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New Molecular Insights Into HLA Immunogenicity

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To my dear parents
And to my beloved Brother and Sister
Who give me the greatest gift ever given
They always believed in me.

To Nicoletta,
Without her this could not be possible

"Stay Hungry, Stay Foolish"
S. Jobs quoting the Whole Earth Catalog

"Non smettere mai di sognare....
I sogni non costano nulla, sono gratis
e se qualcuno cerca di infrangerteli, te ne puoi fottere,
perché sono l'unica cosa che non ti potranno mai portare via.
I Sogni sono la Linfa della nostra Vita, non smettere mai di averne,
qualunque cosa succeda, continua a Sognare..."
M. Mazzoli

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Chapter 1

General Introduction

The term immunogenicity refers to the ability of some molecules to stimulate immune response. In physiological conditions, this response is activated in order to defend the organism from infections caused by microbes and viruses. Defense from these pathogens is evoked by recognition of their molecular components as foreign or non-self antigens, in contrast to antigens expressed by the organism itself referred to as self antigens.

Several non-physiological conditions can elicit pathological activation of the immune system. One of these conditions is the transplantation in which allogeneic antigens expressed by transplanted tissues are recognized as non-self by the autologous immune system, determining unusual activation of immune response. Such immune activity is referred to as alloreactivity.

The major role in this unusual response is played by the Human Leukocyte Antigen (HLA) system, a complex set of polymorphic proteins that acts as immunological target for recognition by T cell receptors (TCR) expressed on alloreactive T cells. Therefore, immunogenicity of such antigens controls alloreactivity after transplantation and constitutes the molecular basis for several clinical events occurring after treatment with this procedure.

The overall aim of this thesis is to disclose the molecular nature of HLA immunogenicity in order to better understand alloreactivity. Below are briefly reported what is known about HLA molecules and

their interaction with TCRs, and their importance on hematopoietic stem cell transplantation (HSCT).

The Human Leukocyte Antigens

The physiological function of HLA system is to present different peptides derived from processing of pathogen's antigens to T lymphocytes in order to evoke a specific adaptive immune response. The HLA system includes a set of 6 highly polymorphic molecules, which are mainly divided into two classes, namely HLA class I and HLA class II [1]. HLA class I molecules are constitutively expressed on the surface of almost all nucleated cells and activate immune response of CD8+ T cells. On the contrary, HLA class II molecules are expressed only on professional antigen presenting cells (APC), including dendritic cells, B lymphocytes, macrophages and some other cell types, and they activate the CD4+ T cell response.

HLA Genetics

The components of HLA system are encoded by genes located into a region of about 4 Mb on the short arm of chromosome 6, namely at position 6p21 [2]. A total of 253 loci have been mapped in this region although many of them have no functional role in immunity [3].

This genomic region is traditionally divided in three minor regions, namely class I, II and III, containing several genes implicated in immunity (Figure 1) [1]. Class I region contains genes encoding for classical (HLA-A, B, C) and non-classical (HLA-E, F, G) HLA class I molecules. In the class II region are located the classical HLA class II

genes HLA-DRA, DRB, DQA1, DQB1, DPA1, DPB1, DMA, DMB, DOA and DOB. Finally, class III region does not contain any known HLA class I and II-like genes; however, it contains several genes implicated in immunity.

One of the most important characteristics of HLA genes is their high polymorphism. A total of 7269 allelic variants (5518 in class I alleles, 1612 in class II alleles, 139 in other non-HLA alleles) have been registered in the IMmunoGeneTics HLA (IMGT/HLA) database (release 3.7 January 2012, http://www.ebi.ac.uk/imgt/hla/) [4]. This huge polymorphism is worldwide distributed in the human population and it is the major factor determining alloreactivity.

Proteins and function

HLA molecules are heterodimers structurally evolved to bind peptides deriving from different source and present them to the receptor expressed on T lymphocytes. Protein structures and functions vary between the two HLA classes of molecules (Figure 2) [1]; as briefly described below.

