Human Immunology 80 (2019) 1–4

Contents lists available at ScienceDirect

Human Immunology

journal homepage: www.elsevier.com/locate/humimm

Editorial

Perspective: HLA functional elements outside the antigen recognition domains

The introduction of high throughput sequencing technologies, namely Next Generation Sequencing (NGS), to the field of Immunogenetics has afforded a number of benefits/advantages deriving from the thorough and accurate characterization of HLA genomic sequences. It is well established that this technology has significantly improved the practice of HLA typing [1], impacting the clinical labs and creating new research opportunities in exploring the entire genomic sequence of the HLA genes for their unrecognized biological significance [2,3]. Our community is now at a stage of assessing the potential benefits of uniformly characterizing the whole HLA gene sequence, or, depending on the context of a clinical or research environment, focusing on particular HLA protein domain or genomic segment. These questions are juxtaposed to issues related to protocol simplicity, cost, operational capabilities and infrastructure or programmatic needs of different labs. Considering the well-documented significance of the antigen recognition domains (ARD) of the HLA molecule for antigen presentation and T cell activation, the question is whether the rest of the exonic, intronic or even 5′ and 3′ UTRs regions of the gene are of any investigative or clinical relevance.

The purpose of this special issue of Human Immunology is to present the work and views of a number of different experts in their respective fields, emphasizing the significance of regions of the HLA molecule other than the (ARD), or even focus on the ARD as this may be most relevant for particular applications. The purpose of this editorial is to attempt the synthesis of an overall picture of HLA immunobiology and offer an opinion, based not only on the viewpoints offered in this special issue but also on our experience and previously published literature, taking into consideration scientific benefits, clinical relevance and operational efficiencies. It is conceivable that the rich complexity of the topic may mean that there will be meaningful and reasonable dissenting views.

The first four articles in this special issue address the multiple functions of the transmembrane (TM) [4] and cytoplasmic domains of the HLA class II molecule [5–7]. Through these comprehensive and compelling reviews, the emerging picture is that the biological and immunological significance of these regions is undisputed. Moreover, it is clear that as we collect data on these segments of the molecule through our NGS characterization of the respective exons, we will be better positioned to explore how different genomic alterations within these segments influence disease susceptibility/progression or clinical outcomes in transplantation.

More specifically, as discussed by Dixon and Roy [4], the class II TM domain possesses multiple motifs that control both α-β chains pairing as well as the binding of membrane cholesterol. In addition, this region of the molecule is also the site of palmitoylation. The authors explain how the TM region of the class II molecule controls both the conformation of the class II molecule's extracellular domain as well as the partitioning of peptide-class II complexes into membrane sub-domains known as lipid rafts. This is central to MHC class II function as it is well appreciated that peptide-class II complexes clustered in lipid rafts are more efficient at activating CD4 T cells than non-raft peptide-class II complexes [8,9]. Hence, the class II TM domain is central to determining the "potency" of individual peptide-class II complex expressed on the cell surface, which can have profound impact on overall immune reactivity. Therefore, it can be concluded that the class II TM domain is not simply a way to link the extracellular and intracellular domains of the molecule, but rather that it plays a much more intricate and interesting functional role in class II immunobiology.

The next article by Thibodeau et al. [7] discusses the role of the cytoplasmic domains of the MHC class II and Ii proteins in controlling MHC class II trafficking. The cytoplasmic domain of the class II molecule controls both the egress of class II-Ii complexes from the endoplasmic reticulum (ER) as well as the trafficking of class II to and throughout the cell's endocytic pathway. Thus, the class II cytoplasmic tail will have a profound impact on both the spectrum of class II-associated peptides and the half-life of expression of each peptide-class II complex. The authors also discuss how MHC cytoplasmic domain polymorphisms could result in allele-specific alterations in class II trafficking and stability. This highlights the potential impact of this non-ARD region of MHC class II on the spectrum of presented antigen-derived peptides and the timeframe that T cells have to recognize these complexes, which would have profound effects on overall immune responsiveness.

