).

2.3.3 Low pSTAT1 levels contribute to basal APM downregulation and CTL evasion in SCCHN cells

To identify the mechanism of IFN- γ -mediated TAP1 expression and restoration of SCCHN cell lysis by CTL (126), we also investigated the role of pSTAT1. Interestingly, SCCHN cells

express IFN- γ R (Figs 10a-c) and treatment of the cells with IFN- γ (100 U/ml, 15 min or 48h) significantly upregulated pSTAT1 and APM component expression compared to basal, untreated conditions (Figs 10d-e; $p < 0.005$ and $p < 0.001$, respectively, two-tailed t-test), demonstrating that the IFN- γ -pSTAT1-APM signaling pathway is intact. Calreticulin, a non-IFN- γ inducible APM component was used as a negative control.

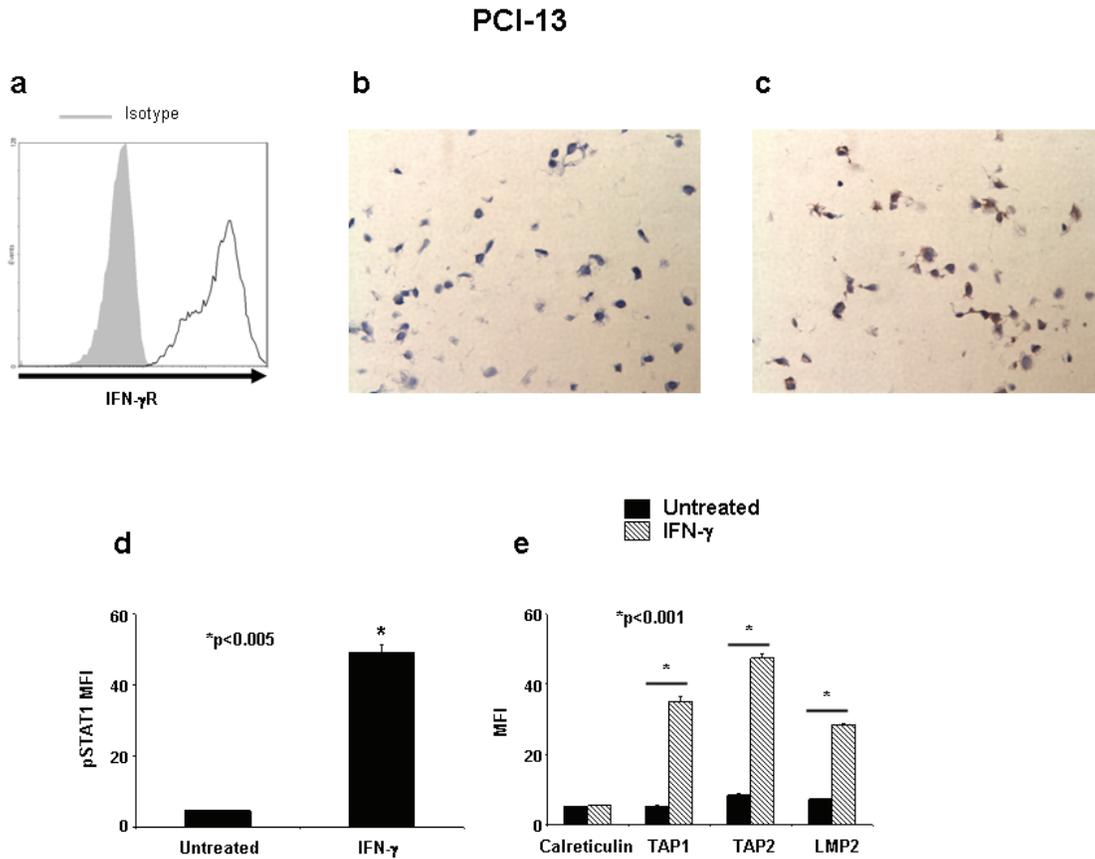


Figure 10. STAT1 signaling is intact in SCCHN cells.

PCI-13 cells were evaluated for basal expression of IFN- γ R by (a) flow cytometry and immunohistochemistry (b) isotype control (40x) (c) IFN- γ R staining (40x). The cells were also treated with IFN- γ (100 U/ml) for either 15 min or 48 h, then analyzed by intracellular flow cytometry for either (d) pSTAT1 (e) or APM component expression respectively. Calreticulin, a non-IFN- γ inducible APM component was included to control for global changes in protein expression following treatment. Data represent at least three independent experiments. Mean fluorescence intensity (MFI) was plotted and error bars indicate standard error (* $p < 0.005$, ** $p < 0.001$, two-tailed t-test).

Moreover, STAT1 siRNA (200 nM, 48h), which specifically decreased STAT1 protein by greater than 75% (Fig 11a), significantly reduced IFN- γ -mediated pSTAT1 and APM component expression (Figs 11b-c; $p < 0.0005$ and $p < 0.002$, respectively, two-tailed t-test). To assess the functional impact of STAT1 expression in SCCHN cells, IFN- γ ELISPOT was

performed. STAT1 depletion significantly impaired TA-specific CTL recognition of IFN- γ treated SCCHN cells (Fig 11d; $p < 0.01$, two-tailed t-test). Similar results were obtained in SCC90 cells. Taken together, these data suggest that activated STAT1 is a crucial mediator of APM component expression, and that low basal pSTAT1 activation contributes to APM-mediated downregulation in SCCHN cells and escape from CTL recognition.

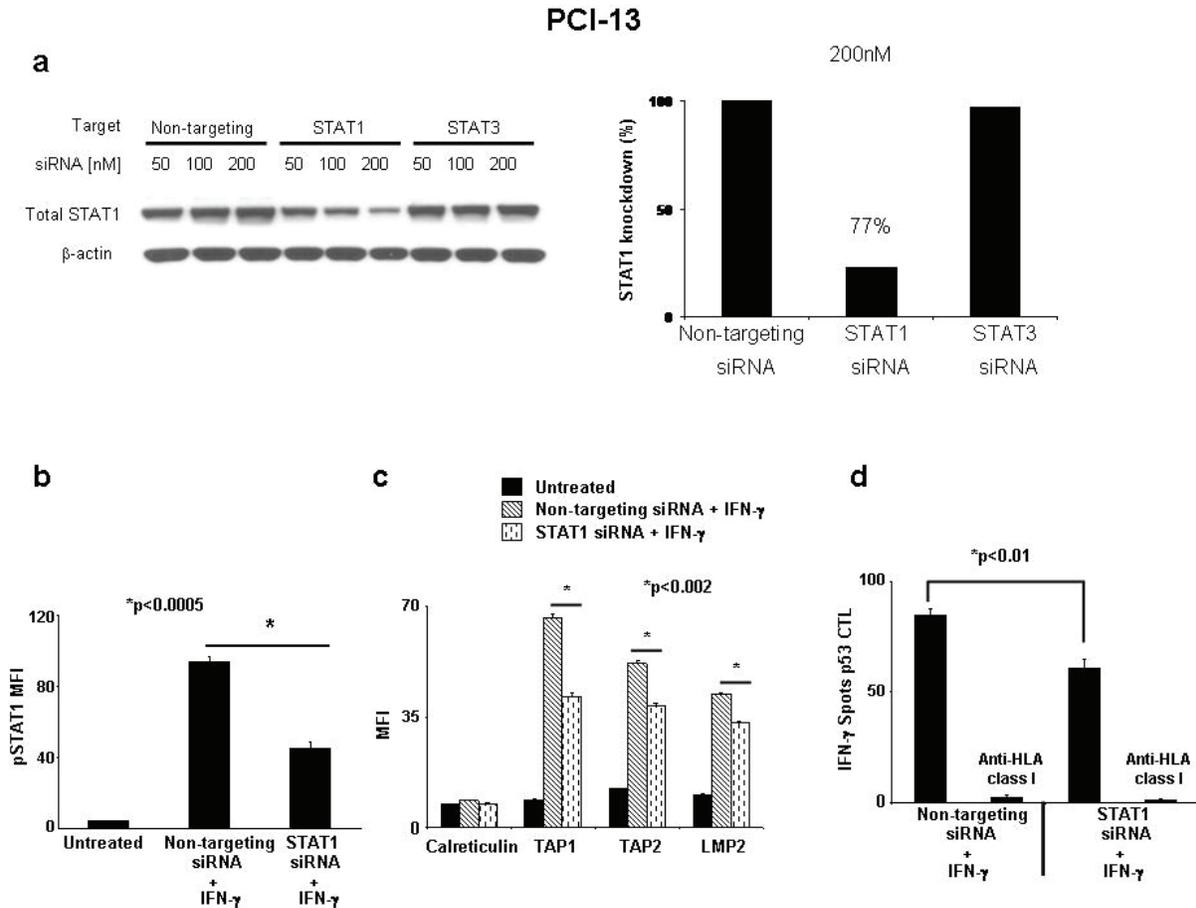


Figure 11. Activated STAT1 is a critical mediator of APM component expression and TA-specific CTL recognition of SCCHN cells.

(a) PCI-13 cells were transfected with the indicated doses of siRNA and knockdown of STAT1 protein was assessed by immunoblot and densitometry analyses. PCI-13 cells were transfected with 200 nM of the indicated siRNA and 24 h after transfection, IFN- γ (40 U/ml) was added for either 15 min or 48 h, then analyzed by intracellular flow cytometry for either (b) pSTAT1 or (c) APM component expression by MFI respectively. Data represent at least three independent experiments. Error bars indicate standard error (* $p < 0.0005$, * $p < 0.002$, two-tailed t-test). (d) PCI-13 cells were transfected with 200nM of the indicated siRNA and 24 h after transfection treated with IFN- γ (40 U/ml) for an additional 24 h. The cells were collected as used as targets and p53₆₅₋₇₃ specific cytotoxic T lymphocyte (CTL) were used as effector cells in IFN- γ ELISPOT assays. An anti-HLA-A,B,C mAb (w6/32) was used to demonstrate that CTL was HLA class I restricted. Error bars indicate standard error (* $p < 0.01$, two-tailed t-test).

2.3.4 IFN- γ and IL-6 induce pSTAT1/3 heterodimerization

Since it has been shown that STAT1:STAT3 heterodimers inhibit STAT1 gene activation (158, 159), this raised the question as to whether these complexes contributed to low APM gene expression. To investigate the potential suppressive role of STAT1:STAT3 heterodimers on basal APM expression, we stimulated SCCHN cells with IFN- γ (100 U/ml, 15 min) or IL-6 (50 ng/ml, 15 min), and co-immunoprecipitated STAT1 and STAT3 from whole cell lysates. These proteins were immunoprecipitated, size fractionated on a SDS gel, and then probed using anti-pSTAT1 (Tyrosine 701), anti-pSTAT3 (Tyrosine 705), anti-total STAT1 and total anti-total STAT3 mAbs. Interestingly, STAT1:STAT3 heterodimers were detected at low levels in the SCCHN cells under basal conditions, and both IL-6 or IFN- γ treatments increased pSTAT1:pSTAT3 heterodimer formation (Fig 12). These data suggest that overexpressed pSTAT3 in SCCHN cells could be interfering with pSTAT1 homodimerization and APM gene activation.

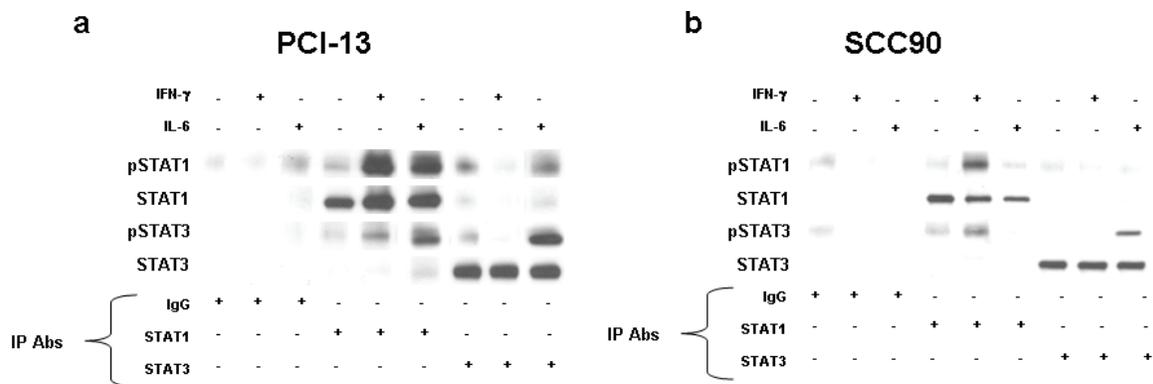


Figure 12. IL-6 and IFN- γ increase pSTAT1:pSTAT3 heterodimerization.

Cells were treated with either IL-6 (50 ng/ml, 15 min) or IFN- γ (100 U/ml, 15min). Whole cell lysates were prepared and immunoprecipitated with anti-STAT1, or anti-STAT3, or an irrelevant control mAb. The immunoprecipitates were size fractionated by SDS/PAGE and transferred to a PVDF membrane. The blots were probed for anti-pSTAT1, STAT1, pSTAT3, and total STAT3 from (a) PCI-13 and (b) SCC90 cells.

