Immunochemical approaches to monitor and modulate the adaptive immune system
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Summary and future perspectives

ADAPTIVE IMMUNITY IN A NUTSHELL

The two branches of the immune system work closely together to detect and eliminate threats. Our immune system has evolved to protect us from infection by viruses and other pathogens, but concomitantly also offers protection from cancer through elimination of cells that express mutated proteins. The first line of defense is provided by the innate immune system via detection of pathogenic or tumor cell fragments[1]. Pattern recognition receptors on innate immune cells recognize pathogen- or danger-associated molecular patterns, such as cell-free DNA, dsRNA or bacterial cell wall constituents[2]. Adaptive immune cells recognize and respond to pathogens or malignant cells more specifically by scanning peptides, small protein fragments. These peptides can be presented at the surface of all nucleated cells by major histocompatibility complex class I (MHCI) molecules, for surveillance by cytotoxic CD8+ T cells[3]. These specialized T cells can scan the presented peptides and distinguish self- from non-self with their highly specific T cell receptors (TCRs) and directly eliminate cells that display signs of infection or mutation. Likewise, the extracellular space is monitored by CD4+ T cells, or T helper (Th) cells, via TCR scanning of internalized extracellular peptides presented on MHC class II (MHCII)[4]. Upon recognition of foreign peptides, CD4+ T cells produce cytokines that induce clonal expansion and activation of B cells so that these can release antibodies.

TCR activation is the first of the three signals necessary for mounting an effective adaptive immune response[5]. The second signal required for sustained activation and proliferation is provided by costimulation[6]. Costimulatory cell surface receptors are expressed on T cells and exert their function upon engagement by their ligands on activated antigen-presenting cells (APCs), such as dendritic cells (DCs). The best-characterized costimulatory pathway involves CD28, constitutively expressed on naïve T cells, and its ligands B7-1 (CD80) and B7-2 (CD86) found on activated DCs[7]. Help provided by CD4+ T cells is crucial for the maturation of DCs and correspondingly increased expression of B7-1 and B7-2[5]. Professional APCs express a variety of additional costimulatory receptors, each with their own cognate ligands[8]. Although activated T cells may undergo several rounds of proliferation when costimulated, clonal expansion, survival and establishment of memory requires a third signal, delivered by cytokines[9]. Absence of this third signal eventually leads to tolerance10. The cytokines needed by CD8+ T cells are interleukin (IL) 12 or type I interferons (IFNs), produced by CD4+ Th cells[11].

A closer look at MHCI antigen presentation and TCR activation

MHCI is loaded with intracellularly-derived peptides, typically of 8-10 amino acids in size, in the endoplasmic reticulum. Two alpha-helices of the MHCI form a groove that contains several binding pockets, into which the side chains of the peptide's amino acids can fit[12]. The peptide-MHCI binding affinity is determined by the interactions of the MHCI heavy chain with the peptide backbone and amino acid side chains[13]. The TCR binds the amino acid side chains of the peptide that protrude out of the binding groove, as well as the exposed residues that make up the MHCI's alpha helices. The extreme selectivity of the TCR ensures that only one particular peptide-MHCI combination leads to activation, warranting the high specificity needed to maintain self-tolerance and prevent auto-immunity.

MHCI genes are divided in three groups, in humans referred to as human leukocyte antigen (HLA) A, B and C. These genes are highly polymorphic, i.e., they contain many variants due to mutation, recombination and conversion. The resulting allotypes can differ in one or multiple amino acids, mainly in the peptide-binding groove, that affect the preferred binding motifs[14]. Each individual inherits one HLA-A, one HLA-B and one HLA-C gene from each of their parents, resulting in 3-6 different HLAs expressed per individual. Through expression of multiple subtypes more fragments of a protein are presented, thus achieving broad immune protection.

Immunotherapy

Improving peptide vaccines through chemical modification
Our immune system is incredibly advanced in detecting and eliminating infected or transformed cells. This ability is gratefully embraced by clinicians: immune therapies that induce or promote anti-tumor or anti-viral responses have proven efficacious against infection as well as cancer. With increasing knowledge therapies are advancing to become increasingly specific and personalized. Activating CD8^{+} T cells has been considered the most straightforward approach, for a cytotoxic response directed specifically at infected or transformed cells will result in elimination of just those cells expressing the antigen, even at distant sites. Various therapeutic strategies can be employed, such as vaccination with antigenic peptides, antigen-coding RNA, peptide-loaded DCs or even antigen-activated CD8^{+} T cells[15]. In particular, the design of peptide vaccines has been of high interest for decades and their efficacy, stability and pharmacokinetics have been studied extensively. Moreover, their synthesis is easy, cheap and flexible[16]. Peptides by themselves are poorly immunogenic and efforts to increase antigenicity

