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Overview of a HLA-Ig based "Lego-like system" for T cell monitoring, modulation and expansion

Mathias Oelke · Jonathan P. Schneck

Abstract Recent advances in molecular medicine have shown that soluble MHC-multimers can be valuable tools for both analysis and modulation of antigen-specific immune responses in vitro and in vivo. In this review, we describe the use of dimeric human and mouse major histocompatibility complexes, MHC-Ig, as part of an artificial Antigen-Presenting Cell (aAPC). MHC-Ig-based aAPC and its derivatives represent an exciting new platform technology for measuring and manipulating immune responses in vitro as well as in vivo. This new technology has the potential to help overcome many of the obstacles associated with limitations in current antigen-specific approaches of immunotherapy for the treatment of cancer, infectious diseases and autoimmunity.

Keywords Class I HLA · $CD8^{+}$CTL · Infectious diseases · Tumors · HLA-Ig dimer · Active and adoptive immunotherapy

Introduction

Identification and characterization of T-cell-mediated immune responses and their immune signature are key elements to understand the cellular immune response. It has previously been shown that cognate monomeric MHC molecules specifically interact with TCR molecules; however, due to the low-affinity nature of this interaction, their usage was limited. To overcome the intrinsic low affinity of the MHC–TCR interaction various approaches have been taken to multimerize the MHC molecules [1, 2]. The most commonly used multimeric MHC molecules are MHC-dimer [3] and tetramer molecules [4]. The

M. Oelke
Department of Pathology, Johns Hopkins University, School of Medicine,
Ross Research Bldg Room 644 B, 720 Rutland Ave, Baltimore,
MD 21205-2196, USA

J. P. Schneck (✉)
Department of Pathology & Medicine, Johns Hopkins University, School of Medicine,
Ross Research Bldg Room 664 G, 720 Rutland Ave, Baltimore, MD 21205-2196, USA
e-mail: jschnec1@jhmi.edu

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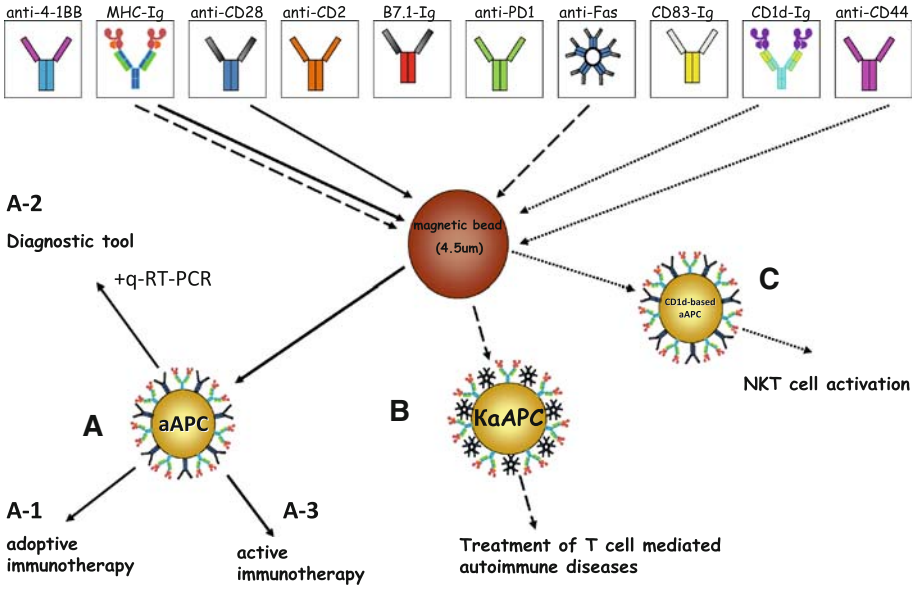

Fig. 1 The aAPC-"Lego-like"-system

development and use of these molecules have previously been previously described and reviewed [5–8]. More recently, by utilizing dimeric MHC-Ig molecules that can be easily loaded with any MHC-restricted peptide of interest, we have developed an artificial antigen-presenting cell for the generation and expansion of antigen-specific CTL [9–11]. This aAPC has been further developed into a bead-based platform technology that allows not only for T-cell activation and expansion but for positive and negative modulation of cellular immune responses. This "Lego-like" platform technology (Fig. 1) represents an easy to assemble, reductionist, system in which different immunological signals can be attached to a central scaffold. The scaffold can be a cell-sized paramagnetic bead as shown in some examples below, but it can easily be exchanged with a bio-degradable bead, a much smaller quantum dot or other desired scaffolds. In this review, we discuss some examples, how this new technology can be used for (a) T-cell activation in vitro and in vivo, (b) T-cell detection, (c) depletion of unwanted T cells and (d) activation of NKT cells.

Artificial antigen-presenting cells, aAPC: an alternative approach to DC

aAPC in adoptive immunotherapy

Adoptive immunotherapy with antigen-specific CTL has been successfully performed in patients using ex vivo expanded cytomegalovirus (CMV)-specific CTL clones as prophylaxis for CMV disease in immunocompromised allogeneic bone marrow transplant recipients [12]. Similarly, adoptively transferred ex vivo expanded CTL has had an encouraging, albeit limited, success in the treatment of Epstein-Barr virus (EBV) and melanoma [13, 14]. The development of effective anti-tumor immune responses is normally limited by an ineffective T-cell response that is unable to eradicate the tumor. This is due to the fact that high-affinity T cells are often hypo-responsive or tolerized, while low-

affinity T cells are unable to eradicate even small tumors. New strategies to amplify anti-tumor activity of low-affinity CTL in vitro and/or in vivo would therefore improve the efficacy of immunotherapy. One strategy has been to use autologous dendritic cells (DC) to activate tumor-specific CTL and overcome tolerance. However, this immunotherapeutic approach is limited by the lack of reproducible and economically viable methods for generating therapeutic numbers of functional, autologous DC. In addition, patient-derived DC are often impaired or dysfunctional due to pretreatment and disease as has been reported in a variety of advanced cancers including breast-, hepatocellular-, prostate-cancer and melanoma [15, 16]. Ultimately, these limitations related to the use of autologous DC highlight the importance of developing alternative approaches such as artificial antigen-presenting cells, aAPC.

Using a reductionist approach, we developed artificial antigen-presenting cells, aAPC, made by coupling HLA-A2-Ig, signal 1, and anti-CD28 mAb, signal 2 (Fig. 1A-1). We initially focused on two clinically relevant HLA-A2 restricted targets, CMV and melanoma, which have widely varying affinities for their cognate TCR. The CMV-peptide pp65 is known to be a high-affinity peptide, whereas the modified melanocyte self antigen, Mart-1-peptide [17], is a low-affinity peptide. Thus, studying these two systems provided insight into the robust nature of the aAPC and its potential therapeutic value [9].

To further explore the growth potential of aAPC-stimulated cells, freshly isolated $CD8^+$T cells from PBMC were stimulated with aAPC for 7 weeks, during which the Mart-1-specific CTL expanded to approximately 10^9 CTL. aAPC expanded CTL were 85% antigen-specific by the third week, remaining at this level throughout the rest of the expansion period. Since the starting population was less than 0.05% Mart-1-specific, this represented minimally a 10^6-fold expansion of antigen-specific cells in less than 2 months.

We analyzed the in vivo tracking and function of aAPC generated Mart-1-specific CTL in an experimental Human/Scid Model. aAPC induced Mart-1-specific CTL survived for at least 15 days and successfully inhibited the development of human melanoma tumors in SCID/Beige mice. Furthermore, using non-invasive, bioluminescence imaging to characterize the trafficking kinetics in a treatment model of subcutaneous melanoma revealed that the CTL distributed initially to the lungs as reported in several studies [18–21] but were then able to localize at the site of HLA-$A2^+$ melanoma tumor as early as 3 days after transfer, where they significantly inhibited tumor growth, while the Mart-1-specific CTL did not track and enrich in an HLA-$A2^-$ control tumor. To summarize, in our preliminary studies, we have studied the ability of HLA-Ig-based aAPC to stimulate clinically relevant CTL populations and modeled those studies on DC-mediated CTL expansion. This has allowed us to compare the efficacy of aAPC to DC-based induction/expansion of antigen-specific CTL. aAPC-based induction was at least as good as, if not better than, DC-based induction and provided significant advantage in case of preparation and use over DC-based expansion.

aAPC for active immunotherapy

While adoptive immunotherapy is a promising approach, it requires in vitro culture of the antigen-specific T cells, which is labor intensive and very costly. Therefore, the development of active immunotherapy, in which the activation and expansion of antigen-specific T cells occurs in vivo, was explored.

Cancer vaccines have not significantly improved the clinical outcome and patient survival [22, 23], even though it is possible to detect tumor-specific CTL in peptide-vaccinated patients. Several factors clearly influence the challenge of an efficient immunotherapy: thymic selection, tumor-released inhibitory cytokines and chemokines, the presence of

regulatory T cells, altered macrophage differentiation, and defects in DCs [24–28]. Another hindrance in developing effective anti-tumor immune responses is often the low avidity of the existing tumor-specific cytotoxic T lymphocytes (CTL) that are unable to eradicate the tumor. We explored the use of aAPC to rescue anti-tumor activity of low-avidity melanoma-specific CTL in vivo.

To overcome issues of low-avidity CTL in tumor immunotherapy, we designed murine aAPC, made by covalently coupling murine ^{pep}MHC-Ig dimers and B7.1-Ig molecules to magnetic beads (Fig. 1A-3) for in vivo administration. We found that aAPC in vivo administration successfully increased the activity and effectiveness of adoptively transferred low-avidity melanoma-specific CTL. In these studies, aAPC administration significantly augmented the in vivo anti-tumor activity of three different antigen-specific CTL, leading to the inhibition of tumor growth in both a lung metastasis and a subcutaneous tumor models [29]. This novel approach represents the first demonstration of an "off the shelf", bead-based aAPC for systemic delivery of both antigen-specific and co-stimulatory signals to tumor-specific CTL. aAPC administration can thus potentially be used to overcome current problems related to low-avidity anti-tumor CTL, therefore increasing the efficiency of both adoptive and active immunotherapy of cancer.

The aAPC-qRT-PCR assay for the detection of antigen-specific T cells in small samples

Acute viral diseases usually present a non-specific set of early symptoms, which do not allow a quick diagnostic based on medical examination without the support of laboratory assays. Often clinicians base their diagnosis on epidemiological viral incidence data and, most times, acute viral diseases end up without a confirmatory diagnostic. However, a specific viral diagnostic of individual cases is extremely important in detecting the early stages of outbreaks to implement control measures in timely manner. The specific diagnostic of acute viral disease is especially important in endemic areas where multiple agents with the potential to generate a deadly global pandemic circulate. The lack of these diagnostics in the clinical routine in such areas results in increased morbidity and mortality and may represent a global threat. The lack of sensitive CMI assay can also represent a potential bio-threat.

Current technologies for analyzing immune cells, such as ELISPOT, intracellular cytokine staining (ICS) and MHC-multimer staining provide opportunities to characterize antigen-specific T cells at the single cell level. However, these methods usually involve isolation of PBMCs, which requires large blood volumes for sample preparation, a major hindrance particularly in young children as well as for high-throughput applications.

Therefore, we developed an aAPC-qRT-PCR assay (Fig. 1A-2) for the detection of antigen-specific memory T cells in whole blood by combining a standardized antigen-specific aAPC stimulation method and a high-sensitivity qRT-PCR assay for amplifying and quantifying IFN-γ gene expression [30]. It is at least as sensitive as current standard assays such as ICS and tetramer analysis but requires much smaller blood volumes. This feature also allows for easy in-field studies in that aAPC can stimulate 50–100 μl of blood, and RNA isolated and stored are shipped for PCR analysis. Therefore, this assay is potentially field-friendly and suitable for studies involving young children. This assay is also potentially for high-throughput analysis. Interferon-γ (IFN-γ) can be used as the readout for measuring low-frequency, antigen-specific T-cell responses to multiple

antigens including both measles virus (MV)-specific responses as well as other clinically relevant infectious diseases.

During the initial evaluation, we found that the new aAPC-qRT-PCR assay has a detection limit of less than 0.004%, which is equivalent sensitive as a standard ICS assay whose lower limit of detection has been reported to range between 0.01 and 0.001% antigen-specific T cells [31–33]. However, to identify 0.001% antigen-specific cells by ICS would require acquisition of several hundred thousand events and a much larger sample volume for analysis than needed for our aAPC-qRT-PCR assay.

Specificity is another important attribute of a good assay. The aAPC-qRT-PCR assay did not result in false positives. All samples that were negative by the standard assays (ICS and MHC-tetramer staining) were also negative by the aAPC-qRT-PCR assay. Although whole blood was used, the IFN-γ detected by the assay, as expected, mainly came from $CD8^+$T cells, because aAPC stimulate only $CD8^+$T cells. $CD4^+$T cells were negative for intracellular IFN-γ in all seven donors stimulated with ^{cmv}aAPC.