2.3.5 IFN- γ -mediated-STAT1 binding to the TAP1 promoter, STAT1 phosphorylation, APM transcription, APM protein, HLA class I and TA-specific CTL recognition is independent of STAT1:STAT3 heterodimerization

Given the presence of pSTAT1:pSTAT3 heterodimers under basal conditions, we investigated whether these complexes could inhibit IFN- γ induced STAT1 binding to the TAP1 promoter, using chromatin immunoprecipitation (ChIP) assays. Pretreatment of SCCHN cells with IL-6 (50 ng/ml, 30 min) to increase the formation of pSTAT1:pSTAT3 heterodimers did not inhibit IFN- γ (1000 U/ml, 30 min) mediated STAT1 binding to the TAP1 promoter compared to IFN- γ treatment alone (Figs 13a-b). Furthermore, the levels of STAT3 bound to the TAP1 promoter remained unchanged with either cytokine treatment, demonstrating that STAT1:STAT3 heterodimers do not bind to the TAP1 promoter (Fig 13). These data illustrate that STAT1:STAT3 heterodimers do not interfere with the IFN- γ -pSTAT1 signaling pathway and that pSTAT1 is a crucial transcription factor in regulating TAP1 gene transcription.

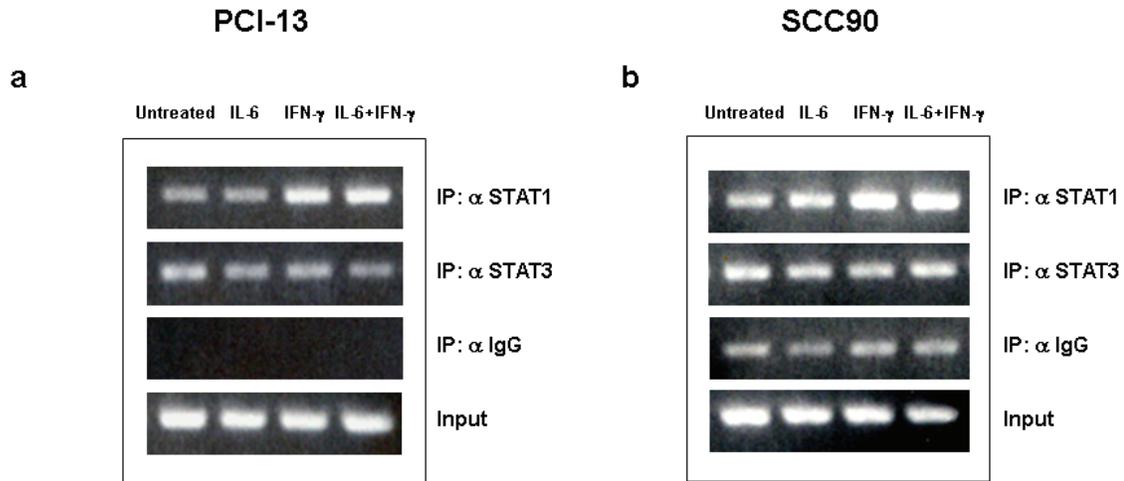


Figure 13. IFN- γ -mediated-STAT1 binding to the TAP1 promoter is independent of STAT1:STAT3 heterodimerization.

(a) PCI-13 and (b) SCC90 cells were untreated, treated with IL-6 (50 ng/ml, 60 min), IFN- γ (1000 U/ml, 30 min), or pre-treated with IL-6 (50 ng/ml, 30 min) then treated with IFN- γ (1000 U/ml, 30 additional min) in the presence of IL-6. The cells were fixed with formaldehyde, quenched with glycine and lysed in SDS lysis buffer. Chromatin was sheared by sonication and probed with anti-STAT1, anti-STAT3, and anti-IgG mAbs. Protein-DNA crosslinks were reversed, and both RNA and protein were removed by enzymatic digestion. DNA was purified and PCR was performed amplifying a canonical GAS sequence in the TAP1 promoter localized to STAT1 binding.

To determine whether increasing pSTAT1:pSTAT3 heterodimerization affected IFN- γ -mediated STAT1 phosphorylation, APM transcription, and APM protein expression, SCCHN cells were pretreated with IL-6 (50 ng/ml, 30 min), then treated with IFN- γ (100 U/ml, 30 min to 48 h) in the presence of IL-6. Under these conditions, IFN- γ -mediated STAT1 phosphorylation, APM component transcription, APM component protein levels and HLA class I were equivalent to cells pretreated with IL-6 prior to incubation with IFN- γ (Figs 14a-h ; p=NS, two-tailed t-test), further supporting the independence of the IFN- γ -pSTAT1 pathway from STAT3.

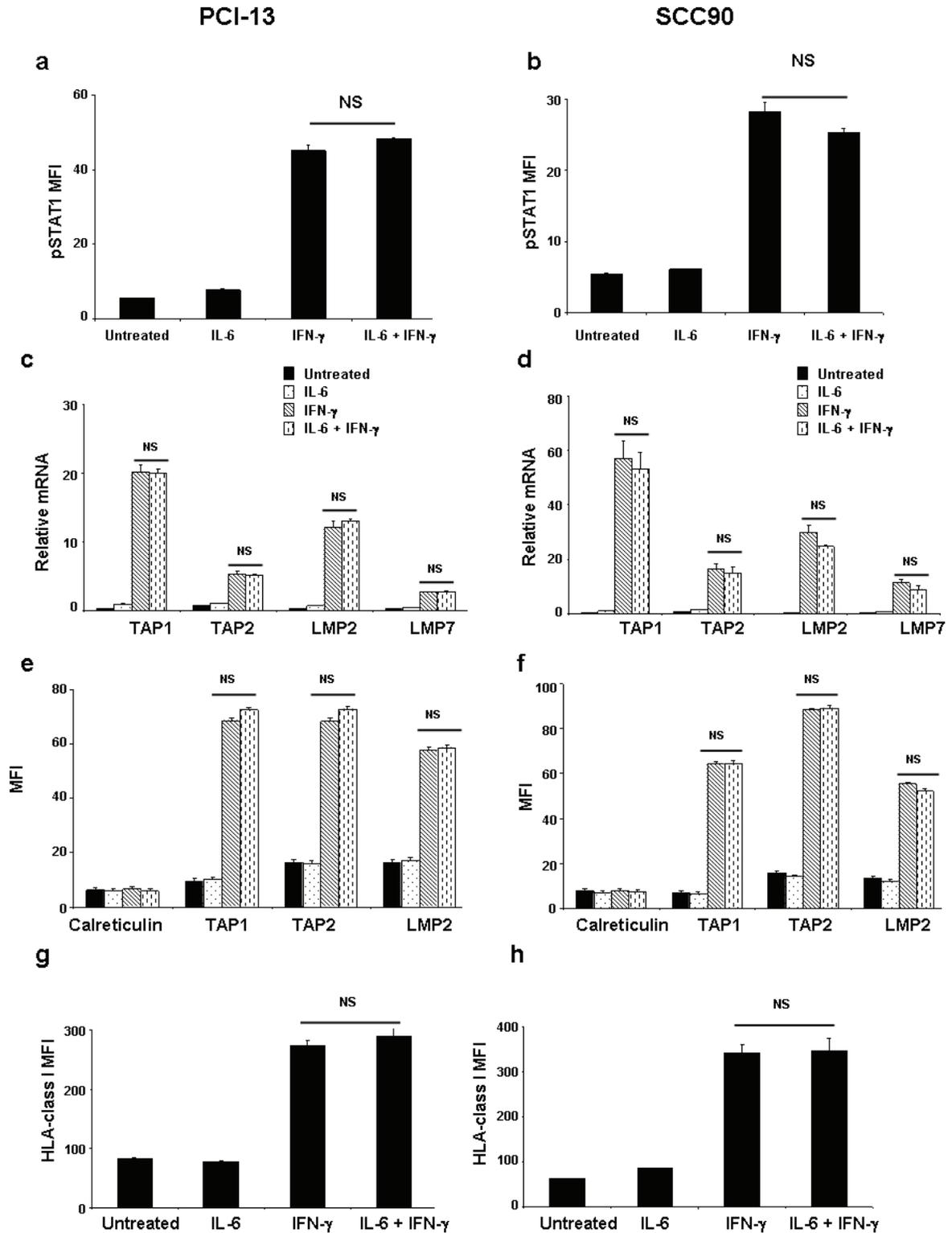


Figure 14. IFN- γ -mediated STAT1 phosphorylation, APM component transcription and protein expression and HLA class I expression is independent of STAT1:STAT3 heterodimerization.

(a) PCI-13 and (b) SCC90 cells were untreated, treated with IL-6 (50 ng/ml, 30 min), IFN- γ (100 U/ml, 15 min), or pretreated with IL-6 (50 ng/ml, 15 min), then treated with IFN- γ (100 U/ml, 15min) in the presence of IL-6. Intracellular flow cytometry was performed measuring pSTAT1. MFI was plotted representing at least three independent experiments (p=NS, two-tailed t-test). Error bars indicate standard error. (c) PCI-13 and (d) SCC90 cells were untreated, treated with IL-6 (50 ng/ml, 5.5 h), IFN- γ (100 U/ml, 5h), or pre-treated with IL-6 (50 ng/ml, 30 min) then treated with IFN- γ (100 U/ml, 5 additional h) in the presence of IL-6. TAP1, TAP2, LMP2 and LMP7 transcription was measured by qRT-PCR as described in the Materials and Methods. Data represent three replicates of at least three independent experiments (p=NS, two-tailed t-test). Error bars indicate standard error. (e) PCI-13 and (f) SCC90 were untreated, treated with IL-6 (50 ng/ml, 48 h), IFN- γ (100 U/ml, 48 h), or pretreated with IL-6 (50 ng/ml, 30 min), then treated with IFN- γ (100 U/ml, 48 h) in the presence of IL-6. Intracellular flow cytometry was performed measuring Calreticulin, TAP1, TAP2, and LMP2 protein. MFI was plotted representing at least three independent experiments (p=NS, two-tailed t-test). Error bars indicate standard error. (e) PCI-13 and (f) SCC90 were treated as described in (e) and (f). Flow cytometry was performed measuring HLA class I. MFI was plotted representing at least three independent experiments (p=NS, two-tailed t-test). Error bars indicate standard error.

Lastly, IFN- γ ELISPOT demonstrated that the beneficial effect of IFN- γ in activating STAT1 and restoring SCCHN cell recognition by TA-specific CTL was unaffected by STAT1:STAT3 heterodimerization (Figs 15a-b; p=NS, two-tailed t-test). These data demonstrated that STAT1:STAT3 heterodimers do not interfere with IFN- γ -pSTAT1 signaling, which is critical for TAP1 gene activation, APM protein expression and TA-specific CTL recognition of SCCHN cells. Thus, a deficiency in STAT1 activation and not overexpressed pSTAT3 is responsible for poor basal APM component expression in SCCHN cells.

SCC90

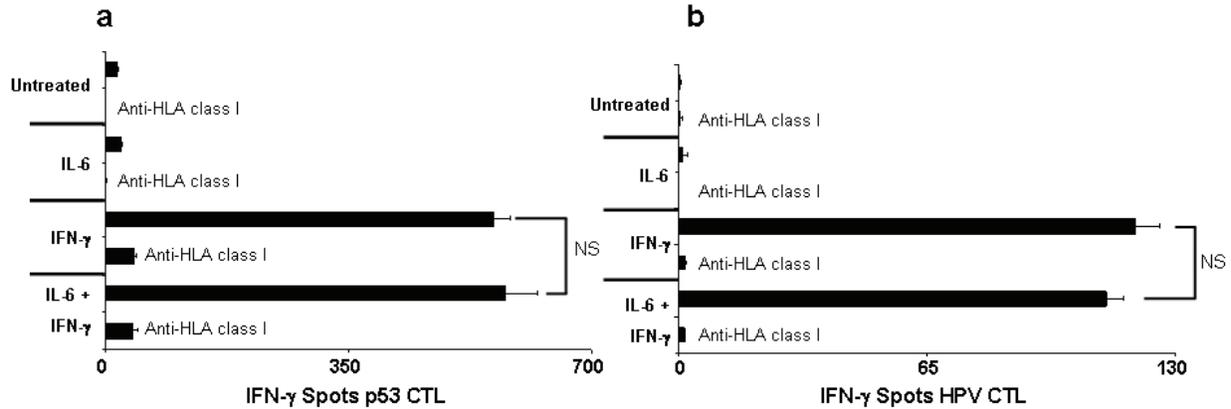


Figure 15. IFN- γ -mediated HLA class I expression and TA-specific CTL recognition of SCC90 cells is independent of STAT1:STAT3 heterodimerization.

