Evaluation of P2X7 Receptor Function in Tumor Contexts Using rAAV Vector and Nanobodies (AAVnano)

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Adenosine triphosphate (ATP) represents a danger signal that accumulates in injured tissues, in inflammatory sites, and in the tumor microenvironment. Extracellular ATP is known to signal through plasma membrane receptors of the P2Y and P2X families. Among the P2X receptors, P2X7 has attracted increasing interest in the field of inflammation as well as in cancer. P2X7 is expressed by immune cells and by most malignant tumor cells where it plays a crucial yet complex role that remains to be clarified. P2X7 activity has been associated with production and release of pro-inflammatory cytokines, modulation of the activity and survival of immune cells, and the stimulation of proliferation and migratory properties of tumor cells. Hence, P2X7 plays an intricate role in the tumor microenvironment combining beneficial and detrimental effects that need to be further investigated. For this, we developed a novel methodology termed AAVnano based on the use of Adeno-associated viral vectors (AAV) encoding nanobodies targeting P2X7. We discuss here the advantages of this tool to study the different functions of P2X7 in cancer and other pathophysiological contexts.

Keywords: P2X7, tumor, Adeno-associated virus, Nanobodies (VHH), animal models

INTRODUCTION

Adenosine triphosphate (ATP) released into the extracellular space represents a well-known danger signal that can signal through two main families of plasma membrane receptors: G protein-coupled receptors, named P2Y receptors, and ATP-gated ion channels termed P2X receptors (1). Among the latter family, P2X7 (also known as P2RX7) forms a homotrimERIC receptor that has attracted much interest in the fields of inflammation and cancer. Activation of P2X7 by relatively high concentrations of extracellular ATP leads to Na\(^+\) and Ca\(^{2+}\) influx, and K\(^+\) efflux. This triggers not only major changes in the cellular ionic content, but also signaling and metabolic pathways involved in cell activation, survival and fate. Prolonged activation of P2X7 leads to the opening of a membrane pore allowing the entry of large molecules of up to 900 Da (2). Whether this membrane permeabilization is due to dilation of the P2X7 channel itself, or the activation of non-selective pores like pannexin-1, gasdermin-D, or anoctamin-6, may depend on the cellular...
when transferred to P2X7 activates tumor cell lines appear to grow faster. Effector functions of T cells (6, 15). In line with the anti-tumor immune responses by unleashing which in turn promote adaptive anti-tumor immune responses also linked to immunogenic cell death and has been associated with tumor growth and maintenance. However, P2X7 activation is with a pro-inflammatory activity which could be beneficial for composition of the tumor microenvironment and the nature

P2X7 functions in cancer are complex and depend on the the role within the tumor microenvironment depending on multiple factors such as its concentration, the abundance of ecto-ATPases, the expression level of P2X7, and the nature of the P2X7 variant expressed by immune and tumor cells.

The functions of P2X7 in immune cells are largely documented. Gating P2X7 on macrophage, microglia, and dendritic cells triggers the formation of the inflammasome, a multiprotein complex that drives caspase-mediated maturation and release of the proinflammatory cytokines IL-1β and IL-18 (10). On T lymphocytes, P2X7 induces the shedding of CD62L and CD27 by metalloproteases (11, 12) and controls the differentiation, proliferation and survival of tissue resident memory T cells (13, 14). Regulatory T cells are known to express P2X7 at high levels, and its activation induces shedding of CD62L and CD27, phosphatidylserine exposure, pore formation, and finally leads to cell death (11, 15). Taken together, activation of P2X7 on myeloid and lymphoid cells converge to promote and amplify inflammation (16, 17). Animals deficient for P2X7 show reduced inflammation in several animal models including experimental colitis (18), collagen-induced arthritis (19), and experimental autoimmune encephalomyelitis (EAE) (20). P2X7 receptor antagonists have been developed by several pharmaceutical companies as potential novel anti-inflammatory drugs (21).

