Natural Killer Cells Preferentially Target Cancer Stem Cells; Role of Monocytes in Protection Against NK Cell Mediated Lysis of Cancer Stem Cells

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Abstract: Mounting effective anti-tumor immune responses by cytotoxic effectors is important for the clearance of tumors. However, accumulated evidence suggests that the cytotoxic function of immune effectors is largely suppressed in the tumor microenvironment by a number of distinct effectors and their secreted factors. The aims of this review are to provide a rationale and potential mechanism for immunosuppression in cancer, and to demonstrate the significance of such immunosuppression in cellular differentiation and tissue regeneration in pathological conditions, and progression of cancer. We have recently shown that increased NK cell function was seen when they were cultured with primary oral squamous carcinoma stem cells (OSCSCs) as compared to their more differentiated oral squamous carcinoma cells (OSCCs). In addition, human embryonic stem cells (hESCs), Mesenchymal Stem Cells (hMSCs), dental pulp stem cells (hDPSCs) and induced pluripotent stem cells (hiPSCs) were significantly more susceptible to NK cell mediated cytotoxicity than their differentiated counterparts or parental cells from which they were derived. We have also reported that inhibition of differentiation or reversion of cells to a less-differentiated phenotype by blocking NFκB or targeted knock down of COX2 augmented NK cell function significantly. Total population of monocytes and those depleted of CD16(+) subsets were able to substantially prevent NK cell mediated lysis of OSCSCs, MSCs and DPSCs. Taken together, our results suggest that stem cells are significant targets of the NK cell cytotoxicity. The concept of split anergy in NK cells and its contribution to tissue repair and regeneration and in tumor resistance and progression will be discussed in this review. Therefore, patients with cancer may benefit from repeated allogeneic NK cell transplantation at the site of the tumor for specific elimination of cancer stem cells.

Keywords: Apoptosis, NFκB, differentiation, immunosuppression, NK, IL-6, split anergy, cancer stem cells.

INTRODUCTION

There is ample evidence for the role of effective immune cell surveillance in the prevention of cancer. Resolution of oral non-Hodgkins lymphoma in a renal allograft recipient was observed after the reduction of immunosuppressive therapy [1]. In a liver transplant recipient rapid progression of oral leukoplakia to carcinoma was observed after immunosuppression [2]. Furthermore, neoplasias of tongue and lip have been widely described in renal transplant patients [3-6], and finally induction of oral cavity cancers was second to liver cancer in patients after bone marrow transplantation [7]. These results indicated the significance of effective immunosurveillance in prevention of malignancies of the oral cavity. Further corroboration is provided by the high occurrence of both virally and non-virally associated lymphomas and Kaposi's Sarcomas in patients with HIV-1 infection [8]. In addition, there is substantial evidence that indicates that immune responses are inhibited by oral tumors, and this may largely be responsible for their induction and progression. This review will focus on the emerging new roles of NK cells in regulation of numbers, resistance and differentiation of cancer stem cells as well as healthy untransformed stem cells. In addition, the significance and role of anergic NK cells, which have largely been ignored or under-appreciated, will be discussed in the process of differentiation and resistance of oral tumors. Thus, we will also focus on the physiological role of NK cells in shaping the size and differentiation of stem cells.

Immunosuppression and tumor escape from immune recognition are thought to be major factors responsible for the establishment and progression of cancer, however, neither underlying physiological significance nor the exact mechanisms by which immunosuppression occurs are well understood. A number of factors responsible for the suppression of NK cell cytotoxicity in humans has been previously identified [9-14]. It is shown that freshly isolated tumor infiltrating NK cells are not cytotoxic to autologous tumors. Moreover, NK cells obtained from the peripheral blood of patients with cancer have significantly reduced cytotoxic activity [15-18]. In addition, NK cell cytotoxicity is suppressed after their interaction with stem cells [19-21]. In contrast, interaction of

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NK cells with the resistant tumors does not lead to suppression of NK cell cytotoxicity [22].

Many mechanisms have been proposed for the functional inactivation of tumor associated NK cells including the overexpression of Fas ligand, the loss of mRNA for granzyme B [14] and decreased CD16 and its associated zeta chain [23].

Many metastatic tumor cells exhibit constitutively elevated NFκB activity [24]. Increased NFκB activity is shown to have a causal relationship to neoplastic transformation, and uncontrolled cell growth in many cell types [24]. Human solid tumors exhibit constitutively activated NFκB [24].

We have previously shown that NK resistant primary oral epithelial tumors demonstrate higher nuclear NFκB activity and secrete significant levels of Granulocyte Monocyte-Colony Stimulating Factor (GM-CSF), Interleukin (IL)-1β, IL-6 and IL-8 [25, 26]. Moreover, the addition of Non-steroidal anti-inflammatory drugs (NSAIDs) which inhibit NFκB have the ability to reverse immunosuppression induced by a tobacco-specific carcinogen [27] in addition to their well established ability to decrease oral dysplasia as well as induction of overt cancer in transgenic animals [28]. In agreement, we have previously demonstrated that inhibition of NFκB by Sulindac treatment of tumor cells increases functional activity of NK cells [29, 30]. In addition, targeted inhibition of NFκB in skin epithelial cells resulted in the induction of auto-immunity and inflammation [31].

The significance and exact mechanism by which NFκB nuclear function in oral tumors modulate and shape the function of key interacting immune effectors is starting to unravel. We have previously shown that inhibition of NFκB by the IκB super-repressor in HEp2 tumors, or in primary OSCCs, or in non-tumorigenic oral keratinocytes (HOK-16B) leads to a significant increase in cytotoxicity and secretion of IFN-γ by the human NK cells [29, 30]. However, the underlying significance and the physiological relevance of NFκB modulation in tumors or in primary cells responsible for the alteration of NK cell cytotoxic function is just being understood. It is clear that the objective in cancer is to enhance the function of cytotoxic immune effectors, while in auto-immunity and inflammation the aim is to inhibit immune effector function. Therefore, dissection of the underlying mechanisms of immune activation when NFκB is modulated in the cells might help design strategies to target each disease accordingly. Indeed, targeted inhibition of NFκB function in both the intestinal epithelial cells and myeloid cells was previously shown to result in a significant decrease in size and numbers of the tumor cells [32].

In this report we review the previous studies from our laboratory and other studies regarding the factors and mechanisms involved in immunosuppression observed in cancer. Furthermore, we will provide evidence which indicates that the stage of maturation and differentiation of the healthy untransformed stem cells, as well as transformed tumorigenic cancer stem cells, is predictive of their sensitivity to NK cell lysis. In this regard we have previously demonstrated that OSCSCs, which are stem-like oral tumors, are significantly more susceptible to NK cell mediated cytotoxicity; whereas, their differentiated counterpart OSCCs is significantly more resistant [26]. In addition, hESCs and hiPSCs, as well as a number of other healthy normal stem cells such as hMSCs and hDPSCs, were found to be significantly more susceptible to NK cell mediated cytotoxicity than their differentiated counterparts [26]. Based on these results, we proposed that NK cells may play a significant role in differentiation of the cells by providing critical signals via secreted cytokines as well as direct cell-cell contact. In order to drive differentiation, however, NK cells first need to receive signals from stem cells or those which have disturbed or defective capabilities to differentiate. In addition, CD14+HLADR- monocytes, fibroblasts or Tumor associated macrophages can condition NK cells to support differentiation of the cells. These signals alter the phenotype of NK cells and cause NK cells to lose cytotoxicity and change into cytokine producing cells. These alterations in NK cell effector function will ultimately aid in driving differentiation of a minor population of surviving, healthy, as well as transformed stem cells. In cancer patients since the majority of NK cells have lost cytotoxic activity, they may eventually contribute to the progression of cancer by not only driving the differentiation of tumor cells but more importantly, by allowing the growth and expansion of cancer stem cells.

IMMUNOSURVEILLANCE IN THE PREVENTION OF CANCER

The theory of immunosurveillance was initially set forth by Burnet [33] to indicate that the key thymus dependent effectors were responsible for eliminating developing cancers [34, 35]. However, the opponents of the immunosurveillance theory strongly criticized the concept primarily due to the lack of data showing elevated susceptibility to cancer in nude mice which had T-cell defects [36, 37]. New data obtained from severely immunocompromised STAT1-/- and RAG-/- mice which have defects in both the innate and adaptive immune effector functions have revived the concept of immunosurveillance, and highlighted the significance of both innate and adaptive immune responses in prevention of cancer [38-41].

Since then, the concept of immunosurveillance has expanded to include immunoediting as an important mechanism for the development of cancer [39, 40]. It was suggested that cancer immunoediting comprises of three phases: elimination, equilibrium and escape [40]. Elimination represents the classical concept of immunosurveillance. However, during equilibrium and escape, the interaction and cross signaling between immune effectors and tumor cells shape the cells by progressive generation of tumors capable of gradual inactivation and death of immune effector cells. The final stages of cancer development may result in the induction of less immunogenic tumors in the presence of fewer immune effectors capable of lysing the tumors. Thus, the pressure exerted by tumor cells and immune effectors on each other may eventually shape the environment to benefit invading tumors. Similarly, certain elements of such interactions are also observed during the interaction of NK cells with healthy non-transformed human stem cells in which case the three phases of interaction may include elimination which marks the decrease in the size of stem cells due to the selection of stem cells by the NK cells, equilibrium which denotes the conditioning of NK cells to lose cytotoxicity and support maturation and differentiation of stem cells, and finally the

resolution phase which denotes elimination of anergized NK cells and differentiation of selected stem cells. Similarities and differences between these phases in cancer and during stem cell maturation will be discussed below.

IMMUNE RESPONSES IN HEAD AND NECK CANCER

Head and neck cancers represent approximately 6% of all new cancers in the United States. Despite many advances in surgical procedures and the availability of new chemotherapeutic agents and optimized radiotherapeutic procedures, survival of patients with head and neck cancers has not improved in the last forty years. There is substantial evidence which indicates that immune responses, which should otherwise suppress or eliminate oral cancer cells, are inhibited by properties and functions of oral cancers. NK cells and cytotoxic T-cells, which play crucial effector functions in the host defense against neoplasia, are functionally inactivated in oral cancers [22, 29, 30, 42-45]. Regressing tumor grafts of oral origin contain significantly larger numbers of functional NK and T-cells than those associated with the primary tumors [46], while patients with metastasis of head and neck cancers have low NK and T-cell activity [47]. Spontaneous apoptosis of circulating peripheral blood T-cells and decreased frequencies of key peripheral blood dendritic cell subsets, attributable to the presence of tumors, are important indicators of immune cell paralysis in head and neck cancers [48].

Immunotherapy with cytokines or adaptively transferred effector cells was found to be ineffective in the treatment of head and neck cancers [49-51], although limited success in immunotherapy of metastatic melanomas or renal cell carcinomas has previously been reported [52]. The reason for this failure of known immunotherapeutic modalities in many cancers including head and neck cancers is poorly understood. It has been hypothesized that a widespread paralysis of cytotoxic effectors residing inside the inflammatory infiltrate of advanced cancer patients is the main reason for poor prognosis [53, 54]. Furthermore, freshly isolated tumor infiltrating lymphocytes are not cytotoxic to autologous tumor cells and show a significantly reduced clonogenicity [15-17, 55]. Functional paralysis of cytotoxic cells have also been reported in a variety of other cancers, notably breast [10, 11, 56, 57], renal [13], and colon cancers [14]. More importantly, depletion of cytotoxic effectors in the tumor milieu has an unfavorable outcome for survival in cancer patients [57-60]. Indeed, a significantly shorter survival rate is reported for colorectal carcinoma patients with little or moderate NK cell infiltration as compared to those with extensive infiltration [61]. A five year survival advantage was also seen with higher CD3 positive tumor infiltrating T-cells than with lower T-cell count in the carcinoma of uterine cervix [58]. In primary cutaneous melanoma a five year survival rate for high tumor associated lymphocyte infiltrate was 77%, with medium lymphocyte infiltrate 53%, and for tumors with absent tumor infiltrating lymphocytes (TILs) 37% [59] suggesting an important function for NK and CTLs in cancer cell rejection.

Defects in NK, T-cells and DCs have been reported in oral cancer patients. Signaling abnormalities, spontaneous apoptosis and reduced proliferation of circulating T-cells, DCs and TILs have also been reported in patients with oral cancers [48, 62]. The percentage of myeloid-derived LIN-DR+CD11c+ DCs is significantly lower in head and neck cancer patients compared to healthy controls [48]. A decrease in the number of DCs in patients was related to the presence of tumor cells since the numbers of myeloid-derived DCs returned to normal levels when the tumors were excised in patients with head and neck cancers [48].

MECHANISMS OF IMMUNOSUPPRESSION BY TUMOR CELLS

Many mechanisms have been proposed for the functional inactivation of tumor associated lymphocytes [9-11, 13, 14]. Soluble products derived from renal cell carcinoma inhibit proliferative capacity of T-cells infiltrating human tumors due to a downregulation of Janus kinase 3 (Jak 3), p56 (lck), p59 (fyn) and zap 70 [13]. Expression of Fas ligand by many human tumor cells including oral tumors has been hypothesized to be a major cause of lymphocyte depletion in the tumor microenvironment [9]. In mice, tumor induced immunosuppression has been associated with a decreased expression of the zeta-chain of the T-cell receptor and the loss of mRNA for granzyme B [14]. Indeed, as observed in mice, the frequency of TCR-zeta positive and granzyme positive lymphocytes are decreased in advanced stage head and neck carcinomas, and the restoration of expression during *in vitro* stimulation suggests the presence of tumor derived suppressive factors [14]. Decreased CD16 and its associated zeta chains are also observed in tumor infiltrating NK cells of patients with cancer [23]. The relative lack of IFN-γ and granulocyte-macrophage colony stimulating factor (GM-CSF), rather than a deficiency of IL-2 by tumor infiltrating lymphocytes (TILs) in breast cancer, has been hypothesized to be a mechanism for impaired immune function [10]. Moreover, the breast cancer associated antigen DF3/MUC1, has been shown to induce apoptosis of activated human T-cells [11]. Overall, both secreted factors and direct cell-cell contact during the interaction of immune effectors with the tumor cells were shown to be responsible for the suppression of immune effector function.

KEY IMMUNOSUPPRESSIVE FACTORS INDUCED IN THE TUMOR MICROENVIRONMENT

Prostaglandin E2 (PGE2)

Immunosuppression linked to enhanced PGE2 synthesis has been documented in many human cancers [63]. Freshly excised human head and neck cancers demonstrated elevated levels of PGE2, transforming growth factor- β1 (TGFβ1) and interleukin-10 (IL-10) secretion which are known to upregulate the expression of Killer Immunoglobulin-like Receptors (KIRs) on the surface of NK, T-cells and DCs and block immune effector function [64]. Furthermore, metastatic head and neck cancers released higher levels of above-mentioned inhibitory factors and lower levels of immune activating factors IFN-γand IL-2 than did their corresponding primary tumors [64]. PGE2 overproduction in the tumor microenvironment was also shown to lead to dendritic cell (DC) abnormalities [65]. Non-steroidal anti-inflammatory drugs (NSAIDs) such as Sulindac, which inhibit PGE2 production

by blocking NFκB-induced cyclooxygenase (COX)-2 production, can reverse immunosuppression induced by a tobacco-specific carcinogen [27], and delay the onset and severity of oral cancer in transgenic animals [28]. In agreement with these observations, we have previously demonstrated that Sulindac treatment of tumor cells increases functional activity of NK cells [30].

Interleukin (IL)-6

IL-6 is secreted constitutively by oral squamous carcinomas [66], and is found to be elevated in oral cancer patients [67]. IL-6 is known to interfere with IFN-γ signaling by inducing Th2 differentiation via activation of NFAT and secretion of IL-4, which subsequently inhibits Th1 polarization via STAT3-induced expression of Suppressor Of Cytokine Signaling (SOCS)-1 in CD4+ T-cells [68]. In support of a role for IL-6 in mediating immune evasion of tumor cells, Menetrier-Caux et al showed that conditioned medium from human renal cell carcinoma cell lines blocked the differentiation of CD34+ bone marrow cells into immature DCs, and this inhibitory effect could be blocked with antibodies against either IL-6 or granulocyte colony stimulating factor (G-CSF) [69]. Furthermore, in other studies, recombinant IL-6 alone could block the differentiation of CD34+ bone marrow-derived cells to functional DCs, supporting a model in which tumor-derived IL-6 enhances cancer progression by impairing host anti-tumor immunity [69].

Vascular Endothelial Growth Factor (VEGF) and Granulocyte Monocyte Colony Stimulating Factor (GM-CSF)

Other factors which have been implicated in immunosuppression in cancers are angiogenic factors such as VEGF [70] and cytokines such as G-CSF and GM-CSF. The finding that neutralizing antibodies to VEGF or GM-CSF could partially reverse the inhibitory effects of tumor cell supernatant on DC maturation demonstrated that these factors could interfere with DC differentiation and function [70]. These results suggested that there may be a strong selection pressure for cells that produce one or more of these factors, because of their ability to avoid immune detection and destruction [69].

Increased numbers of immature DCs were found in the peripheral blood of cancer patients with elevated levels of circulating VEGF [70]. Accordingly, when similar concentrations of VEGF to those found in cancer patients were injected in mice the number of immature myeloid cells and immature DCs were increased in their peripheral blood. Therefore, increased secretion of VEGF in the tumor microenvironment may prevent maturation and differentiation of DCs and contribute to poor anti-tumor immune responses in cancer patients.

Immunosuppression Mediated by Direct Cell-Cell Contact

Contact-dependent immune suppression can occur by engagement of MHC class I molecules on CD8+CD28- suppressor cells with immunoglobulin-like transcript (ILT2 and ILT4) inhibitory receptors on DCs. Blocking of both MHC Class I and ILTs by specific antibodies can reverse immunosuppression [71]. Similarly binding of c-type lectin receptors or Killer Immunoglobulin-like Receptors (KIRs) to MHC Class I ligands inhibit NK cell function [72-75]. In addition, the expression of co-stimulatory molecules, such as B7H1 on tumor cells and inhibitory DCs and T-cells can inhibit T-cell activation and proliferation [76].

Immunosuppressive Effectors in Tumor Microenvironment

The tumor microenvironment consists of a number of heterogeneous cell populations with ability to suppress and limit the function and survival of cytotoxic immune effectors. Patients with cancer often bear high numbers of immature CD14+HLADR- monocytes [77, 78]. Tumor associated Macrophages (TAMs) have previously been shown to significantly influence and limit immune activation in the tumor microenvironment [79, 80]. In addition, Myeloid Derived Suppressor Cells (MDSCs) which are comprised of a number of distinct cell populations of myeloid origin and whose roles in immunosuppression are now well established in both animal and human cancer models have received significant attention in the recent years [77]. T-cell dysfunction is shown to be induced by MDSCs by the secretion of IL10, TGF-β, reactive oxygen species (ROS), arginase and Nitric Oxide synthase (NOS). T-regulatory (Treg) and DC regulatory (DCreg) cells were also shown to have significant immunosuppressive roles in the tumor microenvironment [77]. Perhaps one of the most interesting recent observations regarding immunosuppressive effectors is the identification of Cancer Associated Fibroblasts (CAFs) and Mesenchymal Stem Cells (MSCs) as two potential tumor promoters. Fibroblasts from tumor tissues demonstrate an activated phenotype and have the ability to secrete many immunosuppressive factors such as TGF-β and VEGF among other factors [81]. We have also found that fibroblasts, as well as MSCs and CD14+HLA-DR- monocytes irrespective of their surface expression of CD16 are significantly more susceptible to NK cell mediated cytotoxicity [82], therefore, these cells may condition NK cells to become anergic (please see below). Indeed, in OSCCs the majority of recruited immune effectors are usually found in the connective tissue area where, through cell-cell interaction with the immunosuppressive cells listed above, can inhibit the cytotoxic function of NK cells, leading to increased cytokine secretion capability of the NK cells resulting in differentiation and resistance of oral epithelial tumors (Fig. (**1**)) (please see below).

Role of Transcription Factors in Tumor Resistance

Although each one of the factors indicated above can in part be responsible for the resistance of tumors, previous data obtained from different laboratories [30, 83] indicated that targeting transcription factors may decrease resistance and increase sensitivity of tumors to immune mediated cytotoxicity.

NFκB in Cancer

Many tumor cells exhibit constitutively elevated NFκB activity [24]. Human leukemias and lymphomas as well as human solid tumors exhibit constitutively activated NFκB in the nucleus [24]. However, although previous studies have attributed a significant role for NFκB in oncogenesis and

tumor progression, relatively fewer studies have been conducted to explore the significance of elevated NFκB function in tumor cells in the modulation of immune effector function against the tumor cells [29, 30].

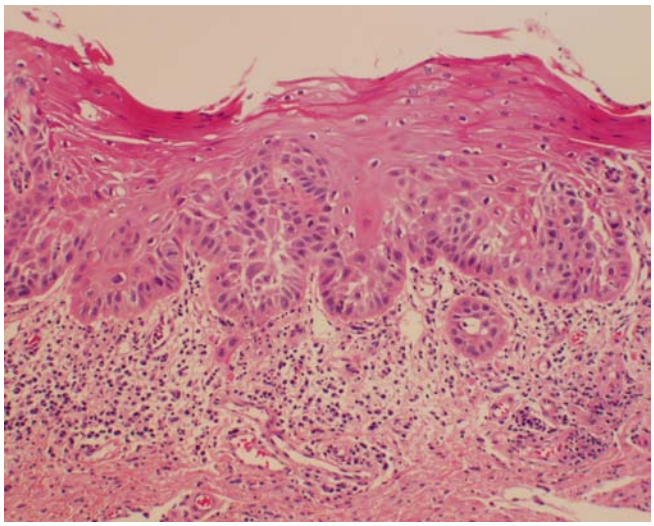

Fig. (1). Immune inflammatory cells are mainly concentrated in the connective tissue area right beneath the epithelial layer of OSCC. The slides from OSCC were prepared and stained with H&E. Significant infiltration of immune effectors right beneath the epithelial layer can be seen in the connective tissue area where the immune inflammatory cells are likely to condition NK cells to lose cytotoxicity and to support differentiation of epithelial cells. Epithelial dysplasia with small microinvasive islands can also be seen in the slide.

Blocking NFκB in Oral Tumors Increases NK Cell Function

We have shown previously that NFκB nuclear function in a primary Oral tumor OSCCs and an established tumor line, HEp-2 cells previously used as an oral tumor model [30, 84-86], modulates and shapes the function of interacting immune effectors [29, 30]. It is believed that HEp-2 cells are Hela contaminants since cells of these lines are shown to contain Hela marker chromosomes (ATCC). Since knock down of NFκB was shown to increase the function of immune inflammatory cells in diverse cell types (please see below) it is not surprising to find similar patterns of immune activation in both oral and non-oral derived cell lines, even in those which have been derived from contaminants such as Hela cells. In addition, the majority if not all cells increase NFκB during their activation and differentiation. A knock down of NFκB is likely to revert the cells, irrespective of their cellular origin, to their less differentiated phenotypes resulting in the potential activation of the immune effectors in order to aid in their differentiation [26]. Thus, inhibition of NFκB by an IκB super-repressor in HEp-2 cell line (Hep2- $I\kappa B_{(S32AS36A)}$) or in primary oral tumors resulted in a significant level of activation of human NK cell cytotoxic function and increased IFN-γ secretion. Similarly, inhibition of NFκB by Sulindac increased the functional activation of NK and enhanced anti-tumor cytotoxic activity [29, 30]. Similar experiments to those performed with HEp-2 and OSCC tumors were also conducted using immortalized but non-tumorigenic human oral keratinocytes (HOK-18). Inhibition of NFκB in HOK-18 also resulted in an increased function of interacting NK cells [26].

NFκB Activation in Tumor Cells Alters the Cytokine/Chemokine Profiles of NK Cells

Inverse modulation of IFN-γ and IL-6 cytokine secretion was seen in co-cultures of NK cells with NFκB knock down OSCCs and HEp2- $I\kappa B_{(S32AS36A)}$ cells indicating that blocking NFκB in these cells serves to switch the balance from Th2 type responses to more of a Th1 type response [26. 29]. Moreover, HEp2- $I\kappa B_{(S32AS36A)}$ cells produced large amounts of pro-inflammatory chemokines MCP-1 and Rantes suggesting that inhibition of NFκB in tumor cells may recruit NK and T cells to the tumor microenvironment [29].

NFκB Inhibition in Mice and Humans Activate Immune Inflammatory Functions

Targeted deletion of IKK-β which inhibits NFκB function in epidermis of mice has previously been shown in one study to lead to inflammatory skin manifestations similar to that seen in patients with Incontinentia Pigmenti (IP) [31]. Elevated levels of cytokines and chemokines have also been demonstrated in the epidermis of patients and animals with Iκκγ and Iκκβ deletions [31, 87].

Blocking of NFκB function by deleting Iκκ-β in intestinal epithelial cells dramatically decreased the rate of tumor formation without affecting the size of the tumors in colitis-associated cancer model [88, 89]. Moreover, deleting Iκκ-β in myeloid cells in the same model system resulted in a decrease in tumor size. These studies also underscore the significance of a cross talk between different subsets of immune effectors with the epithelial cells in induction and progression of the intestinal tumors.

Mice with a keratinocyte-specific deletion of Iκκ–β demonstrated decreased proliferation of epidermal cells and developed TNF-α dependent inflammatory skin disease [31]. In contrast, in other studies in which NFκB function was blocked in dermal keratinocytes by a mutant IκB-α an increased proliferation and hyperplasia [90] and eventual development of cutaneous squamous cell carcinomas of skin were seen if mice were allowed to survive and reach adulthood [91, 92]. In contrast to the results obtained in epidermis, blocking NFκB with a mutant IκB-α super-repressor in HEp2 cells did not change or moderately decreased the rate of tumor cell growth when compared to vector-alone transfected HEp2 cells (unpublished observations). It is of interest to note that in these studies with diverse functional outcomes in keratinocytes, blocking TNF-α function resulted in the prevention of both the neoplastic transformation and the inflammatory skin disease [31, 92]. Thus, it appears that TNF-α is the primary factor mediating the pathological processes in both of these studies. Elevated numbers of immune inflammatory cells recruited to the site of epidemis are likely responsible for the increased secretion of TNF-α. Indeed, we have demonstrated that synergistic induction of TNF-α could be observed when NFκB knock down oral tumors were cultured with either PBMCs or NK cells [29].

NK Cells Lyse Cancer Stem Cells, as well as hESCs, hiPSCs, hMSCs and hDPSCs but not their Differentiated Counterparts

Increased NK cell cytotoxicity and augmented secretion of IFN-γ were observed when NK cells were co-incubated with OSCSCs which released significantly lower levels of GM-CSF, IL-6 and IL-8 and demonstrated decreased expression of phospho-Stat3, B7H1 and EGFR, and much lower constitutive NFκB activity when compared to differentiated OSCCs [26]. More importantly, OSCSCs expressed CD133 and $CD44^{bright}$ oral stem cell markers [26]. Increase in IFN-γ secretion was correlated with a decrease in secretion of IL-6 in co-cultures of NK cells with OSCSCs as compared to those co-cultured with OSCCs. Therefore, from these results a specific profile for differentiated NK resistant oral tumors emerged which demonstrated increased GM-CSF, IL-6 and IL-8 secretion in the context of decreased IFN-γ secretion during their interaction with the NK cells. In contrast, co-cultures of cancer stem cells with NK cells demonstrated increased IFN-γ in the context of lower GM-CSF, IL-6 and IL-8 secretion [26, 82]. In addition, three brain tumor stem cells which were previously characterized [93-95] were found to be significantly more susceptible to NK cell mediated cytotoxicity when compared to their differentiated counterparts which were significantly more resistant (manuscript submitted). Since OSCSCs and brain stem cells were significantly more susceptible to NK cell mediated cytotoxicity we reasoned that healthy, non-transformed primary stem cells may also be susceptible to NK cell mediated cytotoxicity. We demonstrated previously that NK cells lysed hMSCs, hDPSCs, hESCs and hiPSCs significantly. All different types of stem cells became resistant to NK cell mediated cytotoxicity once they were differentiated [26]. In addition, higher sensitivity of hiPSCs to NK cell mediated lysis was also observed when compared to parental line from which they were derived. Taken together these results indicated that undifferentiated cells were targets of NK cell cytotoxicity. Thus, the stage of differentiation of the cells is predictive of their susceptibility to NK cell mediated cytotoxicity.

Targeting NFκB Revert the Cells to More of an Undifferentiated Phenotype and Increases NK Cell Mediated Cytotoxicity against Oral Tumors

Since the degree of differentiation in the cells is predictive of their sensitivity to NK cell mediated cytotoxicity, we reasoned that blocking NFκB in the cells may dedifferentiate and consequently revert the cells to more an of undifferentiated phenotype, resulting in their increased susceptibility to NK cell mediated cytotoxicity. Indeed, blocking NFκB in oral tumors was also found to increase CD44 surface receptor expression, which is one of the hallmarks of stem cells (manuscript in prep). In addition, the profiles of cytokines secreted in the co-cultures of NK cells or PBMCs with NFκB knock down tumors resembled those secreted in the co-cultures of NK cells with OSCSCs or healthy stem cells [26, 29, 82]. Since tumorigenic and non-tumorigenic human oral keratinocytes acquire sensitivity to NK cell mediated lysis when NFκB is inhibited, it is likely that this phenomenon is not specific to cancer, and it may occur during other pathological conditions. Indeed, when human primary monocytes are differentiated to dendritic cells they too became more resistant to NK cell mediated cytotoxicity [26]. Moreover, knock down of COX2 in primary monocytes [26], or in mouse embryonic fibroblasts (manuscript in prep), resulted in the reversion or de-differentiation of the monocytes and fibroblasts respectively, and the activation of NK cell cytotoxicity. Indeed, it is likely that any disturbance in cellular differentiation may predispose the cells to NK cell mediated cytotoxicity. Since STAT3 is an important factor that is increased during differentiation, blocking STAT3 is also critical in the activation of immune effectors [83]. In support of a critical role of STAT3 in immune evasion of tumor cells in humans, we and others have recently shown that glioblastoma multiforme (GBM) tumors display constitutive activation of STAT3 (Cacalano and Jewett, unpublished observation) [96], and poorly induce activating cytokines and tumor-specific cytotoxicity in human peripheral blood mononuclear cells (PBMCs) and NK cells (unpublished observations). Ectopic expression of dominant-negative STAT3 in the GBM cells increased lysis of the tumor cells by the immune effectors and induced production of IFN-γ by the interacting immune effectors (manuscript in prep).

Since NFκB is shown to regulate IL-6 secretion in OSCCs, HOK-16B and HEp2 cells and secreted IL-6 in tumors is known to activate STAT3 expression and function, increases in NFκB nuclear function should in turn induce STAT3 activation and result in a significant resistance of tumors to NK cell mediated cytotoxicity. Therefore, targeting STAT3 or signaling pathways upstream of STAT3, such as NFκB, may dedifferentiate the cells and predispose the cells to NK cell mediated cytotoxicity.

Sensitive but not Resistant Tumors Cause Anergy in NK Cells

We have previously shown that K562, an NK sensitive tumor, causes loss of NK cell cytotoxicity and induce cell death in a small subset of NK cells [22, 97]. On the other hand NK resistant tumors such as RAJI cells induce no or much lower levels of anergy or loss of NK cell cytotoxicity [22, 97]. Furthermore, following NK cell cultures with sensitive tumor-target cells overnight, the target binding NK cells undergo phenotypic and functional changes. Target cell inactivated NK cells express CD16-CD56dim/-CD69+ phenotype [22, 97]. This phenotype has also been observed in several disease manifestations including HIV infection [98]. Significant downmodulation of CD16 receptor expression and decreased NK cell cytotoxic function were also seen in several cancer patients including those of the oral and ovarian cancer patients [99, 100]. In addition, downregulation of CD16 surface receptors on NK cells was also observed when NK cells were treated with CA125 isolated from ovarian tumor cells [101]. The decrease in CD16 surface receptors was accompanied by a major decrease in NK cell killing activity against K562 tumor cells [101]. These observations suggested that CD16 receptors may play an important role in target cell induced loss of NK cell cytotoxicity. Indeed, CD16:Ig fusion proteins are shown to bind to a variety of tumor-target cells indicating the existence of specific ligands for CD16 receptors on tumor cells [102]. Furthermore, we have previously shown that the triggering of CD16 on untreated and IL-2 treated NK cells was found to result in downmodulation of CD16 receptors and in a great loss of

cytotoxicity in NK cells. In addition, a subset of NK cells was programmed to undergo cell death [22, 42, 43, 97]. Cell death of NK cells was shown to be regulated, in part, by endogenously secreted TNF-α from the NK cells [42]. Previous studies by other groups have also shown that IL-2 activated NK cells undergo cell death following cross-linking of the CD16 receptor [103, 104]. Thus, we have coined the term "split anergy" for the responses observed by the NK cells after their interaction with sensitive target cells or after the triggering of CD16 receptors by the antibody in combination with IL-2 treatment. Indeed, three subpopulations of NK cells; namely Free, Binder and Killer NK cells with varying degrees of loss of cytotoxicity were identified after the formation of conjugates with K562 targets [42, 105-108]. Free cells which did not bind or form conjugates with target cells were not inactivated, or exhibited the least inactivation of cytotoxicity, whereas both Binder and Killer subsets exhibited significant loss of cytotoxicity [43, 105-108]. Inactivation of Killer NK cells were somewhat less than the Binder NK cells since they killed their respective targets and thus they were inactivated less than the Binder NK cells which remained bound to their target cells for longer time and therefore, were inactivated the most and had the least ability to mediate cytotoxicity [43, 105-108]. Indeed, when NK cells were dissociated from the NK-K562 conjugates (NK_{DC}) and then treated with IL-2, a subset of NK cells responded to IL-2 activation for cytotoxicity, however, they were less responsive to IL-2 mediated induction of proliferation or secretion of cytokines. In contrast, those which remained conjugated with the tumor cells (NK_C) did not respond to IL-2 activation for cytotoxicity, but did proliferate significantly and secreted large amounts of cytokines [22, 97]. Treatment of NK cells with IL-2 and anti-CD16mAb also induced split anergy by significantly decreasing the NK cell cytotoxicity while increasing the cytokine secretion capabilities of NK cells [42, 109-111]. Furthermore, IL-2 rescued anti-CD16 mAb mediated apoptosis induced in a subset of NK cells [42, 112]. Loss of cytotoxicity in NK cells was exacerbated when NK cells were either treated with $F(ab)'_2$ fragment of anti-CD16 mAb or treated with a combination of MHC-Class I and anti-CD16 mAb, while the same treatments resulted in an increased secretion of cytokines [109, 111]. These results suggested that receptor signaling on NK cells in the presence of IL-2 is likely to result in a decrease in NK cell cytotoxicity while increasing secretion of cytokines by the NK cells. Therefore, three distinct functional outcomes could be observed in NK cells which have either interacted with sensitive tumor-target cells or treated with anti-CD16 mAb in the presence of IL-2 treatment, namely; 1-Loss of cytotoxicity, 2-gain in the ability to secrete cytokines and 3- death in a subset of NK cells.

Split Anergy in NK Cells is Induced by Total Monocytes and those Depleted of CD16+ Subsets of Monocytes

A significant decrease in NK cell mediated cytotoxicity could be observed when MSCs and DPSCs were cultured with either viable or irradiated monocytes before they were exposed to IL-2 treated NK cells. Interestingly, significant lysis of MSCs and DPSCs by untreated NK cells was also significantly and reproducibly blocked by the addition of monocytes [82]. To determine whether CD16- subset of monocytes were also able to inhibit the cytotoxic function of NK cells in a 3 way interaction with the stem cells we sorted this subset of the monocytes and used both the unsorted, and those sorted to remove CD16+ subsets (CD16-) in a 3 way killing assay with the NK cells. Both the total populations of monocytes and CD16- subsets were capable of inducing inhibition of NK cell cytotoxicity against stem cells [82]. We then determined whether decreased lysis of stem cells by NK cells was due to a competitive lysis of monocytes by the NK cells. We confirmed that monocytes were also lysed by the NK cells significantly. Furthermore, when we co-cultured stem cells with monocytes and removed the monocytes from the stem cells we could still observe significant inhibition of NK cell mediated lysis. This argues against the protection of stem cell lysis by NK cells solely by competitive lysis of monocytes. Therefore, even though lysis of monocytes by the NK cells may in part contribute to the prevention of NK cell lysis of stem cells, interaction of monocytes with stem cells can also provide resistance of stem cells against NK cell cytotoxicity. Decrease in NK cell lysis of MSCs and DPSCs was paralleled with a significant induction of IFN-γ. Indeed, when MSCs or DPSCs were cultured with IL-2 treated NK cells alone we could observe significant induction of IFN-γ secretion. However, the highest increase was seen when NK cells were cultured with MSCs or DPSCs in the presence of monocytes. Therefore, although decreased killing of stem cells by the NK cells could be observed in the presence of monocytes, synergistic secretion of IFN-γ by the NK cells in the presence of monocytes and stem cells could be observed, indicating an inverse relationship between cytotoxicity and IFN-γ secretion (split anergy). This was similar to the profiles which we had seen when NK cells were treated with IL-2 and anti-CD16 antibody in which significant decrease in cytotoxicity of NK cells was observed in parallel with increased secretion of IFN-γ (split anergy) [42].

Tumor Microenvironment may Shape the Function and Phenotype of the NK Cells

The above observations prompted us to speculate regarding the significance of interaction of monocytes with NK cells and stem cells. It is plausible that monocytes may serve as shields against NK cell lysis of stem cells. Similar to anti-CD16 antibody mediated effect on IL-2 treated NK cells, monocytes too can shield stem cells from killing by the NK cells by increasing the total IFN-γ release by the NK cells and decreasing the cytotoxic function of NK cells (split anergy), resulting in an increased protection and differentiation of stem cells. Indeed, monocytes also increased TNF-α, IL-6 and VEGF secretion in the co-cultures of stem cells with NK cells which could further augment the induction of NFκB and increased differentiation of stem cells. The shielding effect of monocytes could be a more generalized function of other effectors since NK cells can also target fibroblasts and to a much lesser extent the T and B cells [82]. It would be interesting to see whether NK cells will also be able to significantly lyse MDSCs or PMNs. This may have significant implications regarding the role of NK cells not only in limiting inflammation, but also the significance of other immune effectors limiting the cytotoxic function of NK cells against stem cells raising the secretion of key cytokines for speedy and optimal differentiation of stem cells during inflamma-

tion. This is precisely what is observed in cancer patients in whom global decrease in NK, cytotoxic T cells and monocytes have all been reported [12].

Potential Role of Anergized NK Cells in Differentiation and Regeneration of Tissues

Even though conditioning of NK cells to support differentiation of stem cells is discussed in the context of tumors, induction of split anergy in NK cells, we believe, is an important conditioning step responsible for the repair of tissues during pathological processes irrespective of the type of pathology. In tumors, since the generation and maintenance of cancer stem cells is higher, the majority if not all of the NK cells may be conditioned to support differentiation and repair of the tissues. As such, the phenotype of NK cells in tumor microenvironment as well as in the peripheral blood may resemble that of the anergic NK cells, i.e., decreased NK cell cytotoxicity, acquisition of CD16-/dimCD56-/dimCD69+ phenotype and augmented ability to secrete inflammatory cytokines (Fig. (**2**)). Of course, the degree of decrease in NK cell cytotoxicity may be directly proportional to the load of cancer stem cells. Therefore, our results suggest two very important functions for the NK cells. One function is to limit the number of stem cells by selecting those with a greater potential for differentiation for the repair of the tissues and second to support differentiation of the stem cells and subsequent regeneration of the tissues.

To achieve these tasks NK cells have to acquire two different phenotypes and be conditioned to carry out both functions successfully. CD16+CD56+/dimCD69- subsets of NK cells are cytotoxic and will mediate cytotoxicity depending on which sensitive targets they will encounter first. In respect to the oral squamous cell carcinomas since the majority of immune effectors can be found at the connective tissue area the chances are that they may first encounter and interact with either the other immune effectors or the effectors of connective tissue such as fibroblasts. However, there is also the possibility that NK cells may also first encounter the stem cells at the base of the epithelial layer, in which case by eliminating their bound stem cells, they too can become anergized. Surprisingly, allogeneic CTLs were also found to target Glioblastoma stem-like cells and not their differentiated counterparts (Veronique Quillien, personal communication). By eliminating a subset of stem cells or after their interaction with other immune inflammatory cells or effectors of connective tissue NK cells could then be in a position to support differentiation of selected population of stem cells since they will be conditioned to lose cytotoxicity, induce cytokine and growth factor secretion and gain the CD16-/dimCD56dimCD69+ phenotype (Fig. (**2**)). *In vivo* physiological relevance of above-mentioned observations could be seen in a subpopulation of NK cells in peripheral blood, uterine and liver NK cells which express low or no CD16 receptors, have decreased capacity to mediate cytotoxicity and are capable of secreting significant amounts of cytokines [113, 114]. In addition, 70% of NK cells become CD16 dim or negative immediately after allogeneic or autologous bone marrow transplantation [113]. Since NK cells lose their cytotoxic function and gain in cytokine secretion phenotype and down modulate CD16 receptors after their interaction with tumor cells or the stem cells [22, 42], it is tempting to specu-

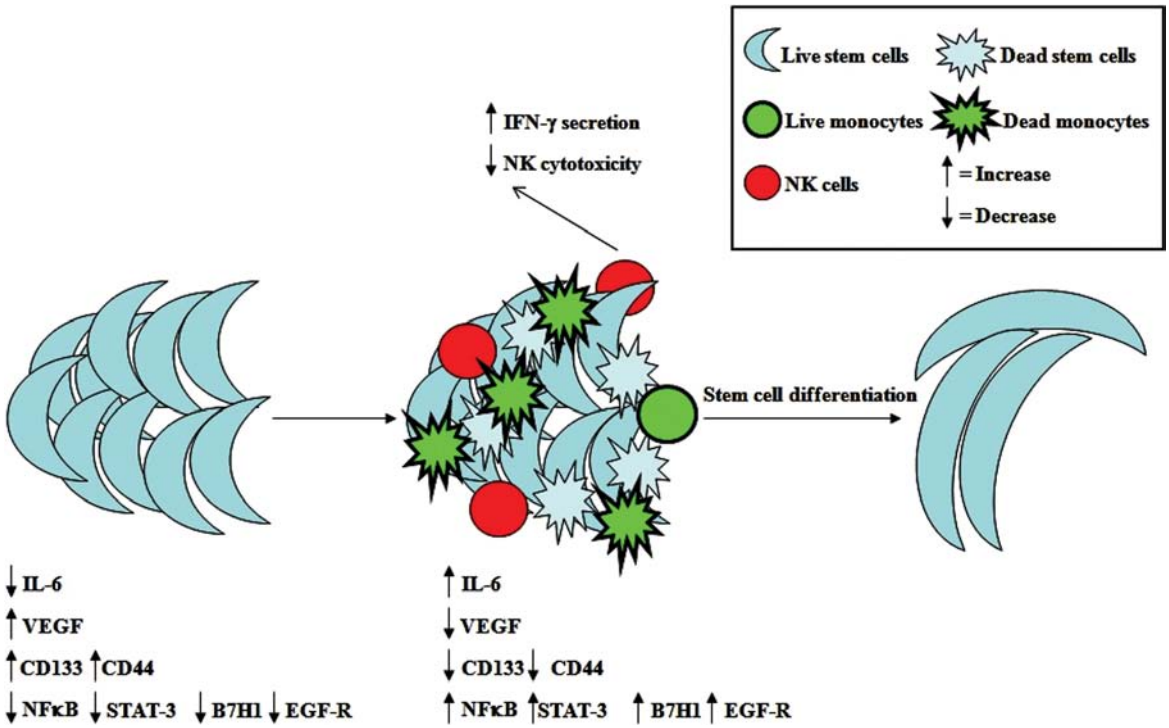

Fig. (2). Schematic representation of hypothetical model of oral cancer stem cell differentiation by NK cells and monocytes. Interaction of cancer stem cells or primary stem cells with monocytes and NK cells results in the loss of NK cell cytotoxicity due partly to the induction of resistance of cancer stem cells by monocytes and indirectly by monocytes serving as targets of NK cells, thus serving as a shield which protects the stem cells from lysis by the NK cells. Loss of NK cell cytotoxicity by monocytes and gain in secretion of IFN-γ results in a significant induction of transcription factors, cytokines and growth factors in stem cells and differentiation of stem cells.

late that *in vivo* identified CD16- NK cells and *in vitro* tumor induced CD16- NK cells have similar developmental pathways since they have similar if not identical functional and phenotypic properties.

The ultimate proof of concept in support of this model was recently obtained in our laboratory. We observed that anergized NK cells were directly responsible for the increased differentiation and resistance of a number of different stem cells including cancer stem cells against cytotoxic effectors (manuscript submitted). In addition, we now have evidence which supports the notion that the induction of anergy in NK cells is an active process which is induced by the triggering of CD16 receptors on the NK cells and is not due to degranulation and exhaustion of cytotoxic granules (manuscript in prep).

Our work collectively suggests that anergized NK cells are as important as the non-anergized NK cells in their effector functions. NK cells are not only important for the removal and shaping of the size of the stem cells but also their differentiation and regeneration of new tissues. The task of NK cells in this regard goes above and beyond their most appreciated function of being the effectors of first line defense against viral infection and malignancies. They too can be effectors of differentiation and tissue regeneration.

CONCLUSION

Recent advances in our understanding of anti-tumor immune responses and cancer biology have revealed a complex dynamic interaction between the immune effectors and the tumor cells. Effectors of the immune system are known to shape tumor cells (immunoediting) and select for cancers with reduced immunogenicity and enhanced capacity to actively induce immunosuppression. However, the same effector mechanisms are likely responsible for the selection of healthy stem cells with enhanced capacity to induce immunosuppression for the ultimate goal of the regeneration of damaged or disturbed tissues. Much work has been done to identify strategies by which tumor cells evade the immune system. Altered expression of MHC molecules which block recognition and activation of T and NK cells are examples of these mechanisms. In addition, tumor cells induce T and NK cell apoptosis, block lymphocyte homing and activation, and dampen macrophage and dendritic cell function by releasing immunosuppressive factors such as Fas, VEGF, IL-6, IL-10, TNF-α, GM-CSF and IL-1β. However, the same effector functions are also important in tissue repair mechanisms induced by the immune effectors. Furthermore, progress has been made in identification of the upstream mechanisms which control the expression of immunosuppressive factors in tumor cells. Two key control elements, NFκB and STAT3 were identified and shown to coordinately regulate the production of multiple tumor-derived immunosuppressive molecules and play a pivotal role in tumor cell immune suppression. The pathways interacting and possibly even amplifying each other underscore the potential for these two signaling modules to repress immune responses. One model for NFκB-STAT3-mediated immunosuppression suggests that NFκB-induced IL-6 expression activates STAT3 in tumor cells and modulates the production of inhibitory cytokines and chemokines resulting in decreased T cell infiltration and activation. However, the same mechanisms are likely to be important for normal tissue regeneration and induction of resistance to NK and T cell mediated cytotoxicity.

Based on the work presented in this review we suggest that NK cells may have two significant functions; one that relates to the removal of stem cells that are either defective or disturbed or in higher numbers than are needed for the regeneration of damaged tissue. Therefore, the first task is to select stem cells that are competent and are able to achieve the highest ability to regenerate tissues. The second important task for NK cells is to support the differentiation of the selected cells after altering their phenotype to cytokine secreting cells (Fig. (**2**)). NK cells may be conditioned to support differentiation of stem cells either by interacting with NK sensitive immune effectors or by effectors of connective tissue as well as with the stem cells. This process will not only remove cells that are damaged and have flaws in the differentiation process, but also it will ensure the regeneration of damaged or defective tissues, while aiding in the resolution of the inflammatory processes. Therefore, processes in which suboptimal differentiation and regeneration of the tissues occurs, a chronic inflammatory process may be established causing continual tissue damage and recruitment of stem cells and NK cells.

The inability of patient NK cells to contain cancer stem cells due to flooding of NK cells by proliferating cancer stem cells and conversion of NK cells to cytokine secreting cells may likely be one mechanism by which cancer may progress and metastasize. Therefore, there should be two distinct strategies by the NK cells to eliminate tumors, one which targets stem cells and the other which targets differentiated cells. In theory this should be achieved by the use of antibodies to surface receptors that are highly expressed on differentiated cells, however, we have also found that NK cell Antibody Dependent Cellular Cytotoxicity (ADCC) is also lower against differentiated tumors when compared to their respective stem cells (manuscript submitted). Therefore, since a great majority of patient NK cells have modified their phenotype to support differentiation of the cells, they may not be effective in eliminating the cancer stem cells. Therefore, cancer stem cells may accumulate and eventually result in the demise of the patient. These patients may therefore, benefit from repeated allogeneic NK cell transplantation at the site of the tumor for elimination of cancer stem cells.

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