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of known epitopes may increase clinical benefit. The longstanding paradigm was that a high peptide-MHC affinity correlates with a high immunogenicity[17]. In line with this assumption, many studies have attempted to increase the immunogenicity of known epitopes by enhancing their affinity[18]. By modifying the anchoring amino acids the affinity for MHCI can be increased, without interfering with T cell recognition, which is mostly dependent on the central amino acids[19]. In **Chapters 2 and 3** we describe the design and use of chemically altered peptide ligands (CPLs), epitopes that are not only modified with naturally occurring (proteogenic) amino acids, but also with chemically modified amino acids. Using such synthetic amino acids can offer several advantages in addition to increased affinity, including improved protease resistance and enhanced bioavailability[20,21].

The study described in **Chapter 2** set out to increase the affinity of epitopes known to bind HLA-A*02:01, the most abundant HLA allele in the Caucasian population, by introducing non-proteogenic amino acids[22]. We made several amino acids substitutions at or around the anchoring positions of a high-affinity influenza epitope and an intermediate-affinity cytomegalovirus epitope. The binding affinities were determined using fluorescence polarization-based assays to learn which substitutions frequently resulted in enhanced binding[23-25]. This information was then used to optimize binding of a number of melanoma-associated epitopes. The enhanced interactions were demonstrated via crystal structures of HLA-A*02:01 in complex with a wild-type antigen or a CPL. These were next loaded on peptide-MHCI (pMHCI) multimers and used to stain peripheral blood mononuclear cells from a melanoma patient. The detected frequencies of antigen-specific CD8+ T cells were similar between pMHCs loaded with the wild-type antigen or with CPLs, indicating maintained interactions with the TCRs. Functional assays demonstrated prolonged activation of clonal wild-type specific CD8+ T cells coincubated with synthetic antigen-pulsed APCs in vitro, and enhanced T cell responses of HLA-A*02:01 transgenic mice in vivo. Finally, the use of CPLs was validated in a clinical setting. CPLs based on a minor histocompatibility antigen (UTA2-1), showed an increased capacity to induce antigen-specific T cell responses that maintained cytolytic function[26].

In **Chapter 3** we moved from a therapeutic to a preventive setting and from cancer to virus[27]. Preventive vaccines differ from therapeutic ones, as they aim at inducing a pool of memory T cells to prevent infection rather than to cure it. The initial immune response is generally slow, but once memory is established the response is fast and infection may even be asymptomatic. Presence of pre-existing T cells has been shown to confer cross-protection against influenza A virus, which is precisely the purpose of vaccination with epitopes from conserved regions[28,29]. Multi-peptide vaccines are generally more successful than single-peptide vaccines, for the obvious reason that such a vaccine activates multiple T cells and hence prepares a more diverse pool of memory T cells to ward off

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even newly emerging subtypes of the same virus[30]. Furthermore, a vaccine that contains peptides that bind different MHC alleles will activate a larger set of T cells and confer broader protection. Therefore the study in **Chapter 3** describes the optimization of binding affinity of a second common allele in the Caucasian population, HLA-A*03:01, in addition to HLA-A*02:01. For both alleles three conserved influenza A virus epitopes of varying immunodominance were selected and modified with a selection of non-proteogenic amino acids derived from the binding studies described in **Chapter 2**. Binding affinities were enhanced to similar levels, regardless of the immunodominance of the wild-type epitope. However, substitutions that increased binding of one epitope did not always translate to another, demonstrating the complexity of predicting binding affinity.

For a selection of the HLA-A*02:01-binding CPLs the capacity of inducing relevant T cell responses was determined using in vitro and ex vivo screening assays. CPL-loaded APCs were cocultured for 24 hours with clonal T cells specific for the corresponding (immunodominant) wild-type antigen. After incubation T cell activation was quantified by measuring IFN-γ secretion, which increased for some, but not all CPLs. In a second in vitro assay reactivity to CPLs was tested in a DC coculture model where CPL-pulsed DCs were cultured with autologous T cells from HLA-A*02:01+ donors, after which IFN-γ production was measured again. This assay showed high variation between CPLs and between donors. In a third assay, designed to decrease inter-donor variance, reactivity of splenocytes from HLA-A*02:01 transgenic mice to CPLs was followed. The responses differed considerably between the assays, which complicated selection of peptides for further investigation. Results obtained in the in vitro and ex vivo assays were then used to select peptides for in vivo testing. For each viral epitope four CPLs with varying binding scores and increased or similar reactivity were compared to their wild-type sequences. The transgenic mice were vaccinated with one of the three wild-type antigens or CPLs. Splenocytes were isolated two weeks post vaccination and restimulated ex vivo in homologous fashion with the same peptide or with the wild-type epitope. Measurements of IFN-γ secretion in response to restimulation revealed that T cell responses were indeed enhanced by vaccination with some of the CPLs, also when restimulated with wild-type antigen, indicating that the induced CD8+ T cells do recognize and respond to the wild-type epitopes.