METHODS TO STUDY THE ROLE OF P2X7 IN VIVO

To investigate the role of P2X7 in vivo, several tools have been developed including genetically deficient animals, pharmacological inhibitors, and specific antibodies. P2X7−/− mice have been widely used to study P2X7 function in vivo since the generation of the first two P2X7 KO strains by Pfizer and Glaxo (30, 31). However, it subsequently became apparent that both lines are leaky: the Glaxo line, in which exon 1 was targeted, still expresses the P2X7k variant on T cells (32, 33) while the Pfizer line, in which exon 13 was targeted, still expresses the P2X7k variant displaying lower functionality (34). This situation could explain reported phenotypic and functional differences as well as conflicting results in disease models using these two P2X7 deficient lines (20, 35, 36). However, novel knockout models and P2X7-floxed mutants derived from the European Mutant Mouse Archive (EMMA) are now available and may facilitate the reevaluation of P2X7 functions in vivo in different disease models (37, 38).

Pharmacological inhibitors of P2X7 like brilliant blue G (BBG) and oATP have shown therapeutic benefit in several animal models including EAE, experimental colitis, inflammatory pain in arthritic animals, or/and antibody-mediated nephritis (36, 39–42). Blocking P2X7 by these small molecule inhibitors has also been shown to inhibit tumor growth in several tumor models that express P2X7 (28, 29). BBG and oATP are rather inexpensive but lack specificity and are associated with off-target side effects. The development of more specific antagonists by several pharmaceutical companies has facilitated the evaluation in the colitis-associated cancer model (18). However, P2X7 is also expressed by most tumor cells, where it is associated with cancer progression. Indeed, tumor cells express P2X7 at low levels and/or express P2X7 variants that are inefficient to promote cell death but which instead exert a trophic activity. In such situations, tonic stimulation of P2X7 by extracellular ATP already present in the tumor microenvironment can stimulate cell metabolism, influences the Ca2+ content of mitochondria and endoplasmic reticulum, increases the intracellular ATP content, protects against apoptosis, and promote tumor growth (24–27). Accordingly, stable transfection of the colon carcinoma tumor model CT26 with a P2X7 expression vector accelerated tumor growth in vivo (28), and P2X7 blockade using oxidized ATP (oATP), a poorly selective but irreversible antagonist, reduced melanoma B16 tumor growth in vivo (29). Taken together, on the side of the tumor, P2X7 promotes cell survival, tumorogenic potential and proliferation, but on the side of immune cells, P2X7 favors dendritic cell activation, presentation of tumor antigens, and initiation of an adaptive immune response. It appears therefore that the net effect of P2X7 is difficult to predict and that the balance could be tilted toward a pro- or anti-tumorogenic outcome, depending on the composition of the tumor microenvironment, on the level of P2X7 expression, and on the nature and functionality of the P2X7 variants expressed by tumor cells.

ROLE OF P2X7 ON TUMOR GROWTH AND ON ANTI-TUMOR IMMUNE RESPONSES

P2X7 functions in cancer are complex and depend on the composition of the tumor microenvironment and the nature of the cells that express this ion channel. P2X7 is associated with a pro-inflammatory activity which could be beneficial for tumor growth and maintenance. However, P2X7 activation is also linked to immunogenic cell death and has been associated with inflammasome formation and dendritic cell activation, which in turn promote adaptive anti-tumor immune responses (22). As mentioned earlier, P2X7 promotes Treg depletion, which increases antitumor immune responses by unleashing effector functions of T cells (6, 15). In line with the anti-tumor activity of P2X7, tumor cell lines appear to grow faster when transferred to P2X7−/− mice (23), and genetic and pharmacological inactivation of P2X7 increases tumorigenesis in expensive but lack specificity and are associated with off-target side effects. The development of more specific antagonists by several pharmaceutical companies has facilitated the evaluation
and confirmation of the role of P2X7 in these diseases (23, 28, 36, 39). However, these antagonists are sometimes more difficult to obtain and are expensive to use, notably in chronic models where they have to be injected every other days for several weeks.

P2X7 MODULATING NANOBODIES

Antibodies represent another emerging class of potent pharmaceutical modulators that are used to block or to potentiate their targets in vivo. Conventional antibodies are composed of two heavy and two light chains that contribute together to the antigen-binding site. Conventional antibodies represent relatively large molecules of ~150 kDa. In direct relation with their size, their biodistribution into tissues is limited (43). In comparison, smaller proteins derived from antibodies but containing only the binding domains fused directly via a linker (i.e., single-chain variable fragment or scFv) offer better biodistribution coefficients. More recently developed nanobodies offer an interesting alternative. Nanobodies are derived from unconventional antibodies found in llamas and other camelids that are composed only of heavy chains. Nanobodies and heavy chain antibodies present similar specificities and affinities as classical antibodies but exhibit a smaller size (80 kDa, 15 kDa) and a better tissue penetration notably into tumors (44–46).

A set of nanobodies modulating P2X7 was selected by phage display from llamas immunized with cDNA expression vectors or P2X7-transfected HEK cells encoding for mouse and human P2X7 (47). In this study, two nanobodies were selected and used to modulate the function of mouse P2X7. Interestingly, one of them potently blocks P2X7 while the other potentiates its activities. These nanobodies, termed 13A7 and 14D5, respectively, were used in vivo to validate the function of P2X7 in disease models. Systemic administration of 13A7 reduced inflammation in mouse models of allergic dermatitis and of glomerulonephritis. Conversely, 14D5 administrated in vivo aggravated disease scores in both animal models (47). Repeated administration of these selected anti-P2X7 nanobodies in vivo undoubtedly represents a novel means to study P2X7 functions in different pathophysiological situations.

AAV-NANOBODIES (AAVnano)

METHODOLOGY

We present and illustrate here the development of a novel methodology using adeno-associated viral vectors (AAV) encoding anti-P2X7 nanobodies for studying P2X7 function in vivo, notably in chronic situations. AAV vectors have widely been used for gene transfer in vivo to elicit long-term expression of the transgenic protein of interest. For instance, a single intramuscular injection of AAV encoding HIV-neutralizing antibodies resulted in their long-lasting systemic production in mice (48) and non-human primates (49). We implemented a similar AAV-mediated gene transfer method to produce in situ anti-P2X7 nanobodies with the aim to durably modulate P2X7 function in vivo. For that, we generated AAV vectors encoding a bivalent P2X7-antagonistic heavy chain antibody designated 13A7-hcAb (nanobody 13A7 fused to the hinge and Fc-domains of mouse IgG1) or a bivalent, half-life extended P2X7-potentiating nanobody dimer designated 14D5-dimHLE (dimer of nanobody 14D5 fused to the albumin-specific nanobody Alb8 conferring half-life extension) (47). The methodology that we developed, termed AAVnano, requires only a single intramuscular (i.m.) injection of the corresponding AAV vector to elicit long-term systemic expression of these nanobody constructs in vivo for at least 120 days (unpublished observations). This avoids the daily injection of nanobodies necessary to maximize the modulation of P2X7 functions and offers the possibility to inhibit or to potentiate P2X7 in chronic models such as chronic inflammation, autoimmune diseases, carcinogenesis, or tumor growth (Figure 1).
FIGURE 1 | Comparison between repeated injection of drugs or antibodies/nanobodies and AAVnano methodology. Theoretical kinetics of the pharmacological agents in serum using different methodologies. (A) Small molecules (blue), antibodies (purple), or nanobodies (green) are generally injected using the i.p. or i.v. routes and necessitate repeated injections usually performed every 1–2 days. (B) Using the AAVnano methodology, a single injection of an AAV vector coding for the nanobody of interest is performed at day 0 using the i.m. route. The nanobody is then directly produced in vivo by the transduced muscle fibers. The nanobody is detectable in the serum 7–14 days post AAV injection and its concentration slowly increases until reaching a plateau 30–60 days post AAV injection. The nanobody produced in vivo can still be present in the serum 120 days post AAV injection.

plasma from these animals confirmed the presence of circulating nanobodies 3 weeks after AAV injection (Figure 2E).

Next, the EG7 tumor cells were injected subcutaneously, and tumor growth was monitored for 50 days. The results show that constant production of the P2X7 antagonistic 13A7-hcAb significantly limited EG7 tumor growth in vivo (Figure 2F). This treatment induced a complete tumor regression in 2 out of 5 mice and significantly improved the survival of treated mice (Figure 2G). These data are in line with previous studies showing that expression of P2X7 by tumor cells represents a trophic and pro-survival factor and that P2X7-blockade can inhibit tumor growth in vivo (24, 25, 28, 29). In contrast, potentiation of P2X7 function using AAV-mediated in situ expression of 14D5-dimHLE did not significantly influence tumor growth in this model (Figures 2E,G). One might have expected the opposite effect than 13A7-hcAb, e.g., promotion of tumor growth. On the other hand, P2X7 potentiation could also promote anti-tumor immune responses by mediating death of regulatory T cells (Tregs) by enhancing their sensitivity to NAD-mediated cell death (NICD). We indeed previously demonstrated that P2X7-dependent NICD of Tregs promotes an anti-tumor immune response in the same tumor model (15). The lack of an effect of 14D5-DimHLE on tumor growth could thus reflect balanced anti-tumoral/pro-tumoral effects in the tumor microenvironment. It will be interesting to determine whether potentiation of P2X7 can enhance anti-tumor immune response when combined with other treatments, e.g., anti-immune checkpoints therapy, immunogenic chemotherapies that increase the release of ATP, or antagonism of CD39/CD73-catalyzed hydrolysis of ATP to immunosuppressive adenosine (51, 52).

Overall, our data support the notion that the AAVnano methodology represents an additional strategy to study the function of P2X7 in vivo, allowing target validation in pathophysiological situations where P2X7 has been implicated, including cancer.

DISCUSSION AND PERSPECTIVES OFFERED BY THE AAVnano METHODOLOGY TO STUDY THE IMPORTANCE OF P2X7 IN DIFFERENT PATHOPHYSIOLOGICAL SITUATIONS

The AAVnano methodology presents several theoretical and practical advantages over other methodologies. As compared to chemical compounds, the nanobodies offer better target specificity. Indeed, nanobodies and antibodies in general are known for their excellent ability to discriminate between closely related targets belonging to the same protein family. This may prevent unwanted off-target effects. In the present study, the
selected nanobodies bind specifically to mouse P2X7 and do not recognize the closest paralogs P2X4 and P2X1 (47). However, high specificity and lack of cross-reactivity with the P2X7 ortholog of other species may limit the use of a nanobody across species in translational studies. A similar limitation has also been observed with chemical compounds that sometimes display different modulating effects and pharmacological properties in different species in translational studies. A similar limitation has also been observed (see above D,E), 106 EG7 lymphoma cells were inoculated subcutaneously in the left flanks. Tumors were monitored every other day during 50 days with a digital caliper, and the tumor volumes were estimated using the formula length × width × (length + width)/2. (F) Tumor volumes in each experimental group are shown for the first 16 days. The statistical analysis was performed using two-way ANOVA (n = 5, **p < 0.01). (G) Mouse survival is illustrated during the entire observation period. The statistical analysis was performed using Log-Rank tests (n = 5, *p < 0.05). Similar results were obtained in 3 independent experiments.

for our engineered nanobody constructs was sufficient to elicit effective systemic levels of the blocking or the potentiating P2X7-specific nanobodies. The AAVnano methodology thus avoids daily administration of the corresponding recombinant nanobodies. Arguably, this may additionally provide more favorable pharmacokinetics by maintaining a stable saturating concentration of the desired nanobody in vivo and by avoiding peak/decline cycles obtained after repeated injection of a recombinant antibody (Figure 1). This may favor a better bioavailability and tissue penetration, which needs to be confirmed experimentally in future studies. AAVnano may provide a nanobody delivery methodology similar to the one obtained with osmotic pump drug delivery systems, which allow a stable equilibrium of the pharmacological agent...
in vivo at a plateau phase (Figure 1). However, as systemic production of transgenic protein in situ using AAV vectors is known to follow slow kinetics, with the apparition of the protein of interest 1 to 2 weeks post-transduction, the equilibrium phase is probably not obtained in vivo before 2 to 3 weeks (54–56). Hence, the AAVnano methodology is intended to be used in prophylactic models, as in the present study, rather than in a therapeutic setting. However, the kinetics of transgenic protein production can be considerably accelerated using recently developed self-complementary AAV (scAAV) vectors that would be more compatible with therapeutic/interventional protocols (56).

One of the features associated with the AAVnano methodology is the permanent and long-term modulation of P2X7 in vivo without any possibility to halt the treatment. While this is adapted to the evaluation of P2X7 functions in chronic diseases, this may appear as an inconvenient in other situations. Additionally, long-term P2X7 modulation (i.e., activation or potentiation) may have unexpected effects on the general physiology of the mice by causing, for instance, immune dysregulation, alteration of barrier functions, or alteration of the microbiota. Knockout mice may possibly not be affected to the same extent by P2X7 deficiency as compensatory mechanisms are presumably induced during embryogenesis, as often observed in knockout animals. In this regard, AAVnano methodology may reveal so far unexpected physiological functions of P2X7 that may be interesting to further explore. One way to avoid such long-term effects would be to use inducible promoters, instead of the ubiquitous CBA promoter used in this study. Such an inducible system, combined with AAV-mediated gene transfer, would allow controlled and timely delivery of the selected nanobody to modulate P2X7 only when required. This type of inducible delivery would be compatible with both, a prolymphatic scheme as well as therapeutic/interventional protocols.

Beyond the cancer field, the AAVnano methodology may be further exploited in target-validation studies and in other disease models where P2X7 has been incriminated. Examples are inflammatory models such as acute and chronic experimental colitis, or autoimmune diseases like rheumatoid arthritis, lupus, or experimental allergic encephalitis. Interestingly, besides skeletal muscles, AAV vectors can be used to transduce efficiently a variety of other tissues including heart, liver, kidney, retina, and the central nervous system (CNS). For example, it is conceivable to use stereotoxic injection of AAV9 vectors into the brain to induce expression of the modulating P2X7-specific nanobodies in the CNS. Since P2X7 is expressed in the CNS, particularly by microglial cells and astrocytes, AAVnano may help to elucidate the role of P2X7 in the pathophysiology of neurological disorders like epilepsy, Alzheimer’s disease, multiple sclerosis, amyotrophic lateral sclerosis, age-related macular degeneration, or cerebral artery occlusion (57). Apart from using tissue-specific conditional P2X7 knockout models, the role of P2X7 in these diseases remains difficult to evaluate as the use of antibodies is limited by the blood-brain barrier. AAVnano may offer a possibility to circumvent this difficulty by exploiting the efficiency of AAV vectors to transduce different regions of the CNS, combined with the good tissue penetration and bioavailability offered by nanobodies. Hence, the AAVnano methodology may pave the way to a better understanding of the role of P2X7 not only in the tumor context, but also in chronic inflammatory/autoimmune diseases and neurological disorders. This may further help the community to evaluate the importance of P2X7 in pathophysiology and as a therapeutic target.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Comité d’Ethique Normandie en Matière d’Expérimentation Animale (CENOMEXA).

AUTHOR CONTRIBUTIONS

MD, FH, FK-N, and SA conceptualization. MD, AS, CA, and SA methodology. MD, CA, and SA investigation. MD, FH, FK-N, and SA conceptualization. MD, AS, CA, and SA writing – original draft. MD, FK-N, and SA writing – review and editing. FH, FK-N, and SA funding acquisition. FH, FK-N, and SA Resources. CA, FK-N, and SA supervision. All authors contributed to the article and approved the submitted version.

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