In addition to controlling class II trafficking and peptide acquisition, the cytoplasmic domain of class II is the epicenter of some elements of MHC class II signaling, such as generation of cyclic AMP (cAMP) and activation of protein kinase C (PKC). These class II-generated intracellular signals are key to the development of a robust humoral immune response. In this special issue Harton discusses the current state of knowledge of signaling emanating from the MHC class II cytoplasmic domain, with a particular focus on B lymphocytes [5]. In addition, the author also discusses the potential impact of allelic variations between class II cytoplasmic domains on class II signaling and humoral immunity. This discussion illustrates the point that differences in immune responsiveness linked to HLA class II molecules don't necessarily need to impact peptide binding or T cell receptor (TCR) engagement, but could alter functions such as TCR-induced MHC class II signaling of antigen presenting cells, providing an alternative framework for the investigation and understanding of the molecular mechanisms underlying the linkage between HLA and multiple human diseases.

When we consider that multiple MHC class II functions such as ER egress and endosomal trafficking and signaling are centered on such a small molecular region (the cytoplasmic domain of each class II chain is

https://doi.org/10.1016/j.humimm.2018.11.005

Available online 14 November 2018

only approximately 15–20 aa in length), it raises the question of whether some of these cytoplasmic domain functions might be mutually-exclusive events when considered at a single molecule level. For example, would ubiquitination of the single lysine (K) residue in the class II β chain cytoplasmic tail, a process that controls class II trafficking [7], prevent the immediately adjacent GP signaling motif from driving cAMP/PKC signaling [5]? This consideration suggests a higher level of sophistication to the regulation of MHC class II function on a molecule-by-molecule basis.

While some may consider MHC molecule signaling a "fringe" area of MHC immunobiology, there is a long history of investigation in the area extending back to the mid-1980's (see [5], this issue) and more recent studies have rekindled new interest in this area of research (reviewed in [10,11]). Moreover, both MHC class I and class II are signaling molecules. While the literature is not as extensive for MHC class I, work from Reed and others has definitively established the signaling capacity of MHC class I molecules and shown a profound impact of class I signaling on transplanted organ survival [12–14]. For class II, a significant number of early studies focused on signaling driven by the molecule's cytoplasmic domain (see [5], this issue). However, class II signaling turned out to be much more complex, involving numerous class II-associated signaling molecules (reviewed in [11]). In this issue, Katikaneni and Jin discuss this aspect of class II signaling, focusing on the B lymphocyte [6]. Here, the authors pay particular attention to class II signaling not exclusively through the cytoplasmic domain but also via associated molecules that possess either stimulatory immune-receptor tyrosine-based activation motifs (ITAMs) or repressive immune-receptor tyrosine-based inhibitory motifs (ITIMs). This organization of multiple molecular units around the membrane proximal region of the MHC class II highlights the need for the immune system to have not just a way to activate a B cell, but a way to maintain a state of quiescence. The authors also provide a discussion of current clinical efforts to use class II signaling elicited by anti-class II monoclonal antibody (mAb) as a way to kill malignant B cells and treat leukemia/lymphoma. Thus, this set of four articles provides an insight into potential mechanisms of dependence of immune responsiveness or non-responsiveness on the class II molecule's ARD-distal segments. As such, any systematic interrogation of sequence polymorphisms in these genomic regions may reveal interesting connections with physiological and/or pathologic conditions.

The following two articles are both related to the HLA-DP locus. The first is a review building on the growing appreciation of the impact of NGS-revealed non-ARD variations. Klasberg et al. report on the pattern of non-ARD variation in more than 300 common and well-documented DPB1 alleles sequenced for the entirety of the DPB1 gene [15]. Their analysis reveals two highly divergent DPB1 allele clades (i.e., the A and G clades), which correlate with the previously defined 3′-UTR SNP rs9277534 A → G variant that is reflective of differences in HLA-DP protein expression. While the 3′-UTR SNP polymorphism may not be directly responsible for the differences in HLA-DP expression (see Shieh et al. [16], which is discussed below), there are over one hundred other HLA-DPB1 polymorphisms in linkage disequilibrium, some of which may actually contribute. This highly detailed look into a large collection of HLA-DPB alleles reveals the daunting task facing investigators as we try to understand the details of the impact of HLA polymorphism on HLA expression and overall immune reactivity.

Complementing the work of Klasberg et al., the second article by Shieh et al. reports on the potential roles of microRNA (miRNA) from across the genome in the regulation of HLA-DP expression [16]. Using a computational approach, hundreds of miRNA isolated from two human B cell lines or primary B cells were screened for possible targeting of polymorphic regions of the HLA-DPB1 gene. The authors identified two major miRNA target regions in the DPB1 gene, intron 2 and the 3′-UTR. This finding raises the possibility that there may be two sites of miRNA action in the potential control of HLA-DP expression: the nucleus where intron 2-targeted miRNA may function and the cytosol where 3′-UTR-targeted miRNA likely exert their effect. The 3′-UTR rs9277534 A → G variant, which has been directly linked to differences in HLA-DP expression (A: low expression, G: high expression) [17] depending on computational stringency conditions, was not the direct target of the miRNAs. Moreover, it was observed that the low expression A alleles are the targets of a larger number of *in silico* miRNA interactions as compared to G alleles, suggesting a greater level of miRNA-driven suppression of gene expression of the A clade of DPB1 alleles. The emerging picture is that regulation of expression of HLA-DP, at least the component that depends on miRNA binding, may be a phenomenon that engages different miRNAs at different numbers under different development states of the cell and possibly different tissues expressing HLA-DP, generating a continuum of possibilities for the control of expression of HLA-DP. These findings extend and complement previously published work on the role of miRNAs for the control of HLA gene expression [18–20] and the role of an HLA-encoded miRNA in the regulation of non-HLA encoded immune gene expression [2] and suggest a previously unappreciated role of miRNA in regulating HLA gene expression specifically and immune cell function more generally.

Further highlighting the high potential impact of NGS-based HLA typing, Sanchez-Mazas and Nunes present four vignettes illustrating the power of this technology as they apply it in their population studies. Their first example relates to studies they have performed on resistance to Malaria in Africa. NGS offered a real advantage in their population-based disease association studies as it provided clarity and precision in the HLA alleles associated with resistance; other antigenic specificities previously thought to be associated with resistance were eliminated. In the second vignette, the authors encourage application of NGS in detecting demographic signatures and population variation as the technology allows for a refined cataloguing of a population's history. Their third example underscores the role of NGS in understanding the evolutionary process that shaped the HLA diversity patterns observed in humans. Their fourth example emphasizes the role of NGS in understanding immune response mechanisms. In this example, they estimate the immune potential of an individual by measuring the relative increase of molecular distance (RIMD) and relative gain in peptide-binding coverage (PGPBC). Accounting for differences within a single locus and among loci, they propose the model of "*joint divergent asymmetric selection*" whereby, thanks to simultaneous involvement of multiple loci, different populations develop similar immune potential independently of limited divergence within a single locus. They believe their work has been aided significantly by performing NGS-based HLA typing. Finally, the authors provide a brief cautionary note on the potential pitfalls that can accompany the application of NGS-HLA typing in population studies, the critical element here being the identification of rare alleles in a population of limited size. Allele frequencies derived by NGS can be misinterpreted when adequate statistical conditions (i.e., large sample sizes) are not fulfilled.

The next article by Petersdorf and O'hUigin details the power of NGS-based analysis of the HLA genes and of the MHC for the study of both coding and non-coding polymorphisms [21]. They summarize our current state of knowledge of non-coding variations and their impact on HLA gene expression, the impact of evolution on shaping HLA gene expression, and the linkage between allele-specific differences in HLA protein expression (regulated by multiple defined and undefined mechanisms) and disease. They also make a strong argument that the phasing of HLA polymorphisms within both coding and non-coding regions through NGS-based approaches will represent an important step forward in understanding HLA immunobiology. Furthermore, a complete haplotype map for the 4 Mb sequence of the MHC will greatly enhance our understanding of concepts of linkage disequilibrium and the necessity that has developed through evolution for the coexistence of many coding and non-coding polymorphisms. The authors also remind us of the clinical significance of phasing the 4 Mb sequence of the MHC in increasing the success of hematopoietic cell transplantation. Considering that "The MHC has [more] associations to human disease

than any other region of the human genome" and that the MHC is "the cornerstone of the immune response," its detailed characterization through NGS will be instructive and necessary in explaining many immunologically related phenomena, whether physiologic or pathologic. They conclude that "the benefits of matching beyond the ARD are immense and quantifiable."

One of the major driving forces behind our investigation of HLA immunobiology is the desire for a better understanding of human health and disease. Therefore, the last two installments in this special issue address the non-ARD-based aspects of HLA biology from a more clinical/applied perspective.

Hurley and Ng discuss the issue of considering non-ARD HLA polymorphisms in clinical medicine [22]. The authors' recommendation is that "clinical typing should continue to focus on typing and matching at the level of the antigen recognition domain (ARD) with the exception of common non-expressed alleles that are distinguished by variation outside of the ARD." The authors also state that "at present there is insufficient data to support extending typing to include regions outside of the antigen recognition domain in clinical decision making." The authors clarify that their focus is on the clinical utility of information beyond the ARD genomic/molecular regions and that the information on ARD-focused HLA assignments can come from different technologies, including Sanger sequencing or NGS. Phasing information can come from NGS, but not always, and Sanger sequencing needs to be supplemented by SSO, SSP or sequence-specific primers. Clearly there is a distinction of the information needed for clinical decisions (ARD vs. whole HLA molecule/gene) and methods to obtain that information. The authors recognize the significant role of NGS for a more complete characterization of the HLA genes/molecules in the research domain, but suggest that this level of characterization is not currently justified for clinical testing. In summary, they base their recommendation to continue to focus clinical typing on the ARD on: "1) the low frequency of variation outside the ARD, especially for non-expressed alleles, suggesting that mismatching will be uncommon; 2) the role of the ARD and linkage of all allelic polymorphisms to the ARD in determining expression variation; (3) data from limited cellular studies of allorecognition; (4) the complexity of typing regions outside the ARD; and 5) the potential confusion with matching and search." [22] However, it remains for our community to utilize the available technologies that provide complete characterization of the HLA genes and demonstrate relevance for clinical applications. Recently, it was announced by Mayor et al. ([23] and https://doi.org/10.1016/j.bbmt.2017.12.612) that better HLA matching, achieved when typing is done at ultra-high resolution that includes exons outside the ARD, introns and untranslated regions, can significantly improve outcomes for recipients of an unrelated donor HCT for a haematologic malignancy. These preliminary studies need to be assessed in the context of the outcomes of both related and unrelated donors with identical HLA profiles to conclusively document the benefits of matching at the allele level. Furthermore, an additional recent report (https://doi.org/10.1016/j.humimm.2018.07.010) regarding the assessment of donor specific antibodies against the α 2 domain of DQα chain, which is outside the ARD, implies the potential danger of relying on P group designations when performing virtual crossmatches or unrelated stem cell searches for patients with complex HLA sensitizations. Some may argue these are rare events, but it is only after we have the means to thoroughly assess these phenomena with our available technologies that the real frequency of these events can be determined. It is our opinion that as NGS is utilized by more and more laboratories, not because we need to assess the non-ARD domains but simply because it is more practical and cost effective, our community will be able to unequivocally determine the relevance of non-ARD domains in clinical practice. Our recent experience (Monos, personal communication) in utilizing NGS HLA typing to support our solid organ transplant programs has demonstrated that more than 40% of cases, whether pre-transplant or post-transplant, need HLA typing at the two-field resolution level to address the many scenarios we encounter in daily practice. We expect further developments in the near future that will shed light to our current practices. Thus, Hurley and Ng appropriately note that their recommendation is "for the present".

The final article in this special issue comes from a practicing clinician who strives to use our greater understanding of HLA immunobiology to understand the immunopathogenesis of sarcoidosis, a multifaceted HLA-linked disease of varying severity. As reviewed by Judson, current thought in the field as to the cause of sarcoidosis is that aberrant HLA class II-restricted antigen presentation leads to inappropriate activation of Th1 and/or Th17 cells, leading to disease-defining granuloma formation [24]. However, there are multiple non-sequiturs that suggest a more complex story, such as the presence of disease-associated HLA alleles in a significant fraction of the general population that does *not* show any sign of disease. Here, it is possible that future research may reveal that HLA polymorphisms outside of the ARD may impact HLA biology or immune cell function and leading to disease, which would have profound impact on the development of new and better clinical treatments.

In summarizing the essence of NGS and its impact in the field of immunogenetics, Sanchez-Mazas and Nunes state in their article: "*if we have the information and we need it, we use it. If we do not need it, we do not use it. But, if we do not have it then we cannot use it, whether we need it or not. This is, basically, the idea of NGS; have more information. If the effort, cost and time to have more information is not prohibitive, then have it*" and we importantly add "*most likely you will use it*".

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