IFN- γ ELISPOT assay were performed using (a) p53₆₅₋₇₃ or (b) HPV₇₋₁₅ specific cytotoxic T lymphocytes as effector cells and SCC90 cells as target cells that have been treated as described in Figure 14 (e-f). An anti-HLA-A,B,C mAb (w6/32) was used to demonstrate that CTL was HLA class I restricted. Error bars indicate standard error ($p=NS$, two-tailed t-test).

2.4 DISCUSSION

Abnormal APM component expression and/or dysfunction frequently occur in human malignancies (179), but this mechanism of immune escape has not been thoroughly investigated. In this study, we document that SCC90 cells express low basal levels of pSTAT1 (Fig 6) as a mechanism of APM component downregulation despite intact IFN- γ R expression and signaling (Fig 10), and that exogenous IFN- γ -mediated STAT1 activation is required to facilitate STAT1 binding to the TAP1 promoter (Fig 13), APM component protein expression (Fig 11c), and TA-specific CTL recognition (Fig 11d). These data support that low basal pSTAT1 levels and not

defects in the endogenous IFN- γ -pSTAT1-APM signal transduction pathway are responsible for the low basal APM component expression observed in SCCHN cells.

Complete inhibition of IFN- γ mediated APM component expression was not observed after significant knockdown of total STAT1 protein. Interestingly, treatment of SCCHN cells with a ten fold lower dose of IFN- γ (10 U/ml) upregulated APM components (Supp Fig 17) suggesting that a threshold level of pSTAT1 is required to induce APM protein expression. Thus, despite the significant reduction of total STAT1 by siRNA, residual STAT1 was sufficiently activated by IFN- γ to mediate APM component expression and CTL recognition, albeit at significantly reduced levels (Figs 11c-d). Interestingly, others have shown reduced IFN- γ levels in the plasma of patients with SCCHN compared to age-matched controls (70, 71), providing a potential clinical explanation for low basal STAT1 activation and APM-mediated immune escape in SCCHN cells.

Others have shown that STAT3 activation by type I IFNs can inhibit STAT1 target gene expression through formation of STAT1:STAT3 heterodimers (158, 159), therefore, we also investigated whether overexpressed pSTAT3, and these heterodimer complexes were responsible for low basal APM component expression in SCCHN cells. Our exclusion for a role of STAT3 in basal APM-mediated immune escape differs from previous reports that provide evidence that STAT3 activation by IL-10 inhibits IFN- γ -mediated STAT1 phosphorylation (155) and downregulates TAP expression in tumor cells (146-148, 183). However, IL-10 did not activate pSTAT3 in SCCHN cells (unpublished data). The biological basis for pSTAT1:pSTAT3 heterodimers in SCCHN is unknown (153). We investigated whether pSTAT1:pSTAT3 heterodimers were responsible for low basal APM component expression and SCCHN escape from CTL recognition. STAT1:STAT3 heterodimers were detected in PCI-13 and SCC90 cells

at basal, untreated conditions, and these complexes were increased after treatment with IFN- γ and IL-6 (Fig 12). Interestingly, IFN- γ -mediated pSTAT1 upregulation also induced heterodimer formation with pSTAT3 (Fig 12), but APM component transcription and protein were still expressed (Figs 14c-f). This finding might be due to the abundant activation of STAT1 by IFN- γ inducing pSTAT1 homodimerization and APM gene activation, and excess pSTAT1 dimerizing with pSTAT3, which is overexpressed in SCCHN cells. Also, STAT3 was found bound to the TAP1 promoter (Figs 13a-b) and this observation is consistent with the fact that STAT1 and STAT3 can bind to the same canonical GAS domain, yet regulate different target genes (192).

IL-6 induced pSTAT1:pSTAT3 heterodimerization did not alter IFN- γ -mediated APM component transcription or protein (Fig 14c-f). To investigate whether the dose of IFN- γ (100 U/ml) used in our studies might be masking a potential negative regulatory effect of IL-6-mediated STAT1:STAT3 heterodimers, we used a 10 fold lower dose of IFN- γ (10 U/ml). Even at this lower dose, IL-6 treatment did not inhibit IFN- γ -mediated APM component expression (Supp Fig 17). Importantly, at baseline, STAT3 depletion by siRNA could not reproduce the beneficial effects of stimulating the IFN- γ -STAT1-APM pathway. Treatment of SCCHN cells with IFN- γ after STAT3 depletion did not augment APM component expression or TA-specific CTL recognition (Fig 9). These data demonstrate that APM component expression and CTL recognition of SCCHN cells primarily require activation of STAT1 with exogenous IFN- γ independent of STAT3.

The mechanism of how SCCHN cells maintain low basal pSTAT1 and APM component expression is still not known. Deficiencies in TAP expression have been documented to occur through a lack of STAT1 and IRF1 phosphorylation (142), mutations in the JAK1 kinase that

prevent its activation and subsequent STAT1 phosphorylation (143), and impaired RNA polymerase II recruitment to the TAP1 promoter (193). Several investigators have identified that members of a small sub-family of non-receptor protein tyrosine phosphatases (PTPs), Src homology-2 domain-containing phosphatases (SHP), which can dephosphorylate Jak1 (194) and STAT1 (168, 169). Providing a stimulus for STAT1 activation such as IFN- γ can correct APM downregulation and enhance CTL lysis *in vitro*, but perhaps a more efficacious therapeutic approach would be a targeted therapy against the negative regulators of STAT1 phosphorylation in SCCHN. A greater understanding of the mechanisms responsible for low basal pSTAT1 and APM expression could augment current T cell based immunotherapies by enhancing the immunogenicity of its tumor cell target.

In summary, these studies identified low pSTAT1 in SCCHN cells as a critical mediator of APM component downregulation and CTL escape. We also investigated a potential role for pSTAT1:pSTAT3 heterodimers in APM component downregulation of SCCHN cells. Our data demonstrate that pSTAT1:pSTAT3 heterodimers do not alter the IFN- γ -pSTAT1-APM signaling axis. The function of the pSTAT1:pSTAT3 heterodimer remains unknown, but these findings indicate a need to directly stimulate the STAT1 pathway to enhance APM expression and reverse CTL evasion by tumor cells. Future studies might include investigating the negative regulators of STAT1 activation whose dysregulated activity might be responsible for low basal pSTAT1 and APM component levels in SCCHN.

2.5 SUPPLEMENTAL DATA

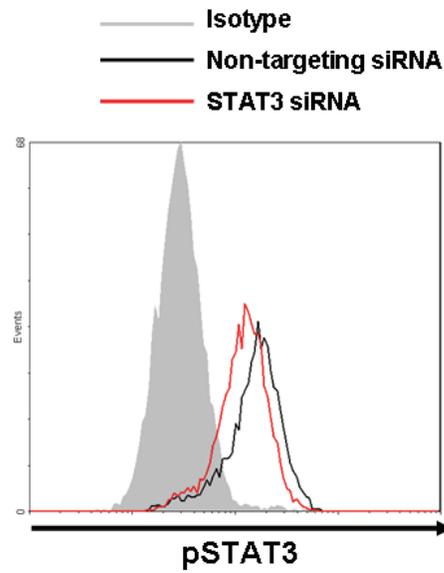


Figure 16. Supplemental Data. STAT3 siRNA decreases pSTAT3 in SCCHN cells.

PCI-13 cells were transfected with the indicated siRNA (30 nM, 48 h) and intracellular flow cytometry was performed measuring pSTAT3.

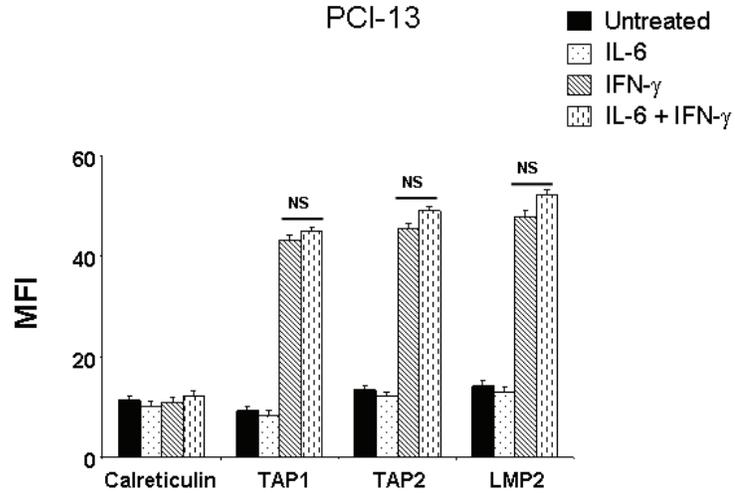


Figure 17. Supplemental Data. IFN- γ (10 U/ml)-mediated APM component expression is independent of STAT1:STAT3 heterodimerization.

PCI-13 cells were untreated, treated with IL-6 (50 ng/ml, 48 h), IFN- γ (10 U/ml, 48 h), or pretreated with IL-6 (50 ng/ml, 30 min), then treated with IFN- γ (10 U/ml, 48 h) in the presence of IL-6. Intracellular flow cytometry was performed measuring Calreticulin, TAP1, TAP2, and LMP2. MFI was plotted representing at least three independent experiments (p=NS, two-tailed t-test).

3.0 SHP2 REGULATES PSTAT1-MEDIATED APM COMPONENT EXPRESSION AND CTL RECOGNITION OF HEAD AND NECK CANCER CELLS

3.1 INTRODUCTION

Tumor immune escape remains a critical obstacle to successful T cell-based cancer immunotherapy (24, 195). Schreiber and colleagues demonstrated that tumor cells undergo a rigorous immune selection process resulting in neoplastic disease that has lost significant immunogenicity (18). In squamous cell carcinoma of the head and neck (SCCHN) and other malignancies, this immune pressure commonly selects for tumor cells that downregulate antigen processing machinery (APM) components, such as the transporter associated with antigen processing (TAP)-1 protein. As a result, tumor antigen (TA) processing and presentation is reduced, leading to escape from TA-specific cytotoxic T lymphocyte (CTL) recognition (126, 128, 132, 133, 135, 196). Interestingly, APM component downregulation correlates with poor prognosis in patients with SCCHN (125), demonstrating that this escape mechanism is clinically significant. Thus, addressing the molecular mechanisms responsible for APM component downregulation is important to promote the success of SCCHN immunotherapy.

Previous studies identified that treatment of SCCHN cells with interferon gamma (IFN- γ) upregulated APM components and restored TA-specific CTL lysis *in vitro* (126). IFN- γ signals transduction occurs by inducing phosphorylation of janus activated kinase (JAK)-1 and

JAK2 and signal transducer and activator of transcription (pSTAT1)-1 (46). pSTAT1 forms homodimers and translocates to the nucleus to induce APM gene activation (114). Given the important role of activated STAT1 on APM protein expression (Chapter 2), we hypothesize that low basal levels of pSTAT1 might be responsible for APM component downregulation in SCCHN cells.

Protein tyrosine phosphatases (PTPs) have been implicated in the inactivation and dephosphorylation of STAT1 (197, 198). In particular, src homology-2 domain-containing phosphatase (SHP)-2 has been suggested as a negative regulator of the JAK-STAT signal transduction pathway (165, 166, 169, 199-202). Furthermore, SHP2 overexpression and/or hyperactivity have been demonstrated in leukemia, breast, cervical, and bladder cancers (167, 173, 203, 204). We posited that dysregulated SHP2 activity may be responsible for maintaining low pSTAT1 levels in SCCHN, thereby reducing APM component expression and mediating escape from CTL recognition.

We investigated the role of SHP2 in mediating APM component downregulation in SCCHN cells. Immunohistochemical analysis was performed on SCCHN tissue to investigate whether SHPs were aberrantly expressed. SHP2 depletion studies were performed on SCCHN cells to evaluate the effect on STAT1 phosphorylation, APM component expression, HLA class I expression, TA-specific CTL recognition, and cytokine/chemokine secretion.

3.2 MATERIALS AND METHODS

3.2.1 Cell lines

The HLA-A*0201⁺ SCCHN cell lines, PCI-13 and SCC90 (205) and HLA-A*0201⁻ cell lines, SCC4 and PCI-15B were characterized and described previously (184). All tumor cell lines were cultured in DMEM (Sigma-Aldrich Inc, St. Louis, MO) supplemented with 10% FBS (Mediatech, Herndon, VA), 2% L-glutamine, and 1% penicillin/streptomycin (Invitrogen Corp, Carlsbad, CA).

3.2.2 Cytokines and reagents

IFN- γ was purchased from InterMune (Brisbane, CA) and sodium orthovanadate was purchased from (Sigma-Aldrich).

3.2.3 Antibodies

Anti-HLA-A,B,C mAb (w6/32) (Ebiosciences, San Diego, CA) and anti-HLA-DR (L243) mAb (Biolegend, San Diego, Ca) were used in ELISPOT assays. LMP2-specific mAb SY-1 (185), TAP1-specific mAb NOB-1, TAP2-specific mAb NOB-2, and calreticulin-specific mAb TO-11, were developed and characterized as described (185, 186). FITC conjugated IgG anti-mouse mAb was used as a secondary antibody for APM staining and purchased from Invitrogen Corp. The intracellular pSTAT staining was performed using PE conjugated irrelevant IgG2a mAb isotype control, PE conjugated phosphorylated tyrosine 701 STAT1 mAb (pSTAT1 Tyr701) and

PE conjugated pSTAT3 Tyr705 mAb purchased from BD Biosciences (San Jose, CA). Western blotting antibodies include anti-pSTAT1 Tyr701 mAb, anti-pSTAT3 Tyr705 mAb (Cell Signaling Tech, Danvers, MA), anti-STAT1 (C-24) polyclonal (pAb) (Santa Cruz Biotech, Santa Cruz, CA), anti-STAT3 (C-20) pAb (Santa Cruz Biotech), anti- β -actin mAb (Sigma-Aldrich Inc), anti-rabbit IgG-HRP (Promega, Madison, WI), anti-mouse IgG-HRP (Biorad, Hercules, CA

3.2.4 Intracellular flow cytometry for APM component and pSTAT1 expression

See section 2.2.4

3.2.5 Immunoblot analysis

See section 2.2.5

3.2.6 Quantitative RT-PCR

See section 2.2.13

3.2.7 Small interfering RNA (siRNA)

SCCHN cell lines were transfected at 30-40% confluence with SHP2 targeting siRNA or a non-targeting siRNA control (Ambion, Austin, Tx), Lipofectamine RNAi max (Invitrogen Corp), and Optimem I (Invitrogen Corp) according to the Lipofectamine RNAi max instructions. Eighteen to twenty four hours after the transfection, cells were washed with PBS and incubated with or

without IFN- γ (100 U/ml) for 15 minutes or 48 hours at 37°C. Then cells were collected and analyzed by flow cytometry, immunoblot, or ELISPOT analyses.

SHP1: 5'-GGUGACCCAUAUUCGGAUCTT' (s) and

5'-GAUCCGAAUAUGGGUCACCTG-3' (as)

SHP2: 5'-GGAGAACGGUUUGAUUCUUTT-3' (s) and

5'-AAGAAUCAAACCGUUCUCCTC-3' (as)

Non-targeting control: 5'-AGUACAGCAAACGAUACGGtt-3' (s) and

5'-CCGUAUCGUUUGCUGUACUtt-3' (as)

3.2.8 ELISPOT assay

ELISPOT assays were performed as described (188). Briefly, multiscreen_{HTS}-HA filter plates (Millipore) were coated with anti-human IFN- γ mAb 1-D1K (Mabtech, Mariemont, OH) (10 μ g/mL in PBS) overnight at 4°C. Unbound mAb was removed by four successive washings with PBS. Plates were then blocked for 1 h at 37°C with DMEM supplemented with 10% human serum. EGFR₈₅₃₋₈₆₁ (188) and p53₆₅₋₇₃ (189, 206) specific CTL were added to wells in triplicate (5×10^4) and then SCCHN cells (5×10^4). Following an 18-24 hour incubation at 37°C, plates were washed with PBS/0.05% Tween 20 (PBS-T), and incubated with biotinylated anti-IFN- γ mAb (Mabtech) (2 μ g/ml) for 4 hours at 37°C. Plates were washed with PBS-T avidin-peroxidase complex (Vector laboratories, Burlingame, CA) (1 hr at RT). Unbound complex was removed by 5 successive washings with PBS-T followed by PBS. Peroxidase staining was performed with 3,3',5'-tetramethylbenzidine (Vector Laboratories) for 4 minutes and stopped by rinsing the plates under running tap water. Spots were enumerated in triplicate wells as a mean \pm standard error using computer-assisted video image analysis software (Cellular Technology

Ltd., Shaker Heights, OH). The HLA class I restriction of the recognition of target cells by the tested CTL was assessed by performing the assay in the presence of an anti-HLA class I specific mAb W6/32 (10 µg/ml); the specificity of the inhibition was assessed using an anti-HLA-DR specific mAb L243 (10 µg/ml).

3.2.9 Immunohistochemistry

Protein levels were evaluated by immunohistochemical (IHC) staining of tumor and adjacent mucosal specimens arrayed in a previously described tissue microarray (TMA) (207). For the studies presented here, the maximum number of evaluable tumor specimens was 46, 16 of these tumors had arrayed adjacent mucosal tissues available for analysis. Tissue microarray quality assessment and morphologic confirmation of tumor or normal histology was performed using one H&E-stained slide for every ten tissue sections.

Arrayed tissues were IHC stained for SHP1 and SHP2 and tissue levels were evaluated semi-quantitatively. Prior to incubation with anti-SHP1 or anti-SHP2 antibodies for 60 minutes at room temperature, antigen retrieval was performed using citrate pH 6 buffer (Dako) followed by incubation with a 3% hydrogen peroxide solution for 5 min at room temperature. The tissue specimens were then blocked with calf serum block (Invitrogen) for 10 minutes at room temperature. SHP1 staining was developed using Dako Dual Envision+ for 30 minutes at room temperature followed by incubation with Substrate Chromagen for 5 minutes at room temperature. Slides were counterstained with Harris Hematoxylin, and cytoplasmic and nuclear staining intensity (0 – 3) and percent of tumor to the nearest 5% were determined separately by a head and neck cancer pathologists (LW and RS). An IHC score was derived from the product of

the intensity and percentage of tumor stained, and IHC scores for each core of a specimen were averaged.

3.2.10 Luminex assay

SCCHN cells were assayed for expression of various cytokines and chemokines using a human cytokine 30-plex panel (Invitrogen) as previously described (208).

3.2.11 Statistical analysis

Data are expressed as a mean \pm standard error of the results obtained in at least three independent experiments. A two-tailed t-test was used to calculate whether observed differences were statistically significant. $P < 0.05$ was considered significant. For IHC studies, differences between paired tumor and adjacent mucosa levels of each protein were evaluated using the signed-rank test. Correlations between proteins were assessed using Spearman's nonparametric correlation coefficient. Threshold for significance was $P < 0.05$.

3.3 RESULTS

3.3.1 Sodium orthovanadate upregulates pSTAT1 in SCCHN cells

Previous work from chapter 2.0 identified pSTAT1 as an important mediator of APM component expression in SCCHN cells. Interestingly, basal levels of pSTAT1 are undetectable in SCCHN cells, but total STAT1 protein is abundantly expressed (Fig 18). To test whether a PTP might be

responsible for this observation, a panel of SCCHN cells, PCI-13, SCC90, SCC4, and PCI-15B, were treated with a broad phosphatase inhibitor, sodium orthovanadate (SOV). After 24 h of treatment with SOV (100uM), pSTAT1 levels were significantly upregulated in these cells by immunoblot analysis (Fig 18). As a positive control, IFN- γ (100 U/ml, 10 min), upregulated pSTAT1 to a similar degree as the SOV treatments (Fig 18). In addition, pretreatment of SCCHN cells with SOV (100uM, 24 h), then incubating with IFN- γ (100 U/ml, 10 min), augmented pSTAT1 expression in all of the SCCHN cells (Fig 18). These data demonstrate that PTPs contribute to the dephosphorylation of STAT1 in SCCHN cells at baseline.

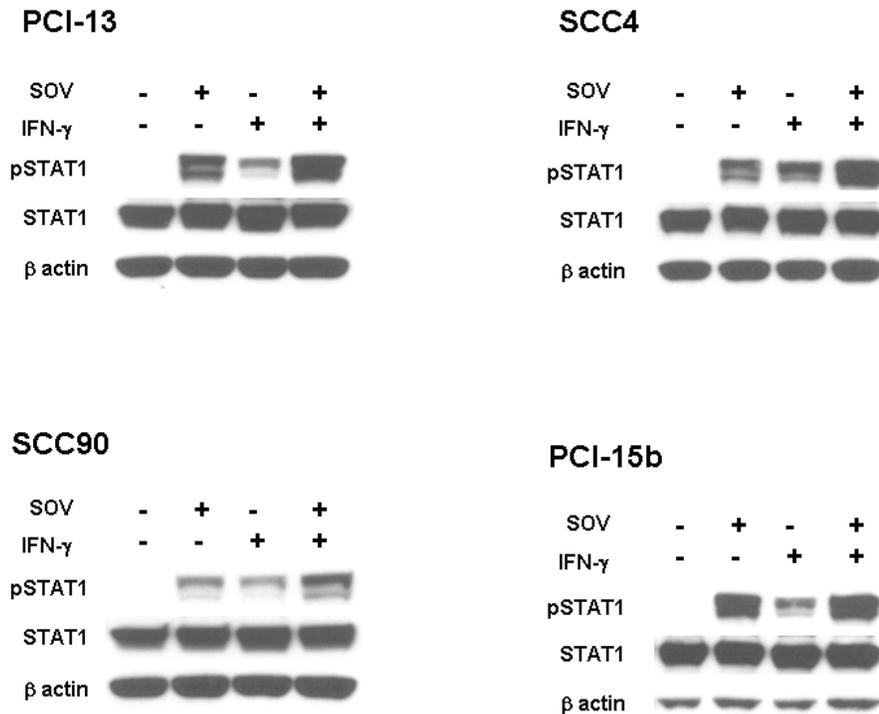


Figure 18. Sodium orthovanadate (SOV) upregulates pSTAT1 in SCCHN cells.

PCI-13, SCC90, SCC4, and PCI-15b cells were treated with SOV (100uM, 24 h), IFN- γ (100 U/ml, 10 min), or pretreated with SOV (100uM, 24 h) then with IFN- γ (100 U/ml, 10 min) and analyzed by immunoblot with the indicated antibodies. β -actin served as a loading control.

3.3.2 SHP2 is overexpressed in SCCHN tissue but not normal mucosa

Both SHP1 (209, 210) and SHP2 (165, 166, 169, 199-202) have been identified as negative regulators of STAT1 phosphorylation. IHC analysis of tumor tissue from patients with SCCHN revealed that SHP2 was significantly overexpressed compared to normal mucosa (Figs 19a-c) but no difference in SHP1 expression was observed (Figs 19d-f). Preliminary investigation as to whether SHP1 inhibition could upregulate STAT1 phosphorylation and APM expression was performed in PCI-13 cells. SHP1 siRNA (100 nM, 48 h) achieved greater than 70% knockdown of SHP1 protein compared to siRNA control (Fig 20b), but did not upregulate pSTAT1 or APM components (Figs 20c-d). These data prompted us to investigate whether SHP2 was responsible for negatively regulating STAT1 phosphorylation and APM expression in SCCHN cells.

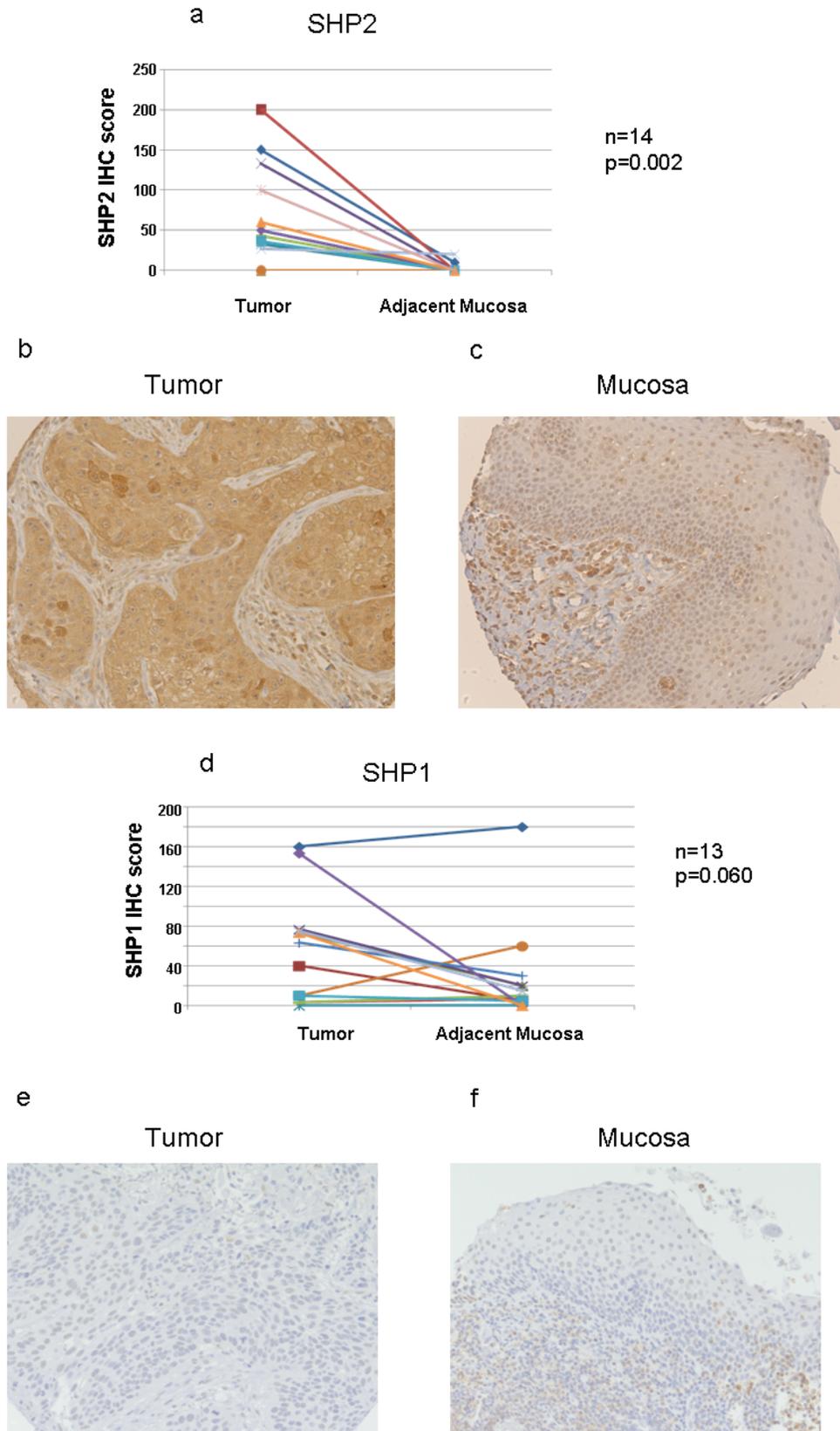


Figure 19. SHP2 but not SHP1 is overexpressed in SCCHN tissue but not in normal mucosa.

SCCHN tumors and normal adjacent mucosa were stained with either an anti-SHP2 (a) or anti-SHP1 (b) mAb and scored. The IHC score was derived by multiplying the cytoplasmic and nuclear staining intensity (0 – 3) by the percent of tumor stained to the nearest 5%. IHC scores for each core of a specimen were averaged. Representative examples of SHP2 staining (b-c) and SHP1 (e-f) from SCCHN tissue (200x) and normal mucosa (200x) are provided.

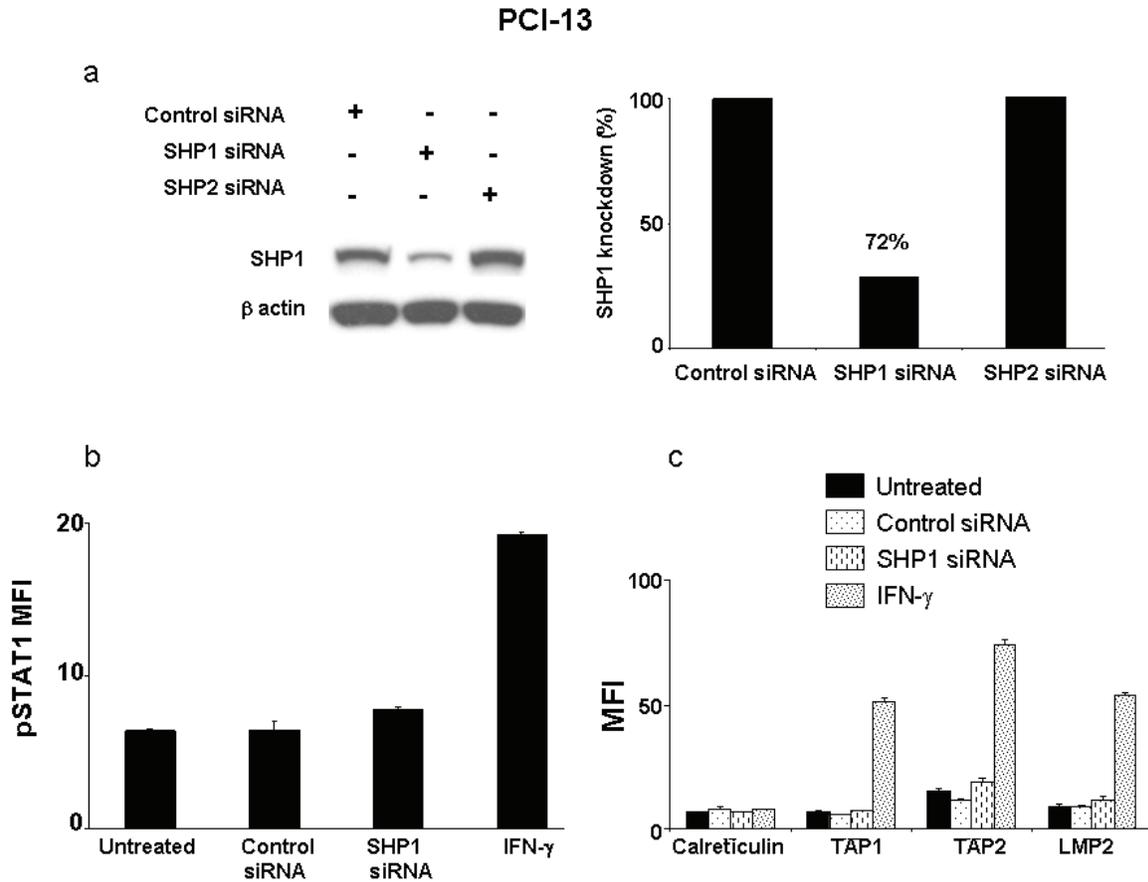


Figure 20. SHP1 siRNA did not upregulate pSTAT1 or APM components.

PCI-13 cells were transfected with the indicated siRNAs (100 nM, 48 h), and greater than 70% knockdown of SHP1 protein was achieved compared to control non-targeting siRNA. Intracellular flow cytometry was performed measuring (b) pSTAT1 and (c) APM components, TAP1, TAP2, and LMP2. Calreticulin, a non-IFN- γ inducible APM component was included to control for global changes in protein expression following transfection. Mean fluorescence intensity (MFI) was measured and error bars indicate standard error. Data represent three independent experiments.

3.3.3 SHP2 knockdown upregulates pSTAT1 in SCCHN cells

PCI-13 and SCC90 cell lines were studied because they express HLA-A*0201 and permit TA-specific CTL recognition using established p53 (189) and EGFR-specific (188) CTL cell lines. Using siRNA (100nM, 48h), selective knockdown of SHP2 protein was achieved in PCI-13 and SCC90 cells by 76% and 66%, respectively, compared to transfection with non-targeting control siRNA (Figs 21a-b). SHP2 knockdown significantly upregulated pSTAT1 expression compared to SCHN cells transfected with control siRNA (Figs 21c-d; $p < 0.002$, two-tailed t-test). IFN- γ (100 U/ml, 10 min) was used as a positive control and induced similar levels of STAT1 phosphorylation as SHP2 siRNA treated cells (Figs 21c-d).

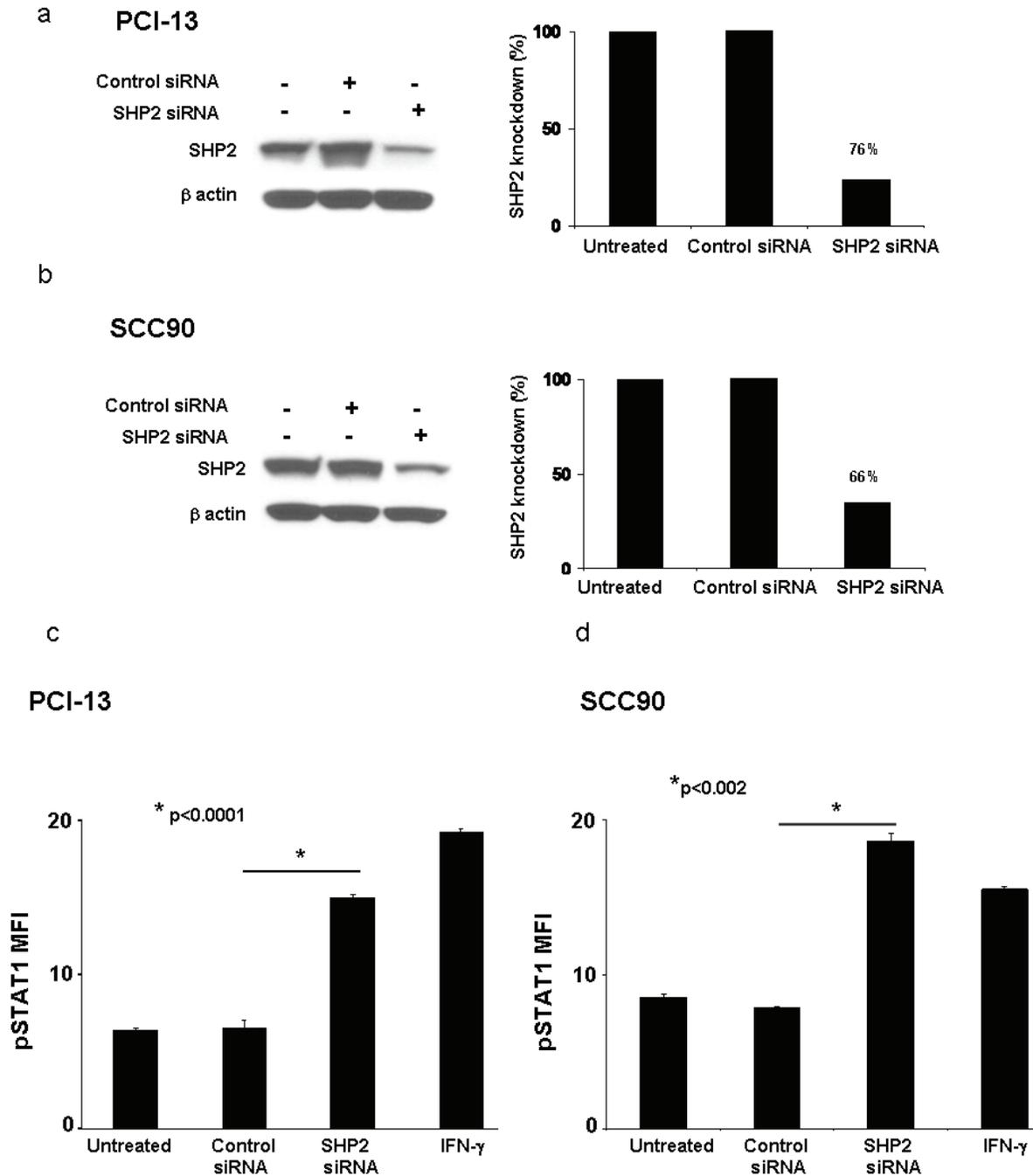


Figure 21. SHP2 siRNA significantly upregulates pSTAT1.

SHP2 siRNA (100nM) achieved 76% and 66% knockdown compared to control siRNA in (a) PCI-13 and (b) SCC90 cells respectively 48 h after transfection. SHP2 siRNA (100 nM, 48 h) significantly upregulated pSTAT1 by flow cytometry in (c) PCI-13 and (d) SCC90 cells compared to siRNA control. The cells were treated with IFN- γ (100 U/ml, 15 min) as a positive control. Data represent three independent experiments ($p < 0.02$, two-tailed t-test).

3.3.4 SHP2 knockdown upregulates APM component mRNA, APM component protein, and HLA class I in SCCHN cells

Next we investigated whether SHP2 depletion and subsequent enhancement of pSTAT1 lead to upregulation of APM component transcription, translation and HLA class I expression in SCCHN cells. SHP2 siRNA (100nM, 48h) transfection induced significant upregulation of APM transcription (Fig 22; $p < 0.05$, two-tailed t-test) and APM component protein in both PCI-13 and SCC90 cells compared to control siRNA (Figs 23a-b; $p < 0.003$, two-tailed t-test). STAT1 phosphorylation induced by SHP2 depletion did not upregulate APM proteins in a non-specific manner since calreticulin expression, a non-IFN- γ -pSTAT1 inducible APM component, was not effected (Figs 23a-b). IFN- γ (100 U/ml, 48h) treatment was used as a positive control for induction of APM component expression. In addition to upregulating APM components, SHP2 siRNA (100nM, 48h) also significantly upregulated HLA-class I molecules compared to control siRNA in SCCHN cells (Figure 23c-d; $p < 0.02$, two-tailed t test).

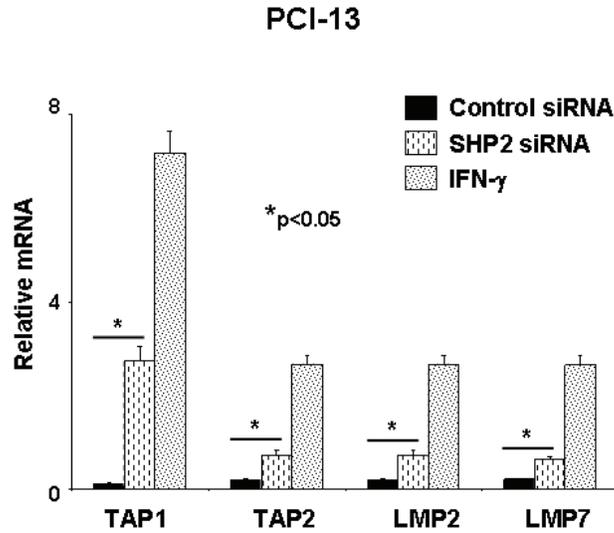


Figure 22. SHP2 siRNA upregulates APM component mRNA.

PCI-13 cells were transfected with the indicated siRNA (100 nM, 48 h) or treated with IFN- γ (100 U/ml, 48 h) as a positive control. TAP1, TAP2, LMP2 and LMP7 transcription was measured by qRT-PCR as described in the Materials and Methods. Data represent three replicates of a single experiment ($p < 0.05$, two-tailed t-test). Error bars indicate standard error.

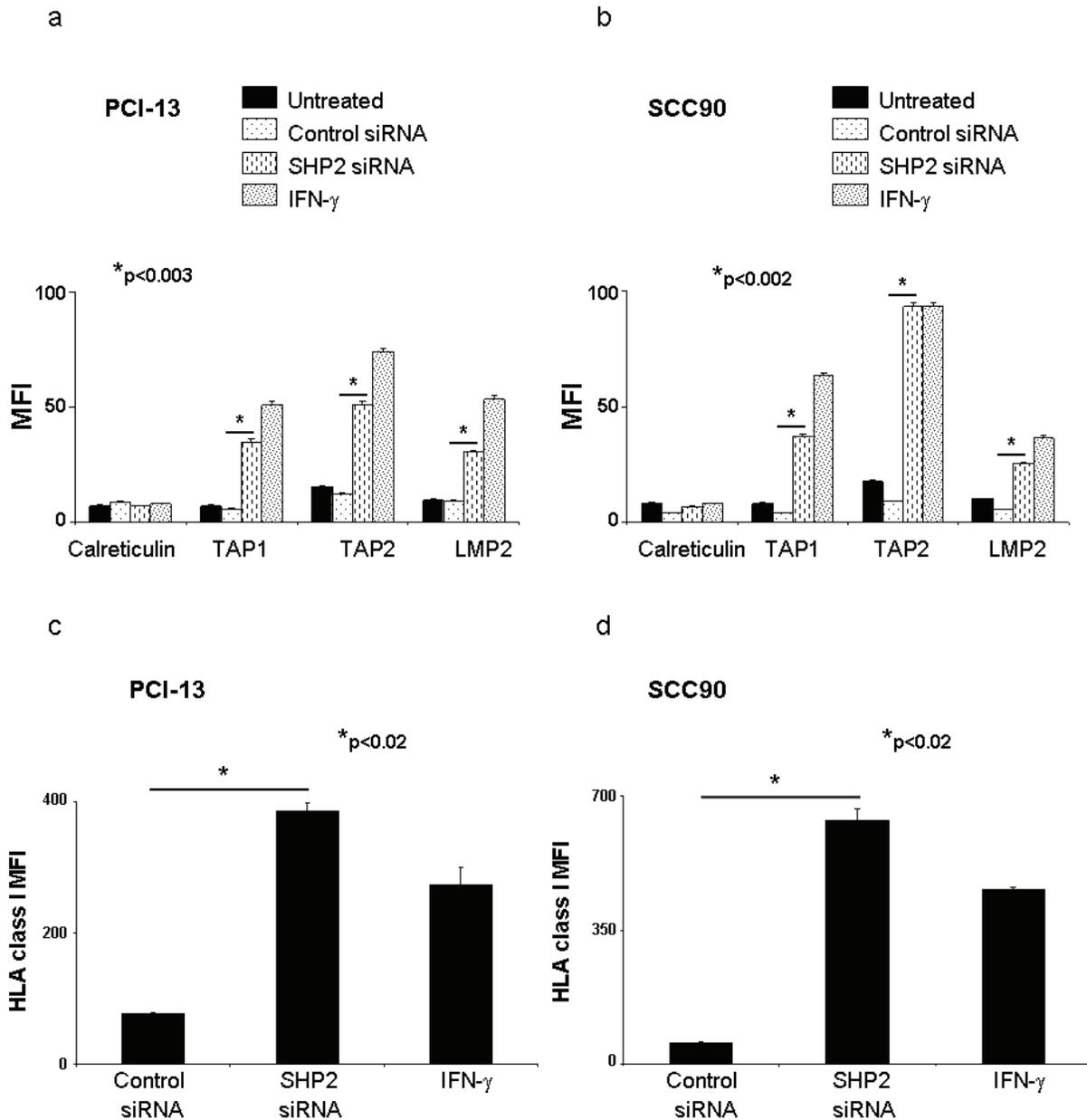


Figure 23. SHP2 siRNA upregulates APM components and HLA class I.

PCI-13 and SCC90 cells were untreated, treated with IFN- γ (100 U/ml, 48hrs), transfected with non-targeting siRNA (100 nM, 48 h) or SHP2 siRNA (100 nM, 48 h). Intracellular flow cytometry was performed measuring TAP1, TAP2, and LMP2 APM components in (a) PCI-13 and (b) SCC90 cells. Calreticulin, a non-IFN- γ inducible APM component was included to control for global changes in protein expression following transfection. Mean fluorescence intensity (MFI) was measured and error bars indicate standard error. Data represent three independent experiments ($p < 0.003$, two-tailed t-test). (c) PCI-13 and (d) SCC90 cells were

treated as described above, and HLA class I expression was measured by MFI. Data represent three independent experiments ($p < 0.02$, two-tailed t-test). Error bars indicate standard error.

3.3.5 SHP2 siRNA reverses APM-mediated immune escape and restores CTL recognition of SCCHN cells.

The observation that SHP2 siRNA significantly upregulated pSTAT1, APM components, and HLA class I, prompted investigation as to whether SHP2 depletion could also restore CTL recognition of SCCHN cells. Indeed, SHP2 knockdown significantly increased EGFR₈₅₃₋₈₆₁ and p53₆₅₋₇₃ specific CTL recognition of **(a)** PCI-13 and **(b)** SCC90 cells, respectively, compared to control siRNA (Figs 24a-b; $p < 0.006$, two-tailed t-test). Blockade of CTL recognition using HLA class I (w6/32) but not HLA class II (L243) mAbs, demonstrated that the CTL were HLA class I restricted.

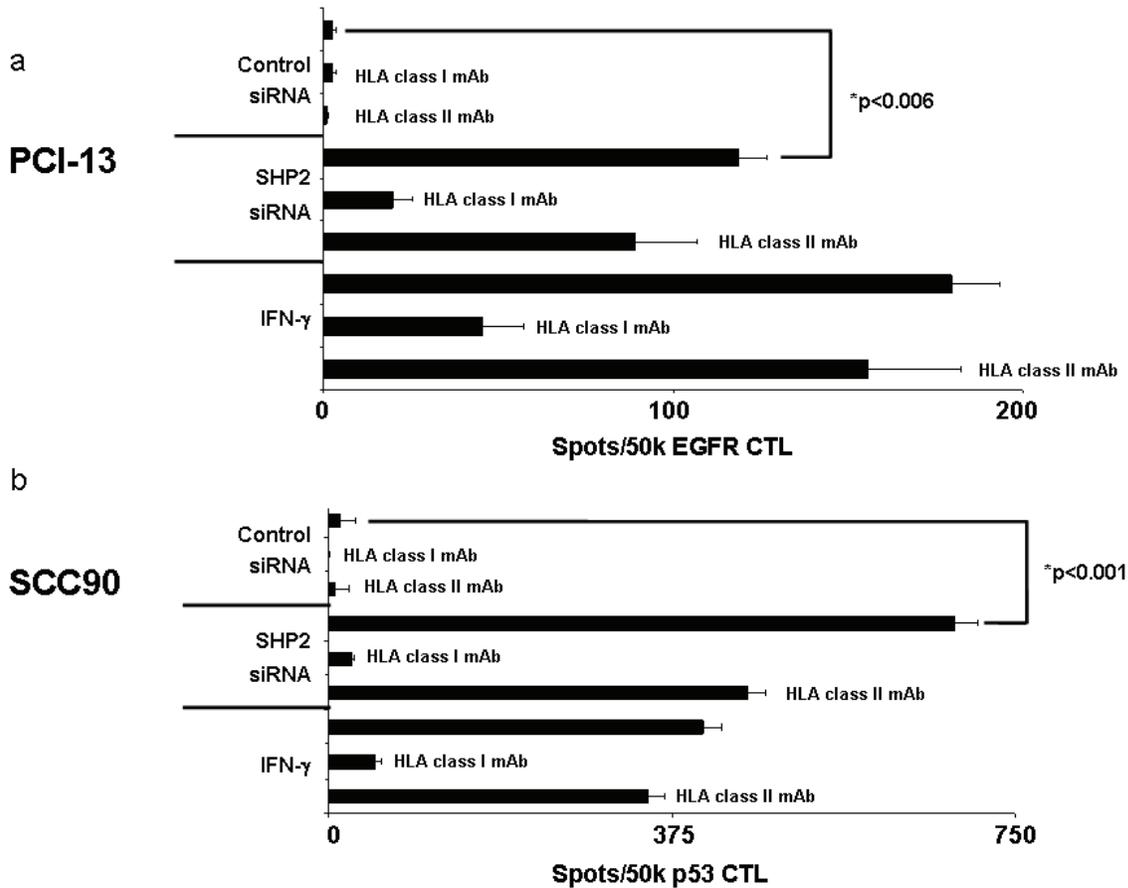


Figure 24. SHP2 siRNA restores CTL recognition of SCCHN cells.

IFN- γ ELISPOT assays were performed to detect TA-specific CTL recognition of **(a)** PCI-13 and **(b)** SCC90 cells and SHP2 knockdown. Cells were untreated, treated with IFN- γ (100 U/ml, 48 h), non-targeting siRNA (100 nM, 48 h) or SHP2 siRNA (100 nM, 48 h). EGFR₈₅₃₋₈₆₁ and p53₆₅₋₇₃ specific cytotoxic T lymphocytes (CTL) were used as effector cells. An anti-HLA class I mAb and an anti-HLA-DR mAb were used to demonstrate that CTL were HLA class I restricted. Data represent a single experiment performed in triplicate ($p < 0.006$, two-tailed t-test). Error bars indicate standard error.

3.3.6 SHP2 knockdown upregulates secretion of chemokines by SCCHN cells.

To investigate other immunologic consequences of SHP2 depletion on SCCHN cells, we performed a multiplex bead immunoassay to evaluate the secretion of several cytokines and chemokines. Interestingly, elevated levels of Regulation upon Activation, Normal T-

Expressed, and presumably Secreted (RANTES) and IFN- γ -inducible protein 10 (IP10) were detected in the supernatants of SCCHN cells transfected with SHP2 siRNA (100 nM, 48 h) (Fig 25). Other chemokines such as monokine induced by IFN- γ (MIG) was not secreted by SCCHN cells after SHP2 knockdown. IFN- γ (100 U/ml, 48h) served as a positive control to induce SCCHN cells to secrete MIG and IP10 (Fig 25). Additionally, cytokines that induce STAT1 phosphorylation such as IFN- γ (Fig 25) and IFN- α (data not shown) were not detected in the supernatants of SCCHN cells after SHP2 knockdown. To the best of our knowledge, SHP2 has not been associated to regulate the secretion of chemokines. Recent studies demonstrated that blocking STAT3 in SCCHN cells (106) and other tumor cells (105) caused secretion of IP10 and RANTES. These data suggest that SHP2 depletion might induce SCCHN cells to secrete chemokines by downregulating STAT3 activity. However, PCI-13 cells transfected with SHP2 siRNA (100 nM, 48 h) showed no significant change in STAT3 phosphorylation, while SHP2 depleted SCC90 cells showed a significant decrease in pSTAT3 (Figs 26a-b; $p < 0.001$; two-tailed t-test).

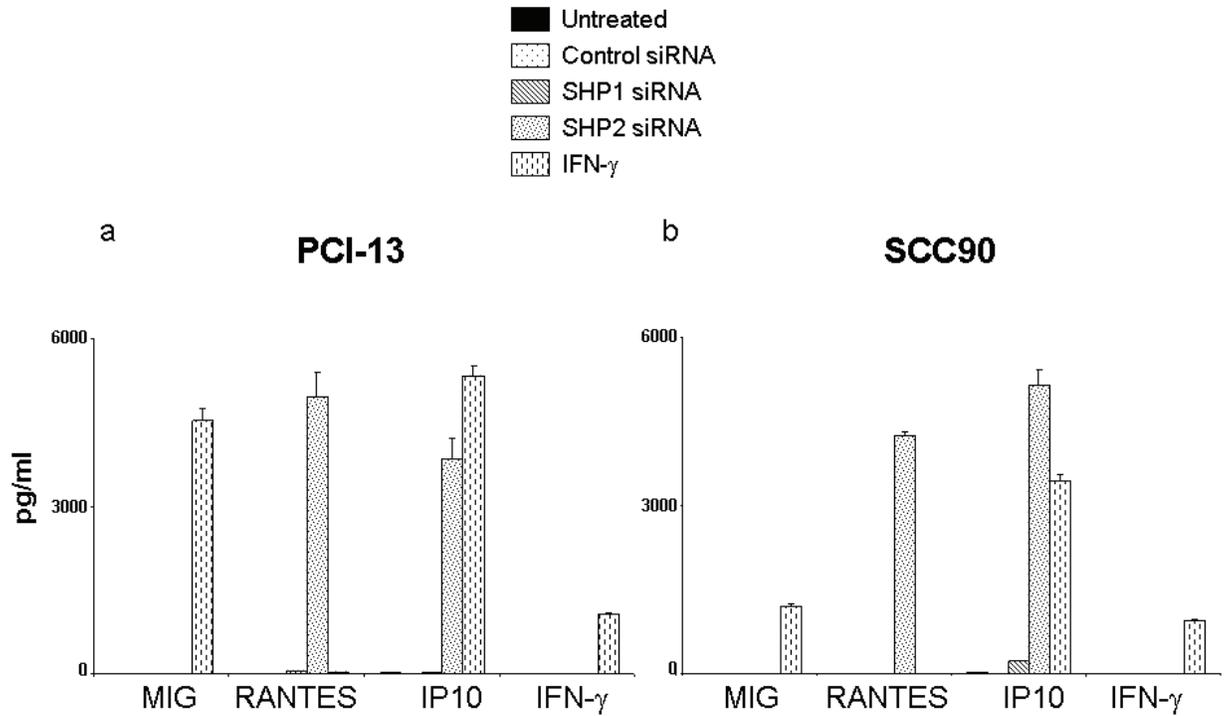


Figure 25. SHP2 siRNA increases secretion of chemokines from SCCHN cells.

Luminex assays were performed on supernatants from (a) PCI-13 and (b) SCC90 cells following transfection with the indicated siRNA (100 nM, 72h) or treatment with IFN- γ (100 U/ml, 48h). Data represent three independent experiments.

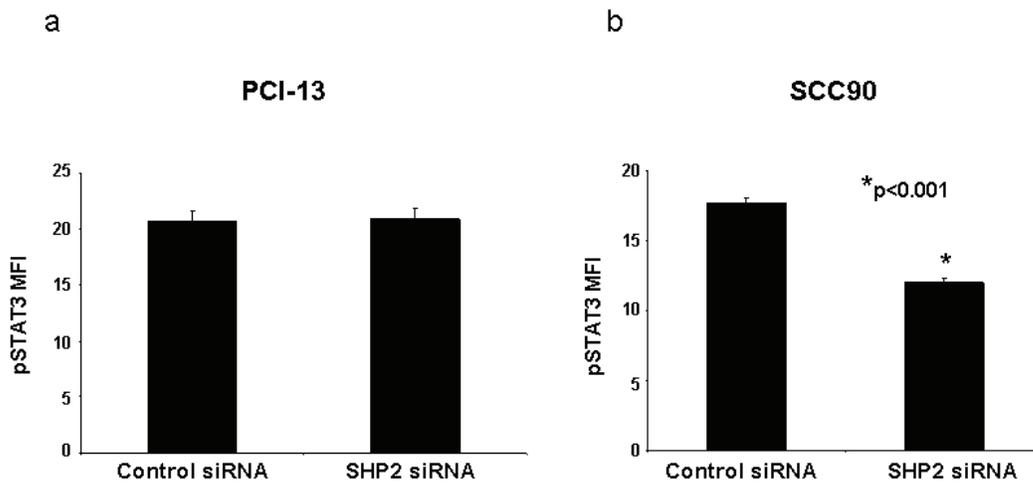


Figure 26. Effect of SHP2 siRNA on pSTAT3 expression in SCCHN cells.

SHP2 siRNA (100 nM, 48 h) was transfected into (a) PCI-13 and (b) SCC90 cells, and pSTAT3 was measured by flow cytometry. Data represent three independent experiments ($p < 0.001$, two-tailed t-test).

3.4 DISCUSSION

We have described a novel mechanism responsible for the observed low levels of STAT1 activation and APM component expression in SCCHN cells. These defects appear to be dependent on SHP2, a SH2 domain-containing tyrosine phosphatase, which was found to be overexpressed in SCCHN tissue (Fig 19). Our results demonstrate that SHP2 dephosphorylates STAT1 (tyrosine 701) in SCCHN cells because knockdown of this protein by siRNA upregulates basal levels of pSTAT1 (Fig 21). Since phosphorylated STAT1 homodimers are important regulators of APM component genes (117), we asked the question if phosphorylated STAT1 in response to SHP2 depletion in SCCHN cells was sufficient to induce APM component expression. Indeed, SHP2 knockdown upregulated APM component transcription (Fig 22), APM protein (Figs 23a-b), HLA class I (Figs 23c-d) and restored CTL recognition of SCCHN cells (Figs 24a-b). Interestingly, knockdown of SHP2 also induced SCCHN cell secretion of RANTES and IP-10 (Figs 25a-b). These findings have important clinical significance because SHP2 targeted therapies could potentially increase the immunogenicity of tumor cells and improve T cell-based cancer immunotherapies.

Mechanisms of immune escape utilized by tumor cells are complex and not mutually exclusive. In SCCHN, APM component downregulation is an important escape (125, 128) mechanism from CTL recognition and correlates with poor prognosis (125, 127, 129, 130). Defects in APM component expression have been identified in several other human tumors such as renal cell carcinoma (132), colorectal carcinoma (133), small cell lung carcinoma (134) and melanoma (135). Thus, investigation of the molecular regulators responsible for APM component defects is clinically important in SCCHN and is applicable to a variety of other tumors.

Previously, we demonstrated that IFN- γ treatment could restore CTL recognition and lysis of SCCHN cells (126), indicating that a regulatory, as opposed to a genetic mechanism was responsible for this escape phenotype. Furthermore, SCCHN cells were found to express abundant levels of total STAT1, but lack phosphorylated STAT1 (Fig 18). These findings suggested that a negative regulator of STAT1 phosphorylation might be responsible for the low pSTAT1 phenotype observed. Others have shown that PTPs can dephosphorylate and inactivate STAT1 (197), therefore we investigated whether PTPs might be responsible for low basal STAT1 phosphorylation in SCCHN cells. Indeed, treatment of a panel of SCCHN cells with a broad phosphatase inhibitor, sodium orthovanadate (SOV), induced STAT1 phosphorylation to levels comparable or greater than IFN- γ treatment (Fig 18). These data suggested that a phosphatase could contribute to the lack of pSTAT1 expression in SCCHN cells, maintaining a basal defect in the pro-apoptotic IFN- γ signal transduction pathway, and promoting immune escape.

SHP2 can dephosphorylate and inactivate STAT1 (165, 166, 169, 199-202) and SHP2 is overexpressed and/or hyperactive in multiple malignancies (161, 167, 173, 203, 204). However, there have been few studies investigating PTPs in head and neck cancer (211-214) and none in terms of regulating STAT1 phosphorylation. To the best of our knowledge, this is the first report to demonstrate that SHP2 is significantly overexpressed in SCCHN tissue (Fig 19) with an important biological effect of its activity. Interestingly, IL-6 is secreted by SCCHN cells in an autocrine/paracrine fashion (58), and it has been reported that IL-6 can activate SHP2 (215, 216), providing a potential mechanism by which SHP2 activity is dysregulated in SCCHN.

SHP2 depletion not only reversed APM component downregulation and restored TA-specific CTL recognition (Figs 23 and 24) of SCCHN cells, it also unexpectedly induced the

secretion of RANTES and IP10 by the tumor cells (Figs 25a-b). Since blockade of STAT3 with siRNA in SCCHN cells (106) or ASO in other tumor cells (105) was able to induce secretion of RANTES and IP10, we postulated that SHP2 depletion might downregulate STAT3 and induce these chemokines to be secreted by SCCHN cells. However several reports demonstrated that SHP2 negatively regulates STAT3 activation (217-219), therefore, SHP2 depletion might increase STAT3 phosphorylation rather than decrease it. Our preliminary findings demonstrate that SHP2 siRNA significantly downregulated pSTAT3 in one of the two cell lines tested (Figs 23a-b). Future studies will investigate whether chemokine secretion in response to SHP2 depletion is STAT3 dependent. Interestingly, neither IFN- γ (Figs 25a-b) nor IFN- α (data not shown) was secreted by SCCHN cells after SHP2 depletion. These data suggest that the observed STAT1 phosphorylation following SHP2 knockdown (Figs 21c-d) is mediated by reducing the sum phosphatase activity of SHP2 in these cells, rather than inducing the secretion of STAT1 agonistic cytokines.

In summary, SHP2 was investigated as a negative regulator of STAT1 phosphorylation and APM components in SCCHN cells. Our data are the first to demonstrate that SHP2 is overexpressed in SCCHN tissue and contributes to APM downregulation in SCCHN cells. We show that SHP2 siRNA can upregulate pSTAT1, APM transcription, translation, HLA class I, and restore recognition of SCCHN cells by TA-specific CTL. We also demonstrate that SCCHN cells secrete RANTES and IP10 in response to SHP2 depletion. Thus, SHP2 depletion both increases the immunogenicity of SCCHN tumor cells, but may also induce recruitment of immune cells to the tumor microenvironment. Future studies ought to investigate the efficacy of a combinatorial immunotherapy approach that utilizes both T cell immunotherapy and SHP2 small molecule inhibitors (220).

4.0 SUMMARY

The inherent heterogeneity of tumor cells, in terms of antigen expression and immune evasion mechanisms, are perhaps the most difficult obstacles to successful cancer immunotherapy. Herein, we investigated molecular mechanisms responsible for SCCHN tumor immune evasion in an effort to create tumor cell targets that can be recognized and destroyed by immune cells and augment T cell-based immunotherapies for cancer.

APM component downregulation represents a major mechanism of immune escape from CTL recognition in SCCHN (125-131), and correlates with poor clinical prognosis (125). APM downregulation has been demonstrated in several other malignancies such as renal cell carcinoma (132), colorectal carcinoma (133), small cell lung carcinoma (134) and melanoma (135), therefore therapeutic interventions that restore APM component expression would have important clinical implications in SCCHN and potentially other cancers.

A recent report demonstrated that treatment of SCCHN cells with IFN- γ restored tumor cell recognition by CTL *in vitro* (126). However data regarding the safety and efficacy of systemic IFN- γ administration to patients with SCCHN is limited (221). At the University of Pittsburgh, a phase I trial is in development where intratumoral administration of recombinant human IFN- γ will be given to patients with SCCHN (42). Although loco-regional administration of IFN- γ will likely reduce toxicity, the risk of initiating potentially fatal cytokine storms in

patients (222) is not trivial, and warrants investigation of alternate measures to increase APM component expression *in vivo*.

The finding that IFN- γ upregulates APM components in SCCHN cells (126), and the knowledge that IFN- γ activates the transcription factor, STAT1, led us to hypothesize that STAT1 activity might be suppressed in SCCHN cells leading to APM component downregulation. Interestingly, SCCHN cells overexpress pSTAT3 (223) but not pSTAT1 (Fig 6). Given the opposing roles of STAT1 and STAT3 in tumorigenesis (Table 1), and several reports that demonstrated that IL-10 mediated STAT3 activation could inhibit basal and IFN- γ induced pSTAT1 and APM component expression (145-149), we investigated the role of STAT3 in STAT1-mediated APM downregulation in SCCHN. Others have shown a cross-regulatory role of STAT1 and STAT3. In the absence of STAT3, IL-6 activated STAT1 for an extended period of time and induced STAT1 target gene expression (151, 152), which normally does not occur. There have also been reports of interferons decreasing STAT3 phosphorylation directly (156, 157) or indirectly through STAT1:STAT3 heterodimer formation, resulting in a reduction of STAT1 and STAT3 target gene expression (158, 159). It is known that these heterodimers can translocate to the nucleus, but what genes they may activate have not been elucidated. We posited that STAT3 directly or indirectly through heterodimer formation, might function to inhibit STAT1 activation and induce APM downregulation in SCCHN cells.

Despite what the literature may suggest and the significant number of high impact papers demonstrating that STAT3 regulates the expression of several important oncogenes in SCCHN and other malignancies, STAT3 was *not* found to inhibit APM component downregulation in SCCHN cells either by directly inhibiting STAT1 phosphorylation or through formation of STAT1:STAT3 heterodimers (Chapter 2). This hypothesis driven research is important and

contributes to the growing body of literature regarding the role of STAT3 in cellular transformation. Although previously published work found that STAT1:STAT3 heterodimers could inhibit STAT1 and STAT3 target gene expression, this work was performed in monocytes that were forced to overexpress STAT3 (158). Furthermore, our data were obtained from tumor cells which are intrinsically different from untransformed cells and better represent what is found in cancer.

Additionally, we discovered that IL-10 induced STAT1 dephosphorylation and APM downregulation in melanoma and murine cells were not reproducible in SCCHN cells. Too often, data that disproves a hypothesis or even one's own work is either unpublished or ignored. These findings are important, and ought to be published and disseminated to the greater scientific community to avoid years of costly research. The stigma of *negative* findings ought to be phased out, and in the long run, they will help advance the field rather than hinder it.

Throughout my graduate training, I have encountered instances where published findings were not reproducible. These experiences highlight a major shortcoming in cancer research, which is the strong reliance on tumor cell lines to inform the biology of cancer. Although tumor cell lines are critically important to cancer research, it is reasonable to suspect that older established tumor cell lines likely exhibit properties quite different from their primary source. After several hundred passages, it is possible that a small subset of tumor cells are unintentionally selected that are characterized by a high proliferation rate and sensitivity to trypsin. Are the data derived from those cells consistent with primary tumors *in vivo*? Are those data applicable only to the cell lines studied? Is it fair to generalize data from two or three cell lines to an entire class of tumors? For example, in SCCHN, do HPV⁺ cells behave biologically similar to HPV⁻ cells? These questions have yet to be fully addressed by the cancer research

field. From personal experience, I can relate to the difficulty of trying to work with primary cells. In terms of SCCHN and other epithelial cancers, the tumors are often exposed to bacteria and require high dose antibiotics or implantation in mice to sterilize the cells. These approaches have a high failure rate and are costly and inefficient. In an effort to avoid confusion and provide quality control, perhaps tumor cell lines ought to be regulated by the NIH and distributed to researchers. In this way, the cell lines could be validated and researchers would have access to the same cells. Nevertheless, inconsistencies in the literature are a part of science, and perhaps a byproduct of real biological differences between cells, or maybe other less scientifically sound reasons.

After extensive experimentation, neither STAT3 nor its heterodimerization with STAT1 altered APM expression in SCCHN cells. However, we identified pSTAT1 is an important mediator of APM component expression (Fig 11) and that SCCHN cells express high levels of total STAT1 and undetectable levels of pSTAT1 (Fig 6). Furthermore, we showed that the IFN- γ -pSTAT1-APM signaling transduction pathway is intact in SCCHN cells (Fig 10). These data suggested that APM downregulation in SCCHN cells might be due to basal dephosphorylation of STAT1 rather than genomic alterations. Since phosphatases have been implicated in negatively regulating STAT1 phosphorylation, we screened several SCCHN cell lines with a broad phosphatase inhibitor, SOV, to determine whether a phosphatase might be responsible for STAT1 dephosphorylation in these cells. Indeed, SOV treatment upregulated pSTAT1 demonstrating that phosphatases contribute to maintaining low basal pSTAT1 levels in SCCHN cells (Fig 18).

Several reports in the literature demonstrate SHP2 negatively regulates STAT1 phosphorylation (165-169). IHC analysis demonstrated significant SHP2 overexpression in

SCCHN tissues compared to adjacent normal mucosa (Fig 19). Selective knockdown of SHP2 protein by siRNA in SCCHN cells significantly upregulated pSTAT1, APM transcription and APM protein (Figs 21, 22, 23). Importantly, HLA class I upregulation was also observed demonstrating that STAT1 regulated proteins other than APM components are upregulated after SHP2 knockdown (Fig 23). Furthermore, preliminary data suggests that SHP2 siRNA induces growth arrest in SCCHN cells (unpublished data), perhaps mediated by STAT1 activation, similar to what is observed after IFN- γ treatment. Ongoing studies are being performed to determine whether SHP2-mediated APM component expression is dependent on STAT1 phosphorylation. We plan to co-transfect SHP2 siRNA and STAT1 siRNA or a STAT1 dominant negative plasmid in SCCHN cells to investigate whether depleting or inactivating STAT1 alters SHP2-mediated APM component expression and cellular proliferation. Furthermore, ChIP analysis will elucidate whether SHP2 depletion increases STAT1 binding to the TAP1 promoter which would suggest that APM component expression is STAT1 dependent. We also recently developed SCCHN cell lines that are stably transfected with either wild-type SHP2, or dominant negative forms of SHP2 (224). The cell lines will be phenotyped for basal and IFN- γ induced pSTAT1 and APM component expression. We expect that the dominant negative SHP2 expressing cells to be sensitized to IFN- γ and upregulate pSTAT1 and APM components greater than WT SHP2 expressing cells. Taken together, these studies would confirm that SHP2 knockdown in SCCHN cells upregulates APM components in a STAT1-dependent fashion.

Perhaps, the most important finding was that SHP2 depletion restored TA-specific CTL recognition of SCCHN cells (Fig 24). Since SHP2 siRNA upregulated both APM component expression and HLA class I expression it is unclear as to whether the increase in recognition is

due to an upregulation in antigen processing or HLA class I expression or both. One way to test this would be to treat SCCHN cells with a proteasome inhibitor, such as MG132 or bortezomib, then deplete SHP2, and perform an IFN- γ ELISPOT assay. If the CTL no longer recognize the SCCHN cells, it would suggest that antigen processing and presentation is important in restoring CTL recognition of SCCHN cells.

Interestingly, Luminex multiplex ELISA analysis suggested that SHP2 depletion induced the secretion of RANTES and IP10 chemokines from SCCHN cells (Fig 25). Importantly, other chemokines such as MIG were not upregulated after SHP2 siRNA transfection, demonstrating that specific chemokines were secreted by the tumor cells after SHP2 depletion (Fig 25). RANTES strongly chemoattracts several immune cells, including eosinophils, basophils, mast cells, monocytes, NK cells, CTLs, naive CD4⁺ T cells, and memory T cells (225). Several studies found that expression of RANTES intratumorally induces infiltration of DCs, CD4⁺ T cells, CTL, and NK cells (226-228) and inhibits tumor growth *in vivo* (229). IP10 has also been found to have antitumor activity by attracting CD4⁺ T cells, CTL, and NK cells to the tumor microenvironment (230). Future studies will include transwell assays to determine if SHP2 depleted SCCHN cells can chemoattract immune cells.

After SHP2 knockdown, we observe STAT1 phosphorylation in SCCHN cells. However, STAT1 phosphorylation is not constitutive in untransformed cells, and it would be unlikely that tumor cells would activate a tumor suppressor protein. Thus, what could account for the observed STAT1 phosphorylation after SHP2 knockdown in SCCHN cells? If our model is correct, that SHP2 is functioning to dephosphorylate STAT1 and inhibit APM expression, it could be that depletion of SHP2 might sensitize SCCHN cells to STAT1 agonists. Perhaps, SCCHN cells secrete STAT1 agonist cytokines at low levels, and after SHP2 knockdown,

sufficient activation of STAT1 occurs to induce APM expression. Moreover, depletion of greater than 75% of STAT1 protein by siRNA did not completely inhibit IFN- γ mediated APM expression in SCCHN cells (Fig 11c) and very low doses of IFN- γ (10 U/ml, 48 h) were sufficient to induce APM expression (Supp Fig 17). These data suggest that APM expression requires very little STAT1 phosphorylation, and we hypothesize that SCCHN cells secrete low basal STAT1 agonists whose activity are amplified when SHP2 is depleted. However, multiplex cytokine analysis did not detect known STAT1 agonists (IFN- γ , IFN- α , IL-6) in the supernatants of SHP2 siRNA transfected SCCHN cells (Fig 25 and data not shown). However these cytokines may be secreted levels below the detection level of the assay. To test our hypothesis, we plan to neutralize known STAT1 agonists after SHP2 depletion in our SCCHN cells and measure the effects on STAT1 phosphorylation and APM expression.

Overall, these promising data warrant future investigation of whether SHP2 inhibition can enhance cancer immunotherapies by increasing the immunogenicity of tumors and inducing the secretion of chemokines to attract tumor infiltrating immune cells. These findings provide the rationale for studies to determine if SHP2 inhibitors can induce tumor regression *in vivo*. Given that SHP2 is ubiquitously expressed and that SHP2 knockout mice are embryonic lethal, toxicity will likely be an issue for future targeted SHP2 immunotherapies for cancer. Thus, administration and delivery of SHP2 inhibitors will need to be carefully considered. Our *in vitro* studies used siRNA technology to deplete SHP2 in SCCHN cells, however there are significant issues regarding siRNA delivery *in vivo*, such as the stability of siRNA and target specificity (231, 232). An alternative approach to siRNA would be loco-regional administration of small molecule SHP2 inhibitors. There have been several reports of small molecule SHP2 inhibitors (220, 233-237) and we are currently testing whether a modified SHP2 inhibitor that is cell

permeable, SPI-112Me (220), inhibits SHP2 activity and upregulates IFN- γ -mediated pSTAT1 in SCCHN cells. Following validation of an effective inhibitor, future *in vivo* studies will be performed to determine the toxicity and off target effects of systemic and intratumoral administration of the drug.

In summary, we report novel findings that SHP2 overexpression in SCCHN mediates APM downregulation, perhaps through STAT1 dephosphorylation, and that SHP2 depletion restores SCCHN cell recognition by TA-specific CTL and induces secretion of RANTES and IP10 (Fig 27). Future *in vivo* studies will test whether SHP2 inhibitors might be a viable therapeutic modality to enhance immunotherapies for cancer.

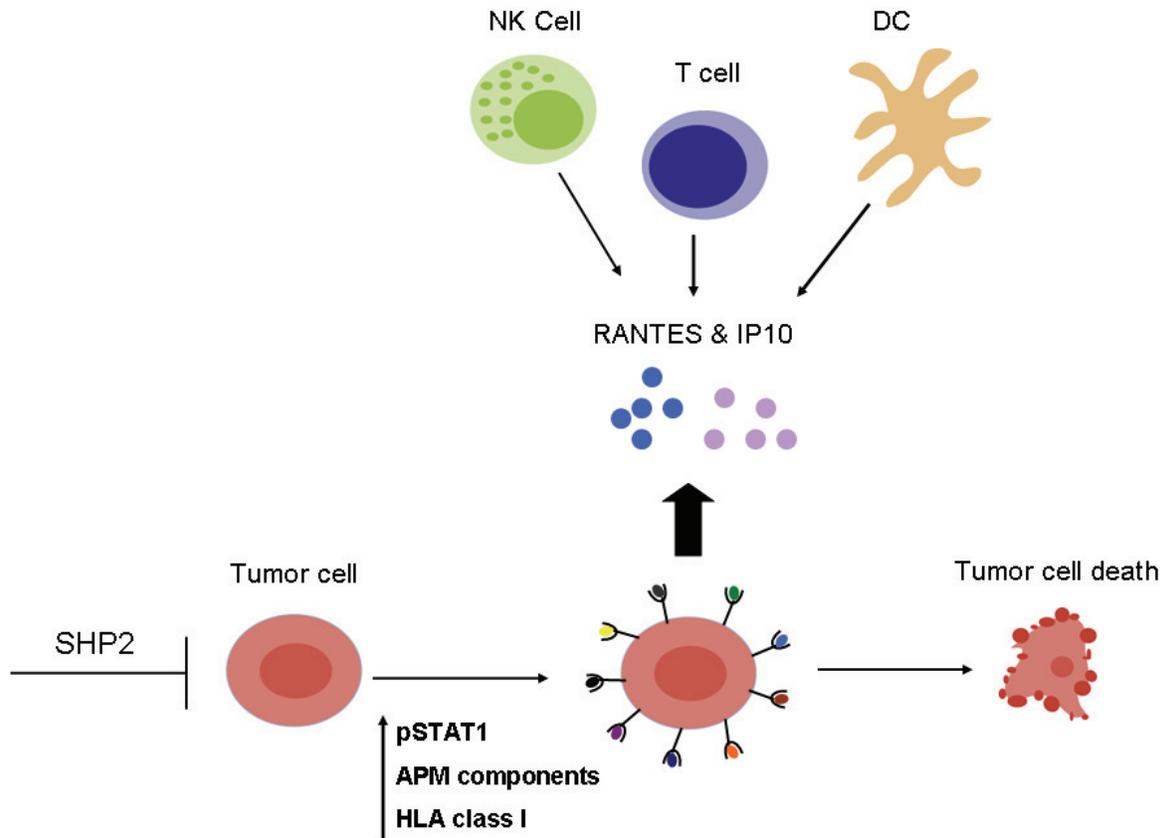


Figure 27. Targeting SHP2 for cancer immunotherapy.

Targeting src homology-2 domain-containing phosphatase (SHP)-2 using siRNA induces STAT1 phosphorylation, upregulation of antigen processing machinery (APM) components and human leukocyte antigen (HLA) class I molecules thereby increasing the immunogenicity of SCCHN cells. SHP2 knockdown also induces tumor cell secretion of IFN- γ -inducible protein (IP10) and Regulated on Activation, Normal T cell Expressed and Secreted (RANTES), which function as chemoattractants for various immune cells including T cells, natural killer (NK) cells, and dendritic cells (DC).

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