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Small Molecule Protein Kinase Inhibitors and their Effects on the Immune System: Implications for Cancer Treatment

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Summary

Oncogenic signaling pathways have emerged as key targets for the development of small molecule inhibitors, with several protein kinase inhibitors already in clinical use for cancer patients. In addition to their role in tumorigenesis, many of the molecules and signaling pathways targeted by these inhibitors are also important in the signaling and interaction of immune cells, such as T cells and dendritic cells. Not surprisingly, there is increasing evidence that many of these inhibitors can have a substantial impact on immune function, both stimulating and downregulating an immune response. In order to illustrate the important role of signaling molecule inhibition in the modulation of immune function, we will discuss the exemplary pathways MAPK, AKT/PI3K/mTOR, and VEGF/VEGFR as well as selected small molecule inhibitors, whose impact on immune cells has been studied more extensively.

Keywords

tyrosine kinase inhibitors; anti cancer; immunotherapy; VEGF; MAPK; mTOR

For several decades, it has been evident that most tumor cells express antigens that can be recognized by specific T cells. It has been postulated that tumor immunity is constantly ongoing and indeed very successful in the context of immune surveillance, where the immune system presumably recognizes single cells or small numbers of tumor cells at the earliest stage of tumor development [1]. However, once cancer cells have organized into a structured tumor environment, the tumor mass becomes a much more complex entity, consisting not only of tumor cells, but many other cell types, such as endothelial cells, stromal cells and several different types of immune cells. These cells communicate either through receptors or through soluble molecules such as cytokines and chemokines, creating an intricate network [2]. Considering the complexity of the tumor microenvironment, it is not surprising that a tumor-specific T cell, after being primed either intrinsically by the autologous tumor or therapeutically by an immune intervention, often cannot mediate tumor destruction. The fact that the presence and frequency of tumor-specific T cells measured in the periphery often does not correlate with the clinical antitumor response is consistent with this observation [3-5]. Many immune therapy approaches currently in clinical use (IL-2, IFN- α) or in preclinical/clinical development (anti-CTLA-4 antibodies) primarily aim to

boost a (presumably weak) tumor-specific T cell response [6]. These therapeutic approaches occasionally work, as is evident from infrequent, but reproducible and sometimes dramatic tumor responses (e.g., high-dose IL-2 and anti-CTLA-4 treatment in metastatic melanoma resulting in durable responses in some patients [7-8]). In recent years it has become increasingly clear that suboptimal tumor immunity is partially a reflection of negative immune regulatory mechanisms in the tumor milieu [2, 9-10]. This recognition adds greater complexity to the design of successful immune therapies: in order for a tumor specific cytotoxic T cell to exert its function, the tolerogenic tumor environment must be overcome.

In recent years, a molecular link between oncogenesis and immune-suppression in the tumor microenvironment has been demonstrated for the STAT3 signaling pathway [2, 11-12]. Signal transducer and activator of transcription (STAT) family proteins, in particular STAT3, play a dual role in tumor progression: i) STAT3 leads to induction of a tumor-promoting inflammatory microenvironment, and ii) STAT3 activation in tumor infiltrating immune cells results in suppression of innate and adaptive immune responses [11, 13].

Another potential link between oncogenic signaling and the immune system has been identified as the upregulation of the MAPK pathway and impaired IL-12 production by dendritic cells (DC) in melanoma [14]. The presence of molecules that are both part of an oncogenic signaling pathway such as MAPK or PI3K/AKT/ mTOR in tumor cells and critical in the regulation of immune cells may have important implications for therapy with small molecule inhibitors in cancer patients. Over the last decade, molecularly targeted therapy as a field for the treatment of cancer has grown dramatically. This has resulted from a recognition that altered intracellular signaling pathways play an important role in the malignant transformation process due to their impact on many different aspects of growth such as cell cycle regulation, apoptosis and angiogenesis [15-16]. The targets of most small molecule inhibitors are phosphorylation sites of kinases (most commonly tyrosine or serin-threonine kinases) that are either associated with growth factor receptors or downstream signaling molecules. Examples of these molecules that are targeted in various combinations by many of the small molecule inhibitors include vascular endothelial growth factor receptors (VEGFRs, VEGFR-1, VEGFR-2, and VEGFR-3), the platelet-derived growth factor receptor (PDGFR) alpha and beta, epidermal growth factor receptor (EGFR), fms-like tyrosine kinase-3 (Flt3), Mammalian target of rapamycin (mTOR), RAF, and cKIT. As for their relevance for immune function, some of these targets and pathways, such as mTOR, have long been recognized as important molecules leading to alteration of the immune response, and indeed have been targeted specifically for that purpose in the case of the mTOR inhibitor rapamycin in transplantation [17-18]. Others, such as the MAPK pathway and the VEGF/VEGFR are not particularly recognized as immune targets for therapeutic purposes, but their role in the function of immune cells has been examined extensively in vitro and in rodent models.

It is conceivable that beyond their effects on the respective pathway in cancer cells (Fig. 1), most targeted agents might have “off target” effects on immune cells, including T cells (Fig. 2) and DC (Fig. 3). Most small molecule kinase inhibitors are currently developed and clinically used with sole attention to their direct effects on tumor cells. As critical signalling pathways and molecules targeted by small molecule kinase inhibitors are present in most

cells, including immune cells, it is reasonable to assume that they also affect immune cells. Recent data have started to substantiate this notion. Since preclinical studies demonstrate both immune stimulatory and inhibitory effects of signaling pathways in immune cells (as will be discussed below), these “off target” effects could either be a welcome and synergistic effect, if an anti-tumor immune response is induced or augmented, or potentially detrimental and antagonistic if a pre-existing antitumor response is compromised. In this review, we will first discuss preclinical data on the impact of MAPK and the PI3K/AKT/mTOR pathway-alteration in immune cells. Both of these pathways have been well studied in immune cells in vitro and in rodent models and are critical signal transduction pathways. Furthermore, these pathways, once upregulated by oncogenes, are important for the mediation of tumor growth. Importantly, they are downstream to many other molecules that are targeted by small molecule inhibitors (such as growth factor receptors on the cell surface). We will also discuss the VEGF/VEGR axis, as it is targeted by many small molecule inhibitors (in addition to antibodies such as bevacizumab and VEGF-TRAP, which will not be discussed in this review) and have emerged as important mediators of tumor immune suppression. We will then discuss four small molecule kinase inhibitors (temsirolimus, imatinib, sorafenib and sunitinib) for which sufficient immunologic data are available and which are used in research studies as well as clinical oncology practice. Finally, we will highlight the preclinical data and the growing body of clinical evidence of their impact on the immune system in cancer patients.

The role of PI3K/AKT/mTOR, MAPK, and VEGF/VEGFR signaling in mediating immune-stimulatory and immunosuppressive effects

PI3K/Akt/mTOR

mTOR is a serin threonine kinase that regulates cell growth, survival, migration, protein synthesis, and other important cell functions. It is positioned downstream of phosphoinositide 3-kinase (PI3K) and Akt (Fig. 1). The fact that mTOR blockade by rapamycin leads to immunosuppression has been known for decades and has been exploited clinically in organ transplantation. Initially, rapamycin's mechanism of immunosuppression was largely explained by its impact on T cell proliferation, which was attributed to the induction of cell cycle arrest in T cells [19-20](Fig. 2). More recent studies demonstrate that the PI3K/AKTmTOR axis is directly involved in the induction of anergy in T cells, independent of the cell cycle [21] (Fig. 2). mTOR inhibition was also shown to induce differentiation of CD4 cells into CD4⁺CD25⁺Foxp3⁺ T regulatory cells and to regulate T cell effector versus regulatory lineage commitment [22-25]. It has become evident that mTOR has a central function in the shaping of an innate or adaptive immune response [26]. In addition to its impact on T cells, mTOR plays a role in the generation, differentiation, maturation, survival, and function of DC (Fig. 3): Toll-like –receptor (TLR) agonist or CD40 antibody-mediated upregulation of CD86 on DC was inhibited by mTOR inhibition [27]. Antigen uptake and presentation in DC, including phagocytosis, macropinocytosis, and endocytosis were shown to be negatively impacted by mTOR inhibition with rapamycin [28-30].

Furthermore, in a recent study, mTOR inhibition was implicated in the suppression of Th17 cell proliferation [31]. In addition to T cells and DC, mTOR blockade also has an inhibitory effect on other immune cells such as B cells, Natural killer (NK) cells, neutrophils, and mast cells [32-35].

In contrast to the above described immunosuppressive effects, mTOR blockade was shown to result in the generation of a Th1 and Th17 T cell response induced by monocytes and mDC that produced increased levels of IL-12 and decreased amounts of IL-10 upon TLR ligation [36-37]. Furthermore, mTOR blockade can lead to increased IL-12 production by LPS-stimulated DC and Th1 cell polarization [36]. It is noteworthy that it was recently reported in a mouse model that mTOR blockade with rapamycin leads to the generation of memory transgenic T cells (OVA-specific OT-1 cells) that were more efficient in generating tumor immunity than the IL-12-conditioned type I effector T cells considered to be the strongest inducers of tumor immunity [38].

It has increasingly been recognised that the PI3K/Akt/mTOR pathway not only affects lymphocyte survival, proliferation and signaling but also affects innate immune cells, including neutrophils, mast cells, monocytes, macrophages and myeloid as well as plasmacytoid dendritic cells (mDCs and pDCs, respectively). Stimulation of TLR7 and TLR9, for instance, has been shown to activate PI3K in human pDCs [39]. This study also demonstrated that PI3K inhibition prevents the nuclear translocation of IFN regulatory factor (IRF)-7, critical for type I IFN production by pDCs. PI3K is furthermore crucially implicated in TLR signaling in monocytes, macrophages and mDC, possibly as feedback mechanism to control the cellular response to pathogens by limiting proinflammatory cytokine production (eg, IL12) and enhancing the synthesis of the anti-inflammatory IL10[40-42]. It has been shown that pharmacological or genetic disruption of PI3K results in excessive IL12 production in murine splenic DCs [41] and PI3K inhibition increased mortality in a murine sepsis model due to an amplified production of proinflammatory cytokines, including IL1 β , IL6, IL12 and TNF α [43]. It was also demonstrated that Fc γ R ligation after TLR4 engagement in murine macrophages activates the PI3K/Akt pathway, inhibits IL12 and promotes IL10 production by controlling nuclear translocation of I κ B α , effects were reversed by PI3K inhibition[44]. Therefore, the PI3K/Akt pathway is a major regulator of innate immunity by controlling the production, accumulation and binding of central transcription factors required for the production of key inflammatory cytokines like IL12 and IL10, supporting the role of PI3K pathway as a negative inflammatory feedback regulator. However, PI3K might regulate different signalling pathways depending on the cell type and the organism and can in some cases even positively regulate some inflammatory mediators.

MAPK pathway

The mitogen-activated protein kinases (MAPK) are comprised of three groups of activation cascades: extracellular-signal-regulated kinases (ERKs), JUN N-terminal kinases (JNKs) and p38 kinases (Fig. 3). While MAPK activity is mainly dependent on extracellular events, the role of these kinases has been elucidated by inhibitors and knock-out mice in recent years. Evidence is emerging that MAPK play a role in many aspects of immune response.

For example, they were shown to be important in the control of DC activation and IL-12 production [45-48] as well as IL-10 production by DC [49-50], suggesting differential roles of signaling pathways in the induction of an activating versus an immunosuppressive DC phenotype. It is intriguing that different aspects of DC maturation (such as expression of surface molecules and cytokine production) and DC maturation by various stimuli such as Toll like Receptor Agonists (TLR), cytokine receptor ligands or CD40 ligand are mediated by different groups of the MAPK pathway with some redundancy and overlap. For example, several studies have shown that p38 is important for the maturation of immature DC into a mature phenotype with LPS or TNF- α , and that constitutive activation of p38 activates DC and enhances tumor immune responses [45, 51-52]. Blockage of the RAF-MEK-ERK pathway by MEK inhibitors was found to enhance LPS and TNF-alpha mediated functional and phenotypic DC maturation, and constitutive activation of ERK was shown to lead to immune suppression mediated by TGF- β and T regulatory cells. On the other hand, blockage of the RAS-MEK-ERK pathway was associated with no significant impact on DC maturation mediated by LPS and TNF-alpha in one of the studies that demonstrated the importance of p38 [45].

In T cells, the role of MAPK has not yet been studied extensively, particularly in humans. Evidence exists that signaling through MEK-ERK is involved in the regulation of cytokine expression, lineage differentiation, cell proliferation, survival, and adhesion of activated T cells [53]. The pathway was found to be important in both regulation of T-cell receptor (TCR) signaling [27] and in functional responses of T cells [54-55]. It was recently shown that ERK signaling inhibits the generation of Th17 cells and affects their effector function in vivo [56].

VEGF/VEGFR

VEGF is primarily known as a mediator of tumor neovascularization [57] (Fig. 1). It is produced by most tumors in response to hypoxia inducible factor (HIF) or upregulation of oncogenes such as c-myc [58-60]. Increased serum levels are associated with a poor prognosis [61]. Different isoforms of VEGF (A-F) bind to transmembrane receptors (VEGFR 1-3), resulting in dimerization and activation through phosphorylation of tyrosine kinases. The role of VEGF in mediating tumor immune suppression has been recognized recently. Specifically, it was found that VEGF affects the maturation of DC precursors into mature DC capable of presenting tumor antigens and inducing a T cell response directed at tumor antigens [62-63]. VEGF promotes the expansion of these 'myeloid-derived suppressor cells' (MDSCs). Treatment of tumour-bearing mice with an anti-VEGF antibody at a dose that did not block tumour growth directly but decreased serum VEGF levels by 90%, led to an increase in mature DC numbers, improved DC function and resulted in a pronounced decrease in tumour growth that was associated with an enhanced tumour-specific CTL response [64-65].

The expression of VEGF negatively correlates with DC numbers in tumor tissue and peripheral blood of patients with different types of cancer, further supporting its role as a potential target for cancer therapy. VEGF can also directly attract immature myeloid cells from the bone marrow into the tumor site, where they develop into immature DC or

(immunosuppressive) tumor-associated macrophages (TAM), under the influence of VEGF and other immunosuppressive soluble factors in the tumor microenvironment such as TGF- β and IL-10. TAM are also directly recruited to the tumor by VEGF. Increased numbers of TAM and elevated levels of VEGF have been found to be associated with metastasis in a melanoma model [66]. It is important to note that in breast cancer patients, the intratumoral presence of TAM predicts outcomes [67-68] and genes characteristic of macrophages (e.g., CD68) have been included as prognostic markers [69]. VEGF can also impair the development of T cells from hematopoietic progenitor cells, thus further impairing immune responses [70]. Combined therapy with a GM-CSF-secreting tumor cell vaccine and VEGF inhibition by a chimeric adeno-associated viral vector expressing VEGFR-1 and VEGFR-2 led to improved survival in a B16 melanoma model. Increased numbers of activated DC and effector T cells were observed, whereas frequencies of CD4⁺CD25⁺ regulatory T cells were decreased in tumors of mice that were treated with the combination of VEGF-blockade and immunotherapy as compared to immunotherapy alone [71]. On the receptor level, recent studies have started to uncover the mechanism of VEGF/VEGFR-mediated immune suppression. VEGFR-2 seems to play an important role shown by a study by Huang et al [72]. The authors showed that Gr1⁺CD11b⁺ immature myeloid cells accumulated in the spleen, whereas CD4 and CD8 cells decreased in mice treated with VEGF; this effect could be completely reversed by blocking VEGFR-2. Furthermore, VEGF-mediated inhibition of T cell development in the thymus was also reversed by VEGFR-2 inhibition. Anti-VEGF has not been proven to improve tumor immune responses in cancer patients to date [73].

Small molecule inhibitors - targeted anticancer agents with increasingly recognized effects on the immune system

Many oncogenic signaling pathways and molecules are specifically targeted with small molecule inhibitors directed at the phosphorylation sites of tyrosine or serin-threonine kinases. Over 50 protein kinase inhibitors are in either preclinical or clinical development or have been approved by the Food and Drug Administration (FDA) for the treatment of several tumor types. Some of the inhibitors selectively inhibit a single kinase, such as the EGFR tyrosine kinase inhibitor gefitinib, resulting in downstream RAS pathway inhibition. Other small molecule inhibitors have multiple targets, such as imatinib, sorafenib and sunitinib (described in more detail below). While the remarkable clinical efficacy of imatinib has revolutionized the concept of selective tyrosine kinase inhibition, the majority of agents in development or clinical use in oncology are not specifically targeted at a driver mutation such as the bcr-abl or c-KIT [74-76]. However, clinical efficacy of targeted therapies can be improved by selecting a patient population based on mutational status of the targeted pathway, as is evident by the much higher response rate to gefitinib in NSCLC patients with somatic EGFR mutations [77] or patients with melanoma harboring mutations of c-KIT [78-79].

The impact of specific small molecule kinase inhibitors on immune cells is being increasingly recognized and is beginning to be systematically studied in vitro and in cancer patients. We will discuss four agents (imatinib, sorafenib, sunitinib and temsirolimus) which are approved by the regulatory agencies in the United States and in most European countries

and which have been studied sufficiently (or in the case of temsirolimus the close analogue rapamycin) with respect to their effects on immune cells to be instructive for the reader.

Imatinib

Imatinib represents the first small molecule inhibitor to be approved by the FDA for use in clinical practice. It was specifically developed to inhibit the tyrosine kinase associated with *abl* in the BCR-ABL fusion protein (the genetic aberration associated with the Philadelphia chromosome in chronic myeloid leukemia, CML). Its remarkable clinical efficacy in CML has changed the management of this disease. Although imatinib was specifically designed as an inhibitor of the above described tyrosine kinase in CML, it also inhibits c-KIT (CD117), PDGFR, the *fms*-like tyrosine-protein kinase 3 (FLT3), and the macrophage colony stimulating factor (m-CSF) receptor. Due to its inhibition of c-KIT, imatinib is now also used in clinical practice as standard first line treatment for metastatic, unresectable, c-KIT expressing gastrointestinal stromal tumors (GIST) and as adjuvant therapy after resection of GIST. It has also shown clinical activity in eosinophilia-associated chronic myeloproliferative disorders that are characterized by activating mutations of either the *PDGFRB* or the *PDGFRA* gene.

In most studies reported to date, imatinib has an inhibitory effect on T cells. In vitro studies showed that imatinib inhibits proliferation of T cells from healthy donors and imatinib treated CML patients [80-82]. Furthermore, expression of the early activation markers CD69 and CD25 was found to be reduced upon exposure to imatinib in vitro [80-82]. In another study, TCR-mediated activation and proliferation of primary T cells and antigen-specific CD8 T cell expansion were markedly reduced by imatinib in a dose-dependent manner [83]. In contrast, in the lymphocytic choriomeningitis virus (LCMV) model, primary T cell responses were not affected by imatinib, whereas memory cytotoxic T cell expansion was inhibited. The authors of the study point out that the impairment of memory T cells by imatinib might compromise the graft versus leukemia effect after allogeneic stem cell transplantation [84]. Reactivation of latent Varicella Zoster Virus (VZV) infection as a result of a compromised cell-mediated immunity (low CD4 cells) was seen in a subset of CML patients treated with imatinib [85]. Furthermore, it was shown that TCR-induced IL-2 production and T cell proliferation was diminished by pharmacological inhibition with imatinib and reduced Th-1 cytokines were detected in CML patients after treatment with imatinib [86-87]. This effect is mediated by inhibition of the TCR signaling pathway, leading to decreased phosphorylation of ZAP70, LAT and ERK in primary T cells [87]. Imatinib was also shown to inhibit Treg function through interference with the TCR signaling pathway mediated by decreased ZAP70 and LAT phosphorylation [88]. Another study revealed that c-KIT tyrosine kinase inhibition with imatinib results in impaired pro T and pro B cell development [89]. Conversely, CML-specific CD4 cells producing TNF- α were recently documented in CML patients who were in remission under imatinib. However, no direct mechanistic link was shown between the emergence of the T cells and imatinib treatment [90].

The effect of imatinib on DC is also controversial. While some studies have described a negative impact on their antigen presenting function, others found either no effect, or even

enhancement of antigen presenting capacity [80, 91-96]. Imatinib was found to increase the NK stimulatory capacity of DC in mice and lead to greater NK cell activation in patients with GIST, correlating with improved tumor response [97]. IFN- γ production by NK cells was an independent predictor of response to imatinib in patients with advanced GIST. Furthermore, a new type of cell, the CD11c^{int}B220⁺NK1.1⁺ IFN-producing killer dendritic cell (IKDC), was recently identified which may account for the immune effect of imatinib [98-99]. Conversely, a different group recently reported increased IL-10 production in macrophage cultures after imatinib treatment, and detected anti-inflammatory M2 type macrophages in immune infiltrates of GIST, suggesting a potential immunosuppressive effect of the drug [100].

Temsirolimus

In renal cell cancer, the Van Hippel Lindau gene (VHL), is mutated in 60 - 70% of cases, leading to its inactivation. VHL is a tumor suppressor gene which regulates the expression of hypoxia inducible factor (HIF), a transcription factor that controls a cell's response to low oxygen levels. The upregulation of HIF in VHL-deficient cells leads to activation of downstream targets such as VEGF-A, PDGF, and TGF- α , explaining its critical role in the tumorigenesis in renal cells. HIF is regulated by mTOR and therefore constitutes a rational molecular target in renal cell cancer. Temsirolimus is a rapamycin-analogue which blocks the kinase activity of mTOR by binding to FK506-binding protein-12 (FKBP12). The FDA approval of the small molecule inhibitor temsirolimus for the treatment of renal cell cancer with high risk features marks another important milestone in targeting an oncogenic pathway [101]. Although its anticancer activity was recognized over 20 years ago, rapamycin was primarily developed as an immunosuppressive agent to prevent allograft rejection in kidney transplants. While a large body of data exists in support of its immunosuppressive effects [19-21, 23-25], the exact mechanism of action is not fully understood. In addition to its inhibitory effect on T effector cells, rapamycin also promotes the expansion of CD4⁺ CD25^{high} Foxp3⁺ regulatory T cells [24-25]. It is unclear why treatment with temsirolimus, a soluble ester of rapamycin with identical immunosuppressive effects, is effective against renal cell cancer, a tumor that is known to be responsive to immunotherapy. Although the effect of temsirolimus on the immune system has not yet been investigated in detail, its similarity to rapamycin would suggest that it also leads to downregulation of a tumor-immune response which potentially counteracts its anticancer effect. This should be carefully considered when planning adjuvant studies using temsirolimus, as it may suppress an intrinsic immune response directed at micrometastases which may protect from cancer recurrence.

Sorafenib

Sorafenib is a multikinase agent that inhibits RAF as well as a number of other cellular targets such as VEGFR, PDGFR, FMS-like tyrosine kinase 3 (Flt-3), c-KIT), and RET receptor tyrosine kinases [102-103]. It is currently being assessed either as monotherapy or in combination with other agents in more than 300 clinical trials for a variety of cancers. The drug recently received FDA approval for use in advanced renal cell carcinoma [74] and hepatocellular carcinoma [75]. The effect of sorafenib on different types of immune cells has been investigated primarily in vitro. It was shown to cause reduced levels of co-

stimulatory and maturation markers on DC upon stimulation with LPS, resulting in a decreased capacity to stimulate allogeneic T cells in vitro and OVA specific T cells in vivo. This effect was mediated via the MAPK and PI3K/AKT pathways and via NF- κ B signaling, and was rapidly reversible after discontinuation of the drug [104]. Conversely, sorafenib counteracted the inhibitory effect of supernatants from a renal cell carcinoma cell line on the function of DC (as measured by stimulation of T cells in a MLR) [105]. Sorafenib was also shown to induce apoptosis in CD4⁺CD25⁺ regulatory T cells in vitro, an effect that was reversed by IL-2 [106]. Another report showed that sorafenib in vitro leads to irreversible inhibition of proliferation of human peripheral T cells at concentrations normally achieved in patients, whereas higher concentrations resulted in apoptosis [107]. Furthermore, downregulation of CD25 expression and production of IFN- γ by sorafenib was seen in human T cells following stimulation with PHA or α -CD3/ α -CD28, including T cells specific for the tumor antigen survivin. Unexpectedly, pERK expression was upregulated in T cells upon TCR stimulation, whereas it was downregulated after protein kinase C activation. This suggests that monitoring of pERK phosphorylation as previously proposed may not be an adequate biomarker for T cell function, as the effect of sorafenib on the MAPK pathway seems to depend on the nature of the T cell stimulus [108].

Sunitinib

Sunitinib is an oral receptor tyrosine kinase inhibitor that inhibits VEGFR, PDGFR, c-KIT, and Flt-3. It is FDA-approved for first line treatment of metastatic renal cell cancer and GIST after failure of therapy with imatinib [76, 109] and is currently being investigated in more than 300 clinical cancer trials.

The impact of sunitinib therapy on different types of immune cells has recently been investigated in renal cell cancer patients, giving rare insight into the in vivo effects of a small molecule tyrosine kinase inhibitor on the immune system in humans. Frequencies of CD3⁺CD4⁺CD25⁺ T regulatory cells, commonly elevated in renal cell cancer patients, were reduced after treatment with sunitinib. This reduction was accompanied by an increase in the frequencies of IFN- γ producing T cells and a decrease in IL-4 producing T cells (upon stimulation with α -CD3/ α -CD28), reversing the Th1/Th2 ratio from a type 2 to a type 1 [110], which is important for the generation of an effective anti-tumor response [110]. In a related study, the authors also found decreased frequencies of CD33⁺HLA-DR⁻ and CD15⁺CD14⁻ myeloid-derived suppressor cells (MDSC) and reduced numbers of CD3⁺CD4⁺CD25⁺Foxp3⁺ T regulatory cells in renal cell cancer patients treated with sunitinib [111]. In another study by the same group, the effect on MDSC seemed to be compartmentalized, as splenic, but not intratumoral, MDSC were reduced after treatment with sunitinib [112]. DC frequencies were assessed in renal cell cancer patients in a separate study after treatment with sunitinib. Myeloid DC subset-1 (MDC-1) were defined as CD11c^{high}, CD19⁻, CD14⁻, BDCA-1/CD1c⁺ MDC-1 and myeloid DC subset-2 were defined as CD11c⁺, CD14⁺, and BDCA-3⁺ [113]. Although myeloid cell frequencies were generally lower after sunitinib therapy, relative MDC-1 and MDC-2 frequencies were increased after treatment with the tyrosine kinase inhibitor. The increase was more pronounced in patients with tumor regression as compared to patients with stable disease or disease progression. In a mouse colon carcinoma model, sunitinib led to decreased numbers of Tregs and MDSC

and decreased immunosuppressive function of MDSC in the periphery of tumor bearing mice. Furthermore, expression of immunosuppressive cytokines and costimulatory molecules TGF- β , IL-10, CTLA-4, and PD-1 was decreased in tumor infiltrating lymphocytes along with an increase in IFN- γ and CTL function. This study suggested that c-KIT mediated the effects of sunitinib on MDSC [114]. Evidence also exists for STAT3 mediating the effect of sunitinib on MDSC. Another recent study showed a decrease in MDSC numbers and a downregulation of STAT5 tumor-associated MDSC of Renca-tumor bearing mice after treatment with sunitinib [115].

In vitro, the effects of sunitinib upon immune cells remain controversial. Hipp et al. reported no direct impact of sunitinib on DC maturation and function in vitro, and no effect on the frequency of vaccine-induced OVA₂₅₇₋₂₆₄ specific CD8 T cells in vivo [104]. In contrast to sorafenib, no effect on signaling pathways in DC was shown with sunitinib in that study. Consistent with the data in humans discussed above, numbers of Foxp3⁺CD25⁺ T cells among CD4 splenocytes were reduced in mice treated with a 14-day course of sunitinib [104]. The discussed in vitro and in vivo effects support a potential role for sunitinib in reversing an impaired T cell effector response. Contrary to this, Alfaro et al. found that sunitinib leads to decreased IL-12 production by DC matured with TNF- α , IFN- α , and poly I:C [105]. It is likely that some of the actions of sunitinib on immune cell populations are the result of direct blockage of receptor tyrosine kinases in immune cells, whereas others result from indirect effects, such as reduction in tumor burden and the associated reduced immunosuppressive environment or, for instance, the inhibition of T cell proliferation via induction of T regulatory cells. Little information is available to date on specific up- or downregulation of intracellular signaling molecules resulting from tyrosine kinase inhibition in immune cells, an area that should be fruitful for future research.

Conclusions and Future directions

There is little doubt that signaling molecules targeted by small molecule kinase inhibitors in tumor and immune cells have therapeutic implications. To date, the majority of data accumulated stems from cell culture systems, where different subsets of immune cells are exposed to inhibitors in vitro for relatively short periods. For cell-based immunotherapy protocols involving ex vivo manipulation of immune cells prior to adoptive transfer or vaccination, in vitro studies are certainly helpful with regard to the impact of small molecule inhibitors on the respective cell type. As illustrated in the discussion of selected small molecule inhibitors, the in vivo effects on immune cells of many of these inhibitors have yet to be studied in detail in cancer patients. The data presented in this review highlight that the impact on the immune system can be both immunosuppressive and immunostimulatory, depending upon the inhibitor itself, the cell type, or the activation status of the immune cell, among other variables. However, it is unknown whether an intrinsic tumor-specific immune response is affected by small molecule inhibitors in a beneficial or detrimental way. As an example, some studies suggest that memory CD8 T cell responses can be inhibited in vitro by imatinib and sorafenib [84, 108, 116], raising the at least theoretical concern for a potentially detrimental effect on the immune response. This highlights the need for comprehensive immune monitoring, including the quantification and functional analysis of regulatory and effector cells in cancer patients treated with small molecule inhibitors.

In both melanoma and renal cell cancer immune cells and their effector molecules play an important role in the natural course of the tumor and as potential targets of therapeutic intervention, and immune interventions have been an important treatment modality for many years. It is remarkable that for both tumors, kinase inhibitors have changed or likely will change the treatment paradigm in the last few years, with sorafenib, sunitinib, and temsirolimus all approved in renal cell cancer and the recent impressive response rates and swift progression through phase 1-3 trials of specific BRAF inhibitors in melanoma [117-118]. The interplay of effects that these agents have on both tumor and immune cells make it critical to incorporate systematic immune monitoring into future clinical trials using new targeted agents, and also to extend recent renal cell cancer studies using sunitinib to additional drugs in clinical use or development, including sorafenib, temsirolimus, BRAF inhibitors and MEK inhibitors. Immune monitoring should include assays testing T cell function such as ELISPOT for IFN- γ and granzyme, ELISA, as well as T cell proliferation and cytotoxicity assays. While the complexity of pathway networks make it challenging to design small molecule inhibitors with both anti-tumor and immunogenic properties, sunitinib appears to be a good candidate which could serve as a model agent in this regard. The limited data available to date suggest that sunitinib appears to have the most favorable immunomodulatory properties.

Prior to designing strategies to co-administer different kinase inhibitors with the goal of blocking complementary pathways and optimize the effect on immune cells, the immunogenic properties of the individual agents should be investigated in greater detail.

Some authors have suggested combining small molecule kinase inhibitors with immunotherapy, exploiting potential beneficial effects of small molecule inhibitors on the (presumably immunosuppressive) microenvironment [119]. In a mouse tumor model, the combination of sunitinib with an immune intervention (IL-12 and 4-1BB activation) resulted in improved survival compared to the immunotherapy alone [114]. Furthermore, it was recently shown that MAPK inhibition can reverse human melanoma immune evasion by rescuing downregulated melanocyte derived antigens, which are important for melanoma recognition by T cells [120]. Of particular relevance is the finding that T cell function was impaired by MAPK pathway interference with a MEK inhibitor, but not with PLX4032, a small molecule kinase inhibitor specific for BRAF^{V600E}, a mutant form of BRAF which is commonly expressed in melanoma. This highlights the potential for immunosuppressive effects of small molecule inhibitors and illustrates the importance of selecting inhibitors with a truly synergistic effect when used in vivo in combination with immunotherapies.

Executive Summary

1. Small molecule kinase inhibition of the MAPK, PI3K/Akt/mTOR, and VEGF/VEGFR pathways in oncology

- MAPK, PI3K/AKT/mTOR and VEGF/VEGFR have emerged as key targets for therapeutic intervention.
- Inhibitors of these pathways have been successfully incorporated into cancer treatments; examples are imatinib (FDA-approved for CML and GIST), sorafenib

(hepatocellular cancer), as well as sorafenib, sunitinib, and temsirolimus (renal cell carcinoma).

- Immune effects of these agents are increasingly recognized, likely as signaling pathways/molecules are shared between tumor cells and cells of the immune system.

2. Immunomodulatory effects of pathway inhibition: both immune stimulatory and immunosuppressive effects are present in different experimental systems

PI3K/Akt/mTOR pathway

- Central function in the shaping of an innate or adaptive immune response Inhibition of the pathway leads to:
 - Decreased T cell proliferation and T cell anergy
 - Induction of Treg, inhibition of B cells and NK cells.
 - Increased IL-12 production by mDC
 - Conflicting data regarding stimulatory versus inhibitory effects on mDCs, Th1 and Th17 cells

MAPK pathway inhibition

- In DC, both negative and positive impact observed depending upon TLR agonist and experimental system
- Important role in T cell receptor signaling and functional responses of T cells

VEGF inhibition

- Augments vaccine therapy in mice, associated with increased numbers of DC and T effector cells as well as decreased Treg frequencies.

3. Small molecule kinase inhibitors, frequently used in cancer treatments, have increasingly recognized effects on the immune system

Temsirolimus (inhibitor of mTOR)

- Immunosuppressive effects of rapamycin, an analogue of temsirolimus, are extensively documented.
- Temsirolimus, although no experimental data available yet, likely has considerable impact on immune responses as its mechanism of action (mTOR blockade) is very similar to rapamycin.

Imatinib (inhibitor of bcr-abl, c-KIT, PDGFR, FLT3 and m-CSF receptor)

- Largely inhibitory effect on T cells. Both stimulatory and down-regulating effects on antigen presenting capacity of DC have been described.

Sunitinib (inhibitor of c-KIT, VEGFR, PDGFR and Flt-3)

- In vivo effects on immune cells evaluated in humans, including in cancer patients

- Impact on the immune system most comprehensively analyzed among small molecule inhibitors in clinical use for cancer
- Overall immune stimulatory effect with Th1 skewing, DC activation, Treg and MDSC suppression.

Sorafenib (inhibitor of VEGFR-2, RAF, PDGFR Flt-3, c-KIT and RET)

- Ambiguous data, showing both immune stimulatory and immunosuppressive effects.

4. Future directions

- Immune effects of small molecule kinase inhibitors likely have therapeutic implications and should be explored further, not only to optimize future treatment regimens, but also to overcome potentially detrimental immunosuppressive effects of current therapies.
- Future strategies may also include the use of tyrosine kinase inhibitors for ex vivo manipulation of immune cells prior to adoptive transfer or vaccination, or for combinatorial approaches with immunotherapeutic modalities.

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Fig. 1.

Small molecule kinase inhibitors and their targets in tumor cells: Signal transduction cascades initiated by growth factors receptors such as epidermal growth factor receptor (EGFR), vascular endothelial growth factor receptors (VEGFRs, VEGFR-1, VEGFR-2, and VEGFR-3), platelet-derived growth factor receptor (PDGFR) alpha and beta, insulin-like growth factor receptor (IGFR) regulate cancer cell proliferation, metastasis, adhesion, apoptosis, invasion, and angiogenesis. The MAPK and PI3K/AKT/mTOR pathways shown here are prominent examples of the complex network of signaling pathways and are targeted by protein kinase inhibitors either directly (e.g. by Temsirolimus) or by agents acting mainly upstream, on the growth factor receptor level (sorafenib, sunitinib, imatinib).

EGFR: EGF receptor; ERK: Extracellular signal-regulated kinase; IGFR: IGF receptor; PDGFR: PDGF receptor; VEGFR: VEGF receptor.

Fig. 2.

Small molecule kinase inhibitors and their targets in T cells: Many signaling molecules and pathways targeted by small molecule kinase inhibitors are also present in T cells. Therefore, these inhibitors have the potential to mediate downstream effects such as cell proliferation and effector functions (cytokine production, cell lysis). Illustrated here are the T cell receptor (TCR)/peptide/MHC interface and some of the key signaling pathways associated with the TCR and other T cell surface receptors such as CD25 (the IL-2 receptor) and CD28 (costimulatory receptor).

ERK: Extracellular signal-regulated kinase; TCR: T-cell receptor.

Fig. 3.

Small molecule kinase inhibitors and their targets in antigen presenting cells: Many signaling molecules and pathways targeted by small molecule kinase inhibitors are also present in antigen presenting cells (monocytes, macrophages, Dendritic cells, B cells). Selected signaling pathways downstream of Toll Like Receptors (TLRs) located in the surface membrane and intracellular compartment membranes are illustrated. Downstream TLR signaling molecules such as MyD88 and TRAF6 can be directly targeted by protein kinase inhibitors such as sorafenib. These adaptor proteins/signaling molecules also interact with the PI3K/AKT/mTOR and the MAPK pathways. As a result, small molecule kinase inhibitors can modulate DC function such as cytokine production and expression of costimulatory molecules.

ERK: Extracellular signal-regulated kinase; TLR: Toll-like receptor.

Table 1

Classical and off targets of small-molecule protein kinase inhibitors.

	PI3K-AKT-mTOR pathway	MAPK pathway	VEGF-VEGFR pathway
<i>Classical target</i>	<i>Tumor cells</i>	<i>Tumor cells</i>	<i>Cell vasculature</i>
	Cell proliferation	Cell proliferation	Neoangiogenesis
	Cell survival	Cell survival	Endothelial permeability
	Apoptosis	Apoptosis	Vascular remodeling
	Invasion	Invasion	
	Metastasis	Metastasis	
<i>'Off' target immune cell</i>	<i>T cells</i>		
Pathway inhibition results in:	↓ cell proliferation ↓ IL-2 production Anergy Differentiation of CD4 cells into CD4 ⁺ CD25 ⁺ FoxP3 ⁺ Treg cells Regulation of T cell effector versus regulatory lineage commitment Suppression of Th17 cell proliferation Generation of memory T cells with strong tumor activity	↑ Th1 polarization ↑ TGF- β Inhibition of Th17 cell generation ↑ Th2 polarization	↓ Treg numbers
Pathway upregulation results in:	Impaired T-cell development	Induction of Tregs ↑ TGF- β Inhibition of Th17 cell generation ↑ Th2 polarization	Impaired T-cell development ↓ CD4 and CDS cell numbers
<i>'Off' target immune cell</i>	<i>Dendritic cells</i>		
Pathway inhibition results in:	↓ and ↑ cytokine production ↓ expression of costimulatory molecules ↓ phagocytosis ↓ endocytosis Survival Generation of Th1 and Th17 response induced by DC with ↓ IL-10 and IL-12	↓ expression of co stimulatory molecules (inhibition of p38) ↑ functional and phenotypic DC maturation (inhibition of MEK)	↑ DC numbers Improved DC function
Pathway upregulation results in:	—	—	Attraction of immature myeloid cells from bone marrow into tumor site ↑ TAM in tumors Maturation of immature myeloid cells into TAMs

	PI3K-AKT-mTOR pathway	MAPK pathway	VEGF-VEGFR pathway
<i>Classical target</i>	<i>Tumor cells</i>	<i>Tumor cells</i>	<i>Cell vasculature</i>
			<p>↓ maturation of DC precursors into mature DCs</p> <p>Negative correlation with presence of DCs in tumor tissue</p> <p>Accumulation of Gr1⁺CD11b⁺ immature myeloid cells in spleen DO Dendritic cell; TAM: Tumor-associated macrophage.</p>

Table 2

Molecular targets and approved indications of small-molecule protein kinase inhibitors.

Inhibitor	Molecular targets	US FDA-approved cancer indications
Imatinib	c-KIT (CD117)	CML
	PDGFRA/B	GIST
	Flt-3	
	m-CSF receptor	
Sorafenib	B-RAF, C-RAF	Renal cell carcinoma
	VEGFR 2,3	Hepatocellular carcinoma
	PDGFR	
	Flt-3	
	c-KIT	
	FGFR1	
	RET	
Sunitinib	VEGFR1–3	Renal cell carcinoma
	Flt-3	GIST
	c-KIT	
	PDGFR	
	RET	
Temsirolimus	mTOR	Renal cell carcinomaCML; Chronic myeloid leukemia; FGFR; FGF receptor; Flt: FMS-like tyrosine kinase; GIST: Gastrointestinal stromal tumor; m-CSF; Macrophage colony stimulating factor; PDGFR: PDGF receptor; VEGFR: VEGF receptor.