- HLA class I [5]. These molecules are heterodimers consisting of two noncovalently linked subunits: a highly polymorphic α-chain, encoded by one of the three classical HLA class I genes, is associated to the invariant chain β_2-microglobulin, a protein encoded by a non-HLA gene. The α-chain contains a transmembrane region that anchors heterodimers on the extracellular side of cell membrane. Moreover, this subunit is folded in three different extracellular domains functionally

relevant: α1 and α2 domains are assembled to form the peptide binding groove, while α3 domain serves as binding site for the CD8 coreceptor expressed on CD8+ T lymphocytes. HLA class I heterodimers are expressed in almost all nucleated cells and they bind peptides deriving from processing and degradation of intracellular proteins. Peptides of 8-9 amino acids in length can be bound into the peptide binding groove stabilizing the heterodimer and allowing its expression on cell surface.

- HLA class II [6]. These molecules are heterodimers of two subunits encoded by two polymorphic HLA genes; both genes are polymorphic although α-chain is less polymorphic than β-chain. The two subunits are very similar and each contains two extracellular domains: α1 and β1 domains, associated to form the peptide binding groove, and α2 and β2 domains, responsible for the binding of CD4 coreceptor, although β2 domain is predominantly involved. These molecules are expressed on specific APC presenting peptides derived from the processing of extracellular proteins. The peptide-binding groove in these molecules is larger than in HLA class I molecules, so that longer peptides (13-25 amino acids in length) can be accommodated. A complex machinery is required for the processing and binding of peptides in these molecules, including other HLA genes such as DM and DO proteins.

Gene expression of both classes is enhanced by cytokines, produced during active immune response.

In both classes, polymorphism is mainly focused on gene regions encoding the peptide binding groove determining different peptide affinities for each allele and, consequently, altering the peptide repertoire presented to TCR.

Figure 1: Location and organization of the HLA complex on chromosome 6 [1].

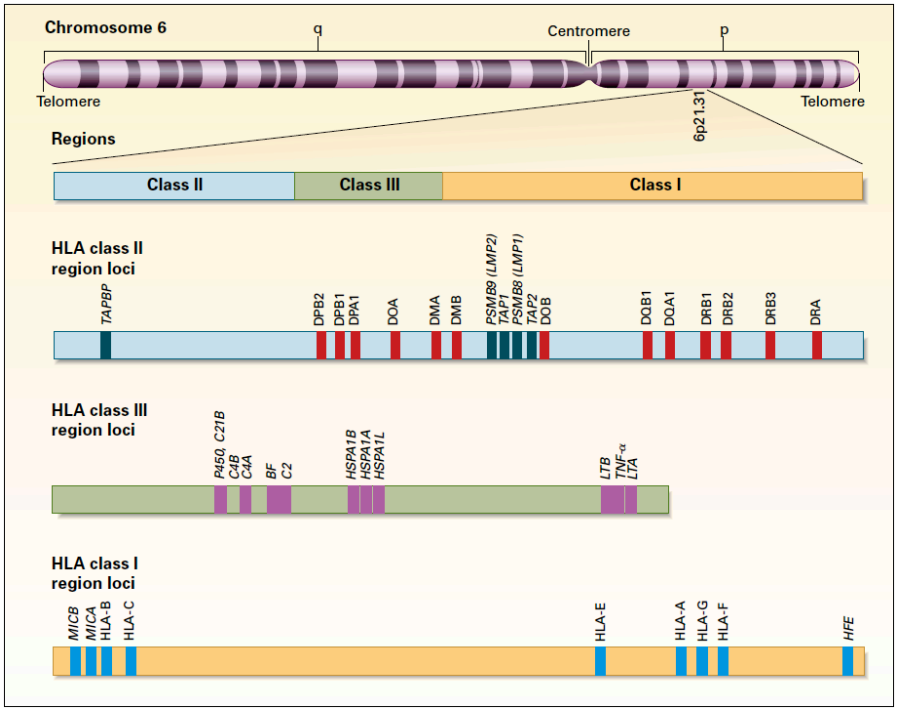
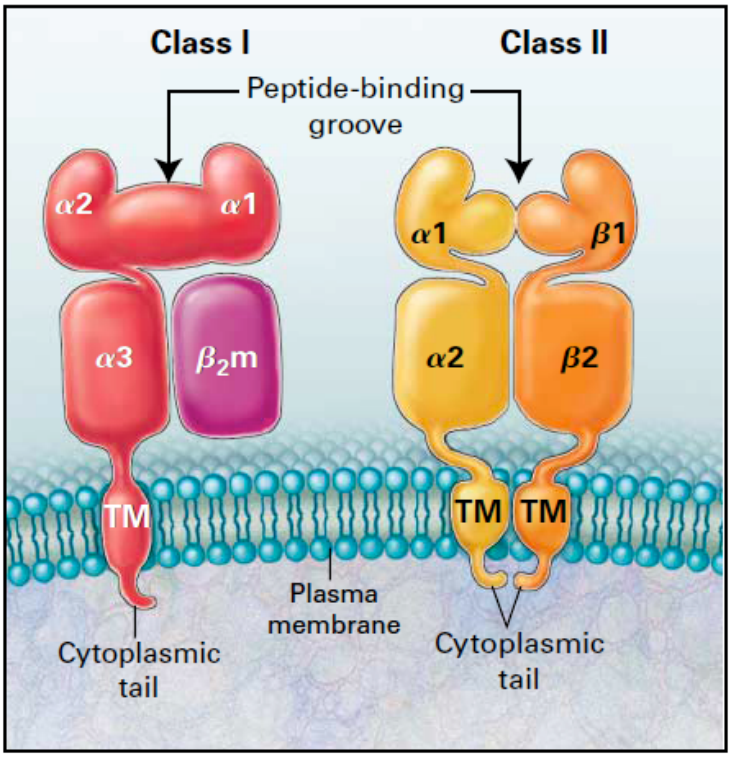