In summary, the aAPC-qRT-PCR assay couples the standardized artificial Antigen-Presenting Cell (aAPC) technique for stimulating $CD8^+$T cells in whole blood with a qRT-PCR for the detection of the antigen-specific induction of IFN-γ mRNA. Thus, aAPC-qRT-PCR overcomes the limitations associated with current assays, because it uses a small sample volume, it does not require prior processing of the sample, and it is sensitive and reproducible. Furthermore, the assay could easily be adapted for monitoring low-frequency T-cell responses to other vaccines and infections.

Killer aAPC, KaAPC, for treatment of T-cell-mediated autoimmunity

Besides cancer, autoimmune diseases are another target for antigen-specific immunotherapy. Current treatment approaches for autoimmune diseases such as diabetes, arthritis and T-cell-mediated liver diseases as well as therapies to prevent transplant rejection are based on global immune suppression. These conventional treatments, including corticosteroids or anti-CD3, suppress the immune system in the attempt to prevent the destruction of self or newly transplanted tissues. While these types of treatments are useful in limiting symptoms and managing pain, they do not result in a cure. Furthermore, other adverse symptoms or side effects not only make patients more vulnerable to infections, but lead to increased incidences of cancers after long-term usage of these immunosuppressive regimens. Therefore, new antigen-specific approaches that leave the immune system intact and able to neutralize opportunistic pathogens with fewer side effects are most desirable.

Previously several cell-based immunotherapy strategies have been developed to specifically modulate T-cell-mediated immune responses. These methods frequently rely on the utilization of tolerogenic-cell-based antigen-presenting cells (APC). However, APC are highly sensitive to cytotoxic T-cell responses, thus limiting their therapeutic capacity. The steadily increasing knowledge of APC/T-cell interactions [3–6], newly identified APC and T-cell subsets and the observation that some tumor cells express Fas Ligand (FasL, CD95L) on their surface to evade T-cell-mediated anti-tumor responses [9–12] has led researchers to design new approaches to induce T-cell inhibition or deletion.

One approach to inhibit or delete auto-reactive CTL in the setting of autoimmune disease and allograft rejection is based on genetically modified dendritic cells (DC) that express the apoptosis-inducing ligand FasL [13–20]. While the results of above approaches are promising, there are the commonly known problems related to the use of autologous DC. In addition, there are other problems related to cellular APC; for example, mature

Killer-APC express the high levels of co-stimulatory molecules that could be counter-productive by stimulating anti-apoptotic processes [22]. Furthermore, while cellular Killer-APC can be pulsed with peptides of interest, they will also present other previously acquired antigens potentially leading to the deletion of unwanted or un-identified T cells. Moreover, all cellular based APC approaches are sensitive to their in vivo and in vitro environment as the cytolytic effector activity of T cells [23] may lead to APC depletion or unwanted changes in cell–cell signaling.

To overcome issues related to the use of cell-based Killer-APC, we have developed a new bead-based artificial Killer-APC by the conjugation of HLA-A_2-Ig dimer molecules and an apoptosis-inducing α-Fas-IgM mAb onto epoxy-beads (Fig. 1B), which could then be loaded with different peptides. Phenotypic and functional characterization demonstrated that KaAPC deleted $CD8^+$ T cells in an antigen-specific fashion, which was directly related to the number of KaAPC present in the co-cultures. In addition, KaAPC selectively eliminated CMVpp65-specific CTL from mixtures of effector memory T cells with unknown specificity, specifically reducing the targeted CMV-specific CTL population, while not significantly effecting the viability of the remaining non-CMV-specific CTL [34].

In contrast to cell-based Killer-APC, KaAPC do not promote the risk of activation of a T-cell response, or induction of tolerance toward other antigens. Furthermore, KaAPC are not eliminated by self- or paracrine-killing due to Fas/FasL signaling, and KaAPC are not targets of the cytolytic effector functions of CTL. In addition, the ease of the KaAPC system allows one to modify the phenotype through simple peptide exchange and large numbers of KaAPC with proven activity can be rapidly and reproducibly manufactured. The apoptosis-inducing signal could be modified as well, by using other ligands or α-receptor-mAb of the TNFR-superfamily [42, 43]. To our knowledge, this is the first report of the generation and functional characterization of bead-based "off the shelf" KaAPC that might be of use in modulating T-cell responses in autoimmune and transplant-related diseases.

aAPC for targeting NKT-cells

Although the field of NKT cells is fairly new, it is expanding rapidly multiple subset of NKT cells with multiple functions exist [35]. NKT cells recognize lipid antigens in the context of CD1d molecules and subsequently produce cytokines that activate cells of both the innate and adaptive immune responses. Therefore, NKT cells are often viewed as one of the components that link the innate and adaptive immune system.

Some reports have shown that NKT cells can have anti-tumor activity [36, 37], while others have indicated a regulatory function of NKT cells suppressing anti-tumor-specific CTL [38]. Furthermore, many studies examining patients with autoimmune disease or cancer have shown that there is a reduction in both NKT cell number and function [39–41]. Supporting these findings, it has been reported that NKT cell activation protects mice against experimental autoimmune diseases [42–44]. Thus, while it is clear that CD1d-restricted NKT cells are an important component of the immune system that has the capacity to either augment or impede specific immune responses, only limited information regarding their different phenotypes and corresponding function is available.

To support growth and analysis of NKT cells, we used our platform technology to developed a CD1d-expressing aAPC (Fig. 1C). CD1d-based aAPC can effectively propagate both canonical (*i*NKT cells) and non-canonical NKT cells in both mice and humans. Importantly, our system allows one to expand the NKT cell population from the PBMC of cancer patients. Furthermore, utilizing the ease and flexibility of our "Lego-like" system to

evaluate the impact of different co-stimulatory-signals on the propagation of NKT cells, we found that anti-CD44 had a strong co-stimulatory effect on the NKT cell hybridomas. Interestingly, anti-CD28 had strong co-stimulatory effect on primary liver NKT cells, compared to stimulation with aAPC expressing anti-CD44. This could be a result of tissue-specific differences in the NKT cell population, as well as a difference in primary cells and hybridomas. Furthermore, when we compared aAPC-based expansion of human NKT cells with the commonly used PBMC-based expansion protocol, we found similar percentages of Vα24+Vβ11+ cells; however, the total cell number was much lower in the group stimulated with pulsed PBMC when compared to aAPC-based NKT cell expansion [45].

Thus, these studies demonstrated that the CD1d-Ig-based aAPC system has the potential to separate and expand different subsets of NKT cells, providing a better understanding of NKT cell biology, and therefore leading to new therapeutic approaches in cancer immunotherapy.

Conclusions

To summarize, by utilizing MHC-Ig dimer molecules, we have initially developed a "Lego-like" artificial Antigen-Presenting Cell, aAPC, for measuring and modulating the cellular immune responses in vitro and in vivo. Initial proof of principle studies has demonstrated the versatility of this new technology in multiple areas. The ease of preparation and the stability of the aAPC and its derivatives represent a distinct advantage over autologous DC. These advantages, with regard to clinical applications, are (1) aAPC can be prepared in large quantities in advance and used "off the shelf" without any further manipulation; (2) aAPC can be stored for long time without the loss of activity and (3) in contrast to autologous DC, no sterile cell culture and cytokines are required to generate the aAPC.

Besides the above explored approaches, there are many more potential applications that could be studied. For example, one can easily introduce other co-stimulatory complexes, such as 4-1BBL or ICAM-1, which have been described to prevent apoptosis and therefore augment T-cell expansion [38, 46]. The exchange of the MHC-Ig with TCR-Ig molecules would allow targeting and modulating not only T cells, but also antigen-presenting cells such as B cells and DC. Furthermore, using TCR-Ig molecules on the surface of the aAPC, one could envision targeting tumor for delivering apoptosis-inducing signals such as FAS ligand.

While the therapeutic aspect of the aAPC is very promising, the diagnostic aspect of the aAPC also has significant potential. As demonstrated, the aAPC-qRT-PCR assay has clear advantages over the current methods for antigen-specific T cell detection. It is currently limited to identifying only one cytokine at a time, an issue that can be overcome through multiplexing of the real-time-PCR protocol, thereby allowing for the detection of more than one cytokine. Furthermore, the possibility to multiplex the stimulation component by using several aAPC loaded with different peptides simultaneously not only will increase the overall sensitivity, but it also enables high-throughput peptide screening. Currently, for all aAPC applications, knowledge of the HLA-restricted antigens is required. However, the decoding of the human genome and the application of novel molecular technologies will permit the rapid identification of more and more antigens in the near future [45].

Thus, in summary, the wide-spread potential applications of this platform technology including but not limited to targeting a large variety diseases from autoimmune diseases to cancer and transplantation, in combination with the robust, reproducible nature of this

approach, highlights its potential to significantly advance the field of customized immunotherapy.

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