The in vitro and in vivo assays demonstrated that vaccination with CPLs could augment CD8+ T cell responses compared to wild-type epitopes, but the most potent CPLs were not necessarily those with the highest affinities. There likely is an optimal window for affinity; a low affinity will result in incomplete activation, whereas vaccination with high-affinity peptides induces higher numbers of T cells, but of lower quality[31-34]. In addition, tumor cells have been shown to upregulate negative regulators of T cell activation in response to prolonged pMHCI-TCR

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contact, thereby blocking full activation[35]. The intricate interplay of factors important for strong TCR activation cannot completely be accounted for in vitro, which is probably why we could not predict from our in vitro experiments which CPLs induced the most potent responses in vivo[36].

CANCER IMMUNOTHERAPY

In many cases cytotoxic responses directed at tumor cells do develop, but due to the low affinity of TCRs to self-antigens, or tumor-associated immune suppression, effective anti-tumor immunity is impaired[32,37-40]. Enhanced tumor-associated antigens to reach an affinity above the threshold for TCR activation can induce activation of effector functions and anti-tumor immunity[41,42]. Peptide vaccines often lack efficiency when provided as a monotherapy, but as an adjuvant to other treatment they may improve outcomes drastically. Formulations composed of long peptides and adjuvants co-activate DCs to induce costimulation and CD4+ T cell help[43].

In combination with (neoadjuvant) checkpoint inhibition the perfect immune-stimulating environment for optimal anti-tumor immunity can be established. Since the Nobel Prize for Physiology and Medicine was awarded to this discovery, checkpoint inhibitors have gained exponential attention. Immune checkpoints are molecules expressed on immune cells that function as negative regulators of T cell activation, thereby maintaining the balance between activation and self-tolerance and thus preventing auto-immunity. Two of the most described and targeted checkpoints in treatment of cancer are programmed death 1 (PD-1), which binds the ligands PD-L1 and PD-L2 on tumor cells, and cytotoxic T lymphocyte-associated protein 4 (CTLA-4), which blocks costimulation by binding the CD28 ligands B7-1 and B7-2[44,45]. A total of six antibodies that block the interaction between PD-1 and CTLA-4 and their ligands have been approved for treatment of various types of cancer, and many clinical trials are still ongoing[46]. Repressing inhibitory immune signals reinstates pre-existing immune responses and therefore as a prerequisite for efficacious treatment cancerous cells have to be on the radar of the immune system. Patients suffering from cancers with a high mutational burden that express high levels of neoantigens therefore generally benefit most from cancer immunotherapy[47]. Vaccination with neoantigens or tumor-associated antigens may boost the first signal for T cell activation and help overcome the bottleneck of immune detection.

As a second prerequisite DC activation is crucial for delivering appropriate costimulatory signals and pro-inflammatory cytokines. Biological or chemical adjuvants that activate pattern recognition receptors on DCs greatly enhance the effect of immunotherapy. A third bottleneck in cancer immunotherapy is

tumor-associated immune suppression. In response to immune detection tumor cells have developed various mechanisms to evade and suppress detection, for example through recruitment of regulatory T (Treg) cells. Inhibiting the drivers of Treg cell recruitment or expansion may potentially relieve immune suppression and enhance clinical benefit. Vice versa agonists of factors involved in Treg suppression may bring about the same effect. Especially combinations of treatment modalities, including standard-of-care radiotherapy or chemotherapy will ultimately lead to maximal clinical benefit. The contribution of biochemistry is relatively unexplored in this field, and we believe it will support cancer immunotherapy as detailed in **Chapter 4**.

VISUALIZING IMMUNE RESPONSES USING pMHCI MULTIMERS

Monitoring an individual's immune status and responses to treatment, as well as mapping of antigenic epitopes as therapeutic targets, can help diagnose disease and design treatment plans. The classical reagents used to visualize and monitor antigen-specific T cell responses consist of MHCI molecules loaded with antigenic peptides of interest[48]. The dissociation rate of pMHCI monomers is generally high and therefore multimerization is needed to achieve strong TCR binding required for further experimental analysis, such as flow cytometry[49]. The MHCI heavy chains are biotinylated and subsequently multimerized on streptavidin conjugated to a fluorophore for detection. Folding of heavy chain, light chain and peptide requires several time-consuming steps that must be performed for each studied peptide. A number of peptide exchange technologies have been developed to increase the throughput of pMHCI multimer generation a number of peptide exchange technologies have been developed[50-52]. Such technologies allow for folding of one large batch of conditional MHCI monomers with a peptide that, upon application of a defined trigger, vacates the binding groove. When performed in presence of a peptide of interest the complex will be loaded with this new peptide. One of the most commonly-used exchange technologies developed in our lab uses a photolabile peptide that is cleaved by UV radiation. However easy, some disadvantages of this technology include photodamage to the fluorophores, proteins or peptides, formation of reactive species and sample evaporation due to heat generation.

    Chapter 5 describes a novel technology that circumvents these drawbacks. This method is based on a previous study on temperature-dependency of peptide affinities for MHCI[53]. At low temperatures the on-rates and off-rates are similar between peptides, but at physiological temperatures the off-rates differ considerably. Based on this finding we designed low-affinity template peptides for the most-studied human- and murine MHCI alleles, HLA-A*02:01 and H-2K^b, by

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modifying the anchoring amino acids of known epitopes. By reducing the quality of the interactions between the peptide and the binding pockets one can form a pMHCI complex that is stable at low temperatures, but dissociates at increased temperatures. The conditional pMHCIs generated in this fashion can thermally be exchanged for any peptide of interest, provided that it has a higher affinity than the template peptide. As a major advantage, in contrast to UV-induced exchange that bleaches fluorophores, thermal exchange can also be performed on multimers, reducing pre-staining handling time significantly. Exchanged multimers stained similar percentages of clonal CD8+ T cells as UV-exchanged multimers or conventional multimers folded with the peptide of interest. **Chapter 6** contains a step-by-step protocol on how to produce and thermally-exchange conditional pMHCI multimers.

The study described in **Chapter 5** successfully verified the applicability of thermally-exchanged pMHCI multimers in a clinically-relevant setting. The exchanged multimers were used for monitoring CD8+ T cell kinetics in a human HLA-A*02:01+ transplant recipient in response to viral reactivation. The frequencies of detected CD8+ T cells were comparable between conventional multimers and those thermally exchanged ad hoc prior to staining, illustrating the efficiency and flexibility of the temperature-exchange technology. We have provided proof-of-principle for two alleles and the design of other exchangeable alleles is ongoing.

In clinical applications the amount of patient material is often limited and hence extensive analysis of T cell specificities may not be possible, since only a certain number of parameters can be measured simultaneously using flow cytometry, depending on the lasers equipped and the fluorophores used. To increase the number of measurable parameters combinatorial coding can be employed, but larger screens require a broader range[54,55]. **Chapter 7** describes a method that provides an essentially unlimited variety of pMHCI labeling, allowing a greatly enhanced screening range for T cells. By labeling each pMHCI with a DNA barcode and fluorescent label, multimer+ CD8+ T cells can be sorted and sequenced, so that many more specificities can be detected in parallel[56]. In this study, we used DNA-barcoded conditional pMHCI multimers to validate true neoantigens predicted from HLA-A*02:01+ human colorectal cancer patients. This particular cancer type is associated with increased microsatellite instability, resulting in DNA mutations that potentially give rise to neoantigens. Neoantigens arise from somatic DNA mutations later in life and therefore no central tolerance has been raised towards them[57]. Combined with the fact that they are only expressed on tumor cells, this makes them extremely suitable immunotherapeutic targets[58]. Because they are patient-specific neoantigens have to be identified per individual, a procedure that with the advent of next-generation sequencing has become readily available. Potential neoantigens were identified by DNA sequencing of tumor and healthy tissue and identification of transcribed antigens

through RNA sequencing[59]. Putative neoantigen sequences were then be matched to the expressed MHCI allotypes using NetMHC.

Proof-of-principle was provided by using DNA-barcoded multimers exchanged for common viral antigens to stain peripheral blood mononuclear cells of healthy volunteers. Some of these specificities were detected in the same volunteers in earlier studies and similar frequencies of CD8+ T cells were detected with exchanged multimers. In a next step multimers were exchanged for predicted neoantigens, derived from DNA sequencing of five colorectal cancer patients, with common viral antigens as control. Responses against some of these viral antigens were detected, but no barcode sequences corresponding to predicted neoantigens were retrieved. Because neoantigen frequencies are usually low it is not surprising that the number of CD8+ T cell specific for neoantigens is lower than those specific for viral antigens. Nevertheless, a hit detected in a previous (coculture) assay was expected to turn up. This particular peptide is a weak binder and may inefficiently be loaded on HLA-A*02:01 multimers, although it has a higher affinity than the template peptide. Likewise, a screen using a murine colorectal cancer model did not provide any hits above the detection threshold. This most likely is due to low cell numbers obtained from the thawed cell suspensions. We expect that careful revision of the experimental set-up will uncover the potential of DNA-barcoded pMHCI screens for discovery of antigen-specific CD8+ T cells responses.

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