Figure 2: Structure of HLA class I and class II molecules [1].



The T Cell Receptor

Specific activation of T lymphocytes derives from recognition of antigenic peptides bound to HLA molecules (p/HLA complex) by specific TCR expressed on the surface of these cells. These molecules are highly heterogeneous in the pool of circulating T cells constituting a large repertoire in order to warrant responses to a wide spectrum of pathogens. However, they are exquisitely specific for foreign antigens and do not recognize self-molecules. This specificity is obtained during thymic development of immune competent cells, through a complex mechanism involving positive and negative selections.

Genetics and protein structure

The TCR is a heterodimer composed of two transmembrane subunits, named α and β, linked by a disulfide bond. Each α-chain and β-chain, encoded by TCRA and TCRB genes respectively, contains two Ig-like domains, one variable (V) and one constant (C), a transmembrane region, and a short cytoplasmic region [7]. The V domain contains 3 strings of residues extremely variable in terms of amino acid sequence. Each string is defined as complementarity-determining region (CDR-1, 2 and 3), and their structural juxtapositions form the TCR portion that specifically recognizes antigens presented by HLA molecules. Different genetic segments, named V, D and J, encode for CDRs, and different variants of each segment are repeated several times along TCR genes [8]. Consequently, during T cell maturation, their complex combination and arrangement allow the generation of a wide repertoire of TCRs with different specificity.

Thymic development of the pre-immune TCR repertoire

Immune-competent T cells clonally express only one TCR able to discriminate between self and non-self. This efficient specificity is achieved after a long maturation process occurring in the thymus [9]. In this primary lymphoid organ, T cell progenitors, coming from the bone marrow, complete the somatic recombination of the TCR gene segments generating functional genes encoding for only one TCR [8]. This process is completely random so that only those cells able to produce TCR with high affinity for self-p/HLA complexes receive survival signals (positive selection) [10] and can proceed to the other maturation steps while any T cell unable to express such TCRs do not receive survival signals and die by neglect. Moreover, some T cells could show a high affinity for self-pHLA complexes. These dangerous cells, prone to be autoreactive, are eliminated before they exit from the thymus by induction of their apoptosis (negative selection) [11]. The resulting repertoire of T cells, survived to this complex selection, undergoes to subsequent terminal maturation and exits from thymus as mature naïve T cells. This complex maturation process generates cells not reactive to the self peptides presented in the context of autologous HLA molecules (p/autoHLA), thus ensuring no autoreactivity. However, they can be able to recognize self peptides loaded by allogeneic molecules (p/alloHLA) determining alloreactivity. Indeed, naïve T cells are frequently cross-reactive to p/alloHLA complexes as previously reported in literature [12-14].

Modulation of TCR repertoire by immune response

Thymic development generates a wide repertoire of T cells expressing different TCRs. This repertoire is further modified after exposure to foreign antigens and activation of the adaptive immune response. Indeed, T cells expressing specific receptors recognize such antigens with high affinity and become activated. This activation results in the clonal expansion of antigen specific T cells followed by their differentiation in effector and memory T cells. Effector cells mediate clearance of the specific antigen while a large number of memory T cells keeps that specificity for further immune responses. Therefore, the effective TCR repertoire ultimately results from the following two processes: thymic development generates a huge heterogeneity of TCRs avoiding autoreactivity, while immune response preferentially expands TCRs useful to fight pathogens infection.

Several studies demonstrated that also pathogen specific memory T cells are commonly cross-reactive to allogeneic HLA molecules [15-17]. In such context, alloreactivity can derive from both naïve and memory T cells, but the key of their reactivity resides on the ability of their TCRs to specifically recognize allo-HLA molecules.

The interaction between pHLA complex and TCR

T cell activation needs specific and high-affinity interaction between the TCR and the complex pHLA. This interaction is particularly complex at the structural level, involving large and conformationally plastic surfaces of the two molecules [18]. Several evidences supported the idea, originally proposed by Jerne, that TCR/pHLA

interaction is due to a germline encoded mechanism [19] [20]. According to this, evolutionally conserved anchor points in the HLA molecule are used to dock TCR with invariant modality. Indeed, the analysis of crystal structures solved until now, showed a recurrent docking mode between these two molecules. In such orientation, TCR-Vα and Vβ contact α-helices forming the walls of the peptide binding groove while their CDR regions scan the peptide loaded inside the groove [21] [22].

TCRs are educated during thymic selection to maintain this modality of interaction only with self-HLA molecules, a condition defined as "HLA-restriction". In such context, allorecognition constitutes an unconventional event in which educated TCR recognize non-self HLA molecules, although increasing evidences support the idea that such unconventional events follow conventional rules.

Theories on allorecognition

The most relevant feature of this phenomenon is its high frequency. Alloreactive T cells are about 100- to 1000-fold more frequent than T cells specific for any single foreign antigens [23], and different models have been proposed to explain such huge difference.

A general distinction is made on direct and indirect allorecognition [24]. The former model describes alloreactivity as consequence of the direct interaction of alloreactive TCR with allogeneic pHLA complexes. In the latter, alloreactivity is a consequence of a conventional self-HLA restricted recognition, in which peptides derived from the processing of allogeneic HLA proteins are presented

in the self-HLA context. The direct mechanism of allorecognition is traditionally considered the most relevant on triggering clinical events after transplantation.

Several attempts to describe the direct alloreactivity have been made leading to two major theories, differing on the contribution conferred to HLA molecules and peptides on allorecognition [25]. The first theory was called “antigen density model” and describes alloreactivity as an effect of activation of alloreactive T cells, expressing degenerate TCRs, able to directly recognize the polymorphism within HLA molecule in a peptide independent manner. In this model, the expression of a huge quantity of HLA molecules on APC compensates the lower affinity of these cells. In the second theory, named “determinant frequency”, allorecognition is peptide dependent and allogeneic HLA molecules, similar enough to the self-HLA, are able to present their peptides as foreign. This phenomenon could be due to a different peptide repertoire for which autologus T cells are not negatively selected or to common peptides that are presented in uncommon conformations.

Several evidences supported this second theory, demonstrating that alloresponse is limited by the endogