Cancer-targeted oncolytic adenoviruses for modulation of the immune system

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Abstract:

Adenovirus is one of the most commonly used vectors for gene therapy and it is the first approved virus-derived drug for treatment of cancer. As an oncolytic agent, it can induce lysis of infected cells, but it can also engage the immune system, promoting activation and maturation of antigen-presenting cells (APCs). In essence, oncolysis combined with the associated immunostimulatory actions result in a "personalized *in situ* vaccine" for each patient. In order to take full advantage of these features, we should try to understand how adenovirus interacts with the immune system, what are the receptors involved in triggering subsequent signals and which kind of responses they elicit. Tackling these questions will give us further insight in how to manipulate adenovirus-mediated immune responses for enhancement of anti-tumor efficacy.

In this review, we first highlight how oncolytic adenovirus interacts with the innate immune system and its receptors such as Toll-like receptors, nucleotide-binding and oligomerization domain (NOD)-like receptors and other immune sensors. Then we describe the effect of these interactions on the adaptive immune system and its cells, especially B and T lymphocytes. Finally, we summarize the most significant preclinical and clinical results in the field of gene therapy where researchers have engineered adenovirus to manipulate the host immune system by expressing cytokines and signaling mediators.

Keywods: Oncolytic adenovirus, Gene Therapy, Innate immune system, Adaptive immune system, pattern recognition receptors, Armed oncolytic adenovirus, Toll-like receptors

Immune system and adenovirus: toxicity or efficacy?

Innate immune responses are triggered when pattern-recognition receptors (PRRs) recognize specific conserved molecular patterns on pathogens. Several classes of PRRs, including Toll-like receptors, NOD-like receptors and various cytoplasmic receptors, recognize distinct microbial components and directly activate immune cells. Exposure of immune cells to the ligands of these receptors activates intracellular signaling cascades that rapidly induce the expression of a variety of both overlapping and unique genes involved in the ensuing immune responses. Activation of PRRs results in the production of large amounts of type I interferons and several other proinflammatory cytokines [1-2]. These responses are important in controlling pathogen replication and they also provide a critical initiation signal which modulates and controls the adaptive immune response [3-4].

Adenovirus is the most commonly used gene therapy vector. It is mostly used in the context of genetic diseases or cancer, or for vaccination [5-18]. Adenoviruses interact with multiple PRRs (**Figure 1**) eliciting a robust cytokines response [19-20]. With regard to conventional gene delivery, virus-induced inflammation can lead to premature vector elimination, and at the doses required for sufficient liver transduction adenovirus can be toxic [19-20]. We and others have hypothesized that we can harness the natural propensity of adenovirus to activate the innate and adaptive arms of the immune system against the tumor. While oncolysis would provide initial cell killing with an associated release of a variety of tumor associated antigens (TAAs), immunological recognition of the virus would provide the required “danger signal” to mature and stimulate antigen presenting cells (APCs, eg. dendritic cells, DCs). Along this line, investigators have recently demonstrated that oncolytic adenoviruses have the capability to stimulate a specific antitumor immune response [21-23].

We hypothesized that oncolysis driven by the viruses plays a double role: 1) lysis of tumor cells resulting in a source of tumor-associated antigens (TAAs) 2) “danger signals” to the immune system to enhance cross-priming of antigen presenting cells (APCs) rather than cross-tolerization (**Figure 2**). Moreover,

oncolytic adenovirus can be armed to specifically stimulate pathways of the immune system for an enhanced antitumor response (**Figure 3**). Importantly, cross-presentation and epitope spreading has been demonstrated, suggesting that cellular anti-viral responses can result in immunity against TAA as well [24]. In particular, memory responses against TAA may be important for long term survival of treated patients [25-27]. Even though the main goal of arming is typically enhancing efficacy, also safety could be increased, if lower doses can be used, or if the arming device results in less systemic dissemination of the virus.

Thus, for optimal modulation of the immune system against the tumor we need to understand what are the key receptors the adenovirus interacts with.

Adenovirus interaction with the innate immune system

Adenovirus and TLRs

The innate immune system recognizes infections through PRRs that detect conserved microbial components called pathogen-associated molecular patterns (PAMPs). PAMPs represent molecules vital for microbial survival such as flagellin, nucleic acid structures unique to bacteria and viruses (CpG DNA, dsRNA), and bacterial cell-wall components such as lipopolysaccharide (LPS), lipoteichoic acid, and peptidoglycan [28]. More than a decade ago, the protein Toll was identified as a key regulator of innate immune signaling in Drosophila melanogaster [29]. Since then, mammalian Toll-like receptors (TLRs) have been recognized for their ability to sense a wide array of microbial and self-ligands at the cell surface and within endosomes [30]. TLRs comprise of 11 different receptors that recognize motifs found on a wide range of pathogens, and activation of TLRs results in the production of large amounts of type I interferons and several pro-inflammatory cytokines. These cytokine responses are important in controlling pathogen replication and they provide an initiation signal for the adaptive immune response.

Adenovirus capsids activate the innate immune system through mechanisms that do not involve viral replication or gene expression [19, 31-36]. For example, following exposure to UV-inactivated adenovirus, human peripheral blood mononuclear cells (PBMCs) rapidly produce many cytokines, including IL-6, IL-

1*β*, granulocyte monocyte colony stimulating factor (GMCSF), IL-8, and TNF-*α* [37]. A similar cytokine profile is also found in the serum of mice and non-human primates following intravenous administration of adenovirus [19, 34, 36, 38]. Also dendritic cells produce large amounts of cytokines and type I interferons immediately after infection with adenovirus [38-40].

Recent literature has started to link these phenomena with specific stimulation of TLRs (**Figure 1**). We showed that adenovirus DNA triggers innate responses in part via TLR9 [36], one of the PRRs located in the endosome and responsible for detecting double-stranded DNA. It was also observed that mice lacking the Myeloid differentiation primary response gene 88 (MyD88), a universal adapter protein used by all TLRs (except TLR3) to activate the transcription factor NF-κB and trigger the immune response, showed significantly reduced cytokine secretion when challenged with high dose of adenovirus, indicating that TLR9 is involved in adenovirus recognition [41]. Later, it was shown that TLR9 and TLR2 are both required to trigger the MAP-kinase dependent inflammatory response characteristic of adenoviral vector administration [42]. This data was confirmed in 2010 by Suzuki and colleagues who showed that TLR2 and TLR9 are responsible for induction of cytokines and gene silencing in mice following adenoviral vector administration [43]. Interestingly, they showed that when *LacZ*-expressing adenoviral vectors were administered intravenously in MyD88 knock-out mice (lacking almost all TLRs-mediated responses) not only was cytokine secretion significantly lower but also transgene expression was significantly prolonged due to absence of a normal immune response [43]. These results highlighted the importance of the TLRs in initiatiation of the innate immune response but also in modulation and shaping of the adaptive response [44]

Adenovirus and cytosolic sensors

Nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) comprise a large family of intracellular PRRs that are characterized by the presence of a conserved NOD [45]. Together with retinoid acid-inducible gene I (RIG-I)-like receptors (RLRs), NODs detect microbial components in the cytosol [46-47]. NLRs containing NOD1 and NOD2 sense the dipeptide γ-D-glutamyl-

meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP), respectively [48]. Both are breakdown products of bacterial peptidoglycan. NOD1 is ubiquitously expressed and occurs in most NLRs, while NOD2 are restricted to monocytes, macrophages, dendritic cells and intestinal Paneth cells [49].

Very little is known to date about the correlation between adenovirus and NOD-like receptors recognition. Recently, Suzuki and colleagues showed reduced proinflammatory cytokine secretion and significantly higher transgene expression when NOD2 KO mice were challenged with non-replicating adenoviral vectors. Moreover, experiments in NOD2/MyD88 double KO mice showed further reduced innate responses to adenoviral vectors compared to responses in singly deleted mice, indicating that NOD2 signaling contributes to the innate responses independently of MyD88 [50].

The NALP proteins are cytoplasmic NLRs [51]. The best characterized is NALP3 (also called cryopyrin, NLRP3), which senses exogenous and host ligands such as bacterial peptidoglycan, ATP or uric acid [52]. NALP3 recruits, via the adaptor protein ASC, the inflammatory caspase-1 into a molecular complex termed the NALP-inflammasome [52]. Once activated, caspase-1 processes pro-IL-1b and pro-IL-18 to their active and secreted forms. Other NLRs that are known to form IL-1b-processing inflammasomes include NALP1 and IPAF, the latter of which directly activates caspase-1 in response to bacterial flagellin [53].

In an elegant study, Petrilli and colleagues showed that internalized adenovirus DNA triggers an innate immune response dependent on the activation of the NALP-inflammasome complex (NALP3 and ASC) [54]. Already earlier, Nociari and colleagues demonstrated that TLR-independent adenovirus DNA recognition led to IRF3 activation and type I IFN and proinflammatory cytokine expression [55]. Also, cytosolic DNA recognized by AIM2 was shown to induce IL-1b secretion through a caspase-1-dependent inflammasome pathway [56].

Among other cytosolic sensors, retinoic acid-induced gene I (RIG-I) plays an important role in promoting IFN production in response to pathogens. RIG-I-like receptors (RLRs), such as RIG-I, MDA5 and LGP2, feature a helicase domain that senses RNA strands. These proteins can distinguish foreign (i.e. viral) RNAs from self-RNAs that feature a 5´ modification. The N-terminal domain contains a caspase activation and recruitment domains (CARD) able to

signal through mitochondrial antiviral signaling protiens (MAVS) [57]. Interestingly, in addition to viral RNA, also DNA is sensed through RLRs. Chiu and colleagues demonstrated that transfection of human cells with different DNA forms, including poly(dA-dT), PCR fragments or DNA oligos activated IRF3. This resulted in the production of IFN- β. The effect was inhibited when RIG-I or MAVS were silenced into the cell line, suggesting an important role of these sensors in the response against viral DNA [58].

Adenovirus and Complement

Together with antibodies, complement plays a role in vector opsonization and clearance. Adenovirus has been shown to activate complement via antibodies in individuals having pre-existing immunity [59]. Also, direct binding to C3-derived fragments has been reported [59]. Such interactions can probably contribute to inflammatory responses associated with virotherapy [60]. In fact, steps have been taken to mask complement-mediated recognition of adenovirus particles with the goal of increasing vector efficacy [60]. Thrombocytopenia is caused by interactions between adenoviral particles and the coagulation system, resulting in platelet activation, binding to endothelial cell surfaces, and formation of platelet-leukocyte aggregates [61]. Finally, Ad vectors directly and indirectly activate endothelial cells primarily via recognition of virus capsid [31].

Adenovirus modification for enhanced innate immunity

Considering how articulated and complex innate sensing of adenovirus is, and considering how various cellular sensors can influence and shape the long-lasting adaptive response, we believe that the innate arm of the immune system can be utilized in the treatment of cancer in an approach that could be defined as ImmunoVirotherapy. It is presently being discovered that we can exploit oncolytic viruses not only for their killing capacity but also for their ability to activate receptors of the innate immune system, translating into specific anti-tumor responses. However, in spite of their robust ability to stimulate innate sensors, human data suggests that oncolytic adenoviruses *per se* are not

usually able to elicit immune responses capable of fully eradicating metastatic tumors, possibly due the highly immune suppressive nature of advanced cancers

Hence, researchers have now entered a new era where they are genetically manipulating adenoviruses to enhance activation of some specific innate receptors. In this respect, an interesting approach is generation of an oncolytic adenovirus expressing the pan-TLR adaptor protein MyD88. Tantalizingly, intratumoral injection of Ad-MyD88 into established tumor masses enhanced adaptive immune responses and inhibited local tumor immunosuppression, resulting in significantly inhibited local and systemic growth of multiple tumor types. Further, Ad-MyD88 infection of primary human dendritic cells, tumor-associated fibroblasts, and colorectal carcinoma cells elicited significant Th1-type cytokine responses, resulting in enhanced tumor cell lysis and expansion of human tumor antigen–specific T cells [62].

Adenovirus interaction with the adaptive arm of the immune system

Adenovirus and B cells

As consequence of the activation of the innate recognition receptors, a rapid increase in several cytokines, particularly IL-6, IFN-α/β, RANTES, IL-12 (p40), IL-5, G-CSF and GM-CSF is observed [38]. Furthermore, a complex set of interactions between the innate and the adaptive immune system results in activation of CD4+ and CD8+ T cells, and B cells [38]. Type I IFN signaling is important for T help-dependent antibody formation by B cells. IFNs also induce DC maturation by up-regulating co-stimulatory molecules such as CD80, CD86, and CD40. Neutralizing antibodies against IFN-α and IFN-β have been found to be effective in blocking both innate as well as adaptive immune responses to viral vectors [63].

Understanding the humoral immune response to adenovirus is of importance for gene delivery for at least two reasons: 1) presence and prevalence of neutralizing antibodies to adenovirus (NAB) might hinder the efficiency of the transduction while 2) the presence of NAB might influence the outcome of the therapy at post-tranductions steps.Epidemiological studies on NAB in different

populations have shown that mostly people in the world already carry some levels of antibodies in their serum, although some geographical variation does occur [64]. It however, needs to be emphasized that the specificity and immunogenicity of adenovirus type 5 NAB elicited by natural infection or by immunization with an adenoviral vector is quantitative and qualitative different [65]. In this study, 1904 participants were enrolled in a cross-sectional serological survey at seven sites in Africa, Brazil, and Thailand to assess neutralizing antibodies (NAB) for adenovirus types Ad5, Ad6, Ad26 and Ad36. Clinical trial samples were used to assess NAB titers from the US and Europe. The proportions of participants that were negative were 14.8% (Ad5), 31.5% (Ad6); 41.2% (Ad26) and 53.6% (Ad36). The study was conducted to correlate high Ad5 titers and the inefficiency of Ad5-based vaccine for HIV.

It would be very interesting to conduct a similar study correlating the efficacy of oncolytic adenovirus treatment in terms of overall survival (OS) or progression free survival (PFS) with the pre-existing NAB presence. Thus far, we have not seen correlation between oncolytic virus treated patients and efficacy [66-67]. Interestingly, we saw lower NAB levels than have been reported in previous studies. One month after the treatment high NABs against Ad5, an RGD-4C modified Ad5 capsid, or a Ad5/3 chimeric capsid were seen in only < 20, < 50 and < 20 % of patients respectively. In other words, low or negative titers were present in 80, 50 and 80% of patients. Differences between different reports could be due to geographical factors, the patients studied (cancer versus healthy), methodological issues (sensitivity and specificity of the test used) or a combination thereof.

Adenovirus and T cells

CD4+ and CD8+ T cells cross-reactive against different adenovirus serotypes have been found among human peripheral blood mononuclear cells (PBMCs) [68-69]. Adenovirus-specific CD4+ T cells often recognize epitopes conserved among different serotypes, with the majority of people developing a long-lived memory response.

Further, adenovirus-specific secretion of IFN-γ from PBMCs has been reported to occur within 12 hours of exposure, suggesting prior activation of

adenovirus-specific CD8+ cells. Transduction of APCs by adenoviruses contributes to CD8+ responses, which can be directed against both viral gene and transgene products and are dependent on CD4+ help [70].

It has in fact been demonstrated that already adenovirus *per se*, given its ability to interact with a variety of receptors of the innate immune system, is able to trigger a T cell immune response in context of cancer therapy. Tuve and colleagues, using a model of syngeneic mammary-cancer have shown that intratumoral injection of replication-deficient, transgene-devoid adenovirus induces responses at two different anatomical sites: the tumor-draining lymph nodes and the tumor microenvironment. The lymph nodes supported the generation of both *neu*- and virus-specific T effector cells, while inside the tumor microenvironment only adenovirus-specific T cells expanded. Importantly, Ad-specific T cells were anti-tumor-reactive despite the presence of active regulatory T cell-mediated immune tolerance inside tumors. Moreover, efficacy was increased by pre-immunization regardless of NAB [71].

The structure of the adenovirus features a plethora of immunogenic signals that are sensed by the innate immunity and translated into adaptive responses. For this reason, adenoviral particles can act as adjuvant becoming a powerful cancer vaccine platform. We further developed this concept and demonstrated that, even in absence of oncolysis, the physical combination of virions and MHC-I epitopes results in antigen-specific responses. Hence, peptide-coated conditionally replicating adenoviruses (PeptiCRAds) are able to stimulate dendritic cells and activate antigen specific T-cells resulting in reduced growth of treated and non-treated melanomas. We were also able to confirm this results in humanized mice, which represents the most advanced model available to study the synergy between oncolytic adenoviruses and the immune system [72]. Our results suggest that oncolysis is not necessary for anti-tumor activity, however we believe that it provides important additional pro-inflammatory stimuli to the immune system as proved by our studies in humanized mice.

Syrian hamsters provide key advantages when studying oncolysis due to the ability of human adenoviruses to replicate into hamster tumor cells. By using this model, Wang and colleagues were able to show that the efficacy of oncolytic adenoviruses is largely mediated by T-lymphocytes. They

demonstrated that viral replication inside tumors is enhanced by depletion of T-cells, however, this abrogates the efficacy of virus injections. Hence, the presence of T-lymphocytes is necessary for the anti-tumor efficacy of oncolytic adenoviruses in this model [73]. Siurala and colleagues also investigated the synergy between oncolytic adenoviruses and lymphocytes. They observed that the combination of oncolytic Ad5-D24 with adoptive transfer of tumor infiltrating lymphocytes (TILs) reduced the growth of HapT1 pancreatic cancer in syngeneic Syrian hamsters [74].

We have treated more than 200 cancer patients with armed and un-armed oncolytic adenoviruses, and have observed activation of CD8+ T cells against both virus and tumor epitopes [75-80]. In addition, we have recently described different factors that might predict the response of patients to oncolytic virotherapy. We found that administration route, absence of liver metastasis or low neutrophil-to-lymphocytes ratio were significantly correlated with a better prognosis [81]. Thus, our data suggests that adenovirus can be a useful platform for combining immunotherapy with gene therapy as "immunovirotherapy".

Oncolytic Adenovirus Armed with Immunomodulatory Transgenes

In spite of emerging data showing induction of anti-tumor specific immune response elicited by oncolytic adenoviruses *per se*, a strong anti-tumor immune response capable of complete rejection and eradication of advanced tumors is rarely seen. Tumors initiate from normal tissues and thus most TAA are resemble self-antigens which results in lower immunogenicity in comparison to heterologous epitopes such as PAMP [82-83]. Further, since tumors typically grow over a decade of more in the presence of an intact immune system, a tremendous amount of immunoediting (ablation of immunogenic clones) has usually occurred [84-85]. Also, as mutations accumulate, and tumor cells resemble normal cell less and less, an increasing amount of immune suppression is required [86-88]. Thus, the biggest challenge for cancer immunotherapy in general and immunovirotherapy in particular could be manipulation of the tumor microenvironment in favor of immune responses rather than tolerance. In this

regard, encouraging results have been achieved using oncolytic adenoviruses armed with transgenes for modulation of both the innate and adaptive immune systems (Table 1). Moreover, viruses encoding monoclonal antibodies, such as anti-HER2 Trastuzumab, have been evaluated. The main advantages of this approach are intra-tumor production and decreased side-effects caused by the bio-drugs expressed by the oncolytic vector [89].

Cytokine-expressing adenoviruses

Cytokines are used by the immune system for cross-talk between different cell types and are thus easily harnessed as arming devices. One widely used cytokine in this respect is interleukin 12 (IL-12) [90-105]. This cytokine is an interleukin naturally produced by antigen presenting cells (APCs) in response to antigenic stimulation. As a consequence of interaction with its receptors (IL-12R-β1 and IL-12R-β2) L-12 activates natural killer cells (NK) and T lymphocytes (T cells) enhancing their cytotoxic activity. T and NK cells produce IFN-γ in response of IL-12 activation. Oncolytic adenoviruses expressing IL-12 have demonstrated to enhance T cell and NK activation several tumor model in mice and human [90-91, 93-109].

One interesting report focused on Syrian hamsters, which is one of the few models considered semi-permissive for human adenovirus [110]. An oncolytic adenovirus expressing IL-12 driven by the viral E3 promoter was capable of curing syngeneic pancreatic tumor in conjunction with an anti-tumor immune response measurable by T cell proliferation[111]. This work also suggested partial cross-reactivity between mouse and Syrian hamster cytokines since they used murine Il-12. In another study, Gabaglia *et al*. reported that the treatment of human PC3 prostate xenograft or TRAMP-C1 tumors with the combination Ad5IL-12 and mifepristone produced significantly better therapeutic efficacy in comparison to controls [112]. . In particular, they found that combination therapy increased the capacity of tumor sentinel lymph node lymphocytes to produce granzyme B in response to tumor cells. Finally, combination therapy groups had fewer CD4+/FoxP3+ T regulatory cells in local nodes.

A clinical trial using an IL-12 expressing adenovirus reported twenty-one patients (nine with primary liver, five with colorectal, and seven with pancreatic cancers) treated with a total of 44 injections. Ad.IL-12 was well tolerated, and dose-limiting toxicity was not reached, nor were adverse events cumulative. Frequent but transient adverse reactions, including fever, malaise, sweating, and lymphopenia, seemed to be related to vector injection rather than to transgene expression.. In four of 10 assessable patients, a significant increase in tumor infiltration by effector immune cells was apparent. A partial objective remission of an injected tumor mass was observed in a patient with hepatocellular carcinoma. Stable disease was observed in 29% of patients, mainly those with primary liver cancer [113].

Granulocyte-macrophage colony-stimulating factor (GMCSF) is among the most potent inducers of anti-tumor immunity [114]. It acts through several mechanisms, including direct recruitment of natural killers (NK) and APCs such as dendritic cells (DC) [115-116]. GMCSF can also specifically activate DCs at the tumor site to increase their expression of co-stimulatory molecules to enhance cross-priming and T cell activation rather than cross-tolerance. We showed that Syrian hamsters challenged with a syngeneic pancreatic tumor developed, after treatment with the GMCSF-expressing virus, a specific anti-tumor response capable of protecting animals from successive challenge by the same tumor but not by different tumors [117]. Since a virus armed with human GMSCF was more immunogenic than an unarmed virus, these results also suggested that human GMCSF is active in Syrian hamsters. Importantly, preclinical results were followed up by treatment of humans, resulting in data suggesting induction of a tumor-specific immune response also cancer patients, as measured by ELISPOT and pentamer staining [117-118]. A recent study evaluated the efficacy of an oncolytic (D24 mutation) and chimeric Ad5/3 virus expressing GMCSF in combination with standard-care chemotherapy against mesothelioma[119]. Unfortunately, evaluating the immunological effects of the combination was not possible since the study featured a immune deficient xenograft murine model. Nevertheless, the researchers found that the oncolytic adenovirus could induce immunogenic cell death *in vitro* suggesting that the oncolytic activity might re-shape the tumor microenvironment.

IL-23 is a cytokine similar to IL-12 and in fact they share their p40 subunit. However, IL-23 has a preference for memory CD4+ T cell [120-121]. Recently, IL-23 together with IL-6 and TGF-b1 have been implicated in the mechanism that stimulates naive CD4+ T cells to differentiate into Th17 cells, which are distinct from classical Th1 and Th2 cells [122]. Th17 cells produce IL-17, a proinflammatory cytokine that enhances T cell priming and stimulates the production of proinflammatory molecules [123]. Reay and colleagues showed that three intratumoral injections of adenovirus expressing IL-23 significantly increased animal survival and resulted in complete rejection of 40% of tumors, with subsequent generation of protective immunity and tumor-specific cytotoxic T lymphocytes. In addition, they showed that the antitumor activity of IL-23 was independent of IL-17, perforin and Fas-ligand, but dependent on interferon-γ, CD4 and CD8 T cells [124].

Interferon -expressing adenovirus

Interferons (IFNs) are small protein made and released by the host cell to counteract the effect of pathogens, in particular viruses. IFNs are roughly divided into two subclasses: type I (*alpha*, α and *beta*, β) [125] and type II (also called late IFNs) such as INF-γ [126]. There is extensive data showing that IFNs are not only important in protection of normal tissues against pathogens, but they have also been shown to have anti-tumor activity directly on tumor cells and through activation of the immune system [127-128].

Although generating an adenovirus that expresses IFNs might seem counterintuitive due to the anti-viral activity of the latter, rationale is present due to near-universal deficiency of tumors to IFN signaling [129-130]. Thus, arming with IFN can increase the therapeutic window between actions on normal and tumor tissues and has been successfully used in several cancer type [131]. Interestingly, Santodonato and colleagues showed also anti-tumor activity in IFN-resistant tumors in mice when treated with an IFN-alpha expressing adenovirus [132]. Interestingly, it has been also reported efficacy and anti-tumor immunity with IFN-α expressing adenovirus even in very difficult tumors as

pancreatic cancer [133]. Similar results were also obtained in Syrian hamsters[133]. More recently it has been proposed that anti-tumor immunity can depend on the route of administration [134] and that IFN-α resistant tumors can be killed throughtumor immunity, oncolysis and autophagy [135]. The efficacy of an adenovirus encoding for INF-γ has been evaluated in a phase II clinical trial in patients with cutaneous B-cell lymphomas (CBCL). Complete or partial responses were observed in 85% of patients and one patient experienced stable disease. In addition, all skin biopses showed a decrease of the lymphoid infiltrate in the lesions [136]. The same IFN-γ armed adenovirus (TG1042) was tested in combination with adoptive T-cell transfer therapy in 13 patients with unresectable stage III or stage IV melanoma. Three patients presented a complete regression, two patients experienced partial response and one patient presented a stable disease; the median overall survival was 21.1 months [137].

An alternative but equally attractive approach is represented by expression of IFN-beta. Already in 2001 Odaka et al. showed that a non-replicating adenovirus expressing murine IFN-β was able to eradicate intraperitoneal and distant syngeneic mesothelioma tumors by inducing anti-tumor immunity, as shown by reactive CD8+ T cells and loss of activity in group of mice depleted of CD8+ T cells [138]. [139]. An interesting comparison between human and mouse IFN-beta expressing adenovirus in different tumor models was reported by Qin *et al*. [140]. Although many actions of IFN are through the adaptive arm of the immune system, an interesting role has also been proposed for macrophages [141]. A promising proof-of-concept study was performed by Park *et al*. who reported that a combination of oncolytic adenovirus Ad5D24RGD and a non-replicating adenovirus coding for IFN-b resulted in a high local concentration of IFN-beta. Importantly, local release of tumor antigens by oncolysis induced a strong antitumor immune response[142]. These preclinical reports have been followed up in several clinical trials in human patients [143-146].

Surface Protein expressing adenoviruses

The basic theories of immunology predict that when an antigen-presenting cell (APC) such as a dendritic cell (DC) is presenting an antigen to a T cell, it has the ability to decide whether or not there will be immune response or anergy of that specific cell. Normally, peptides derived from endogenously expressed proteins are presented by APC in the context of MHC class I (MHC I) to CD8+ T cells, whereas peptides obtained from exogenously derived proteins are normally loaded onto MHC class II (MHC II) for presentation to CD4+ T cells. However, exogenous antigens can be also loaded onto MHC I for ‘‘cross-presentation’’ to CD8+ T cells [147]. In tumor-draining lymph nodes both cross-priming and cross-tolerization have been reported, tumor antigen–specific T-cell proliferation has been detected, but the numbers of T cells proliferating are typically too low, and therefore the overall effect of CD8+ T-cell activation does not always result in inhibition of tumor outgrowth [148].

To enhance T cell activation it has been proposed to over-express co-stimulatory factors that act directly on T cells. CD154, better known as CD40L, is one of the most used. Normally it binds to CD40 on APC, which leads to many effects depending on the target cell type. In general, CD40L plays the role of a co-stimulatory molecule and induces activation in APC in association with T cell receptor stimulation by MHC molecules on the APC. In our laboratory we have generated an oncolytic virus expressing CD40L. This approach has shown remarkable efficacy in animal models as well as good safety and evidence of activity in human patients [149-150].

A similar approach is the expression of CD80, which is also called B7-1. This is a membrane protein especially expressed by B cells, monocyte and APCs, that provides a powerful co-stimulatory factor for T cell activation and survival. B7-1 is the ligand for CD28 and for CTLA-4. Along this line it has been shown that an adenovirus expressing IL-7/B7.1 induces rejection of transplanted tumors in mouse model[151]. The authors conclude suggesting that cancer vaccines can be effective against “minimal residual disease” but additional experimental procedures must be found against established non-transplanted tumors. A similar approach has been also described in the same year by a different group that generated an adenovirus expressing IL-12/B7.1[152]. They showed that the efficacy of this virus was dependent on NK cells as well as T cells, and loss of efficacy was in fact observed in NK- or T cell- depleted

animals. Moreover they showed that the efficacy of this virus was further enhanced by combination with radiotherapy [152]. More recently, a virus expressing a newly discovered member of B7 family, B7-H3, was reported. The mouse protein shares about 88% amino acid identity with the human. Unlike B7-H1 and B7-H2, its mRNA is broadly expressed in lymphoid and non-lymphoid organs[153]. B7-H3 has been shown to costimulate the proliferation of CD4+ and CD8+ T-cells and to stimulate interferon-γ (IFN-γ) production and cytolytic T-cell activity[154]. A study also demonstrates that adenoviral B7-H3 transfer is able to induce a specific cellular antitumor immune response leading to primary tumor regression and reduction of secondary metastasis *in vivo* [155].

Final Remarks

For the past decade, many gene therapy approaches have attempted to fight, avoid or suppress the immune system in order to retain efficacy similar to seen in nude mouse models. However, many investigators in the cancer field have realized that an alternative approach could be to ally with it to stimulate and shape the immune response towards the tumor. In the context of cancer therapy, adenovirus is an attractive agent due to its extraordinary ability to interact with different pathways of the innate and adaptive immune system.

Not only is there scientific interest in elucidating how different wild type viruses or their therapeutically-aimed derivatives induce different types of immune responses, but this information also has significant applied potential. For example, it was recently proposed that in contrast to the commonly used adenovirus serotypes 5 or 35, vectors based on serotype 28 suppressed proinflammatory cytokine secretion while eliciting strong type I interferon secretion, ultimately resulting in greater vaccination efficacy [156]. Thus, the quality rather than the quantity of the immune response could be an important determinant of the overall outcome. The authors speculated that the differential TLR sensing and signaling by this virus in comparison with the other serotypes in the study resulted in optimal cytokine secretion by DCs and subsequently superior activation of the adaptive immune response. In another study, despite higher uptake and subsequent maturation of DCs, Ad35 was shown to interfere

with NFkB activation and result in reduced activation of CD4+ T cells compared to Ad5 [157].

While many immunostimulatory approached have been proposed to have desirable qualities for tumor immunotherapy, they may be insufficient on their own to achieve full and long-lasting tumor control. In particular, emerging data suggests that induction of immunity may not be enough, due to the immune suppressivity of the tumor environment. Thus, selective combinations of immune stimulation with reduction of suppression are being explored. As an example, GM-CSF synergized with B7-1 to provide superior tumor control in a mouse model of metastasizing melanoma [158]. It is still relatively unclear, however, how each agent shapes the immune response and how best to combine different modalities for maximal net anti-tumor effect. We foresee this line of research to gather increasing interest in the coming years. However, the biggest problem in the field relates to animal models. Regulation and function or different pathways tend to vary between animals and few signaling molecules cross species. Thus, models tend to be poorly predictive. Although this is especially problematic in the oncolytic adenovirus field since human viruses do not replicate productively in most species, the Syrian Hamster may be of some utility, if immunological reagents can be developed for it. Nevertheless, given the complexity of immunological pathways, human translation is critically important but unfortunately difficult and expensive.

Despite the above-mentioned concerns regards pre-clinical models, the pharmaceutical industry has invested many resources into the study of advanced virus-based immunotherapies, as demonstrated by the acquisition of Oncovex^{GMCSF} (now T-vec) [159] by Amgen Inc. for 1 billion US dollars (http://www.amgen.com/media/media_pr_detail.jsp?releaseID=1519312). Amgen completed a randomized phase 3 study (OPTIM) in patients with stage IIIb, IIIc or IV melanoma with unresctable but accessible lesions. Interestingly, the trial compared also pre-treated or treatment-näive patients. Patients receiving T-vec experienced increased durable responses compared with the control group (patients receiving GM-CSF alone) [160]. Patients with lower stages of melanoma had increased both durable response rate (DRR) and objective response rate. In addition, treatment-näive patients had

increased DRR compared to patients receiving T-vec as second-line therapy [161]. The success of T-vec in melanoma encouraged researchers and clinicians to study other types of cancer and new trials have been designed to test the combination of this oncolytic virus with immune checkpoint inhibitors such as ipilimumab (anti-CTLA4; NCT01740297) or pembrolizumab (anti-PD1; NCT02263508). The positive results in clinical studies finally led to the approval by FDA of T-vec for the treatment of advanced melanoma on 27 October 2015 [162]. These studies have important consequence also for the research based on adenoviral vectors. In fact, the development of optimized treatment schedule, dosages and immunological analysis are common challenges. We believe that the results and the re-newed enthusiasm towards this field will be at the basis of future clinical trials featuring oncolytic adenoviruses.

Overall, it is remarkable how rapidly rationally designed oncolytic viruses have entered clinical use, perhaps facilitated by a century of work with non-modified lytic viruses [163]. The first oncolytic adenovirus product was approved in China after a mere decade after the first preclinical report [164], and has subsequently acquired safety and efficacy data in more than 5000 patients . In the West, the efficacy of an oncolytic adenovirus mediated cytotoxic gene therapy with intensity modulated radiation therapy was studied in a randomized, multisite, prospective, controlled phase II/III trial (NCT00583492). The study, completed in 2014, revealed that the combination of gene therapy and radiation therapy resulted in a 42% relative reduction of biopsy positivity in patients with intermediate-risk prostate cancer. In addition, several oncolytic adenoviruses delivered promising results in preclinical models and are under evaluation on clinical studies [80, 150, 165]. The amount of data collected in different clinical trials so far, points to an immune-mediated efficacy of oncolytic adenoviruses. Therefore, the whole field is now strongly supporting the concept of “oncolytic vaccines”, highlighting the importance of innate and adaptive responses elicited by the viruses. This aspect is evolving through the work of researchers that take into account the evident links between immunology, oncology and virology fields.

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Conflict of interests

Akseli Hemminki and Otto Hemminki are shareholders in Oncos Therapeutics Ltd. and TILT Biotherapeutics Ltd.

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Figure legends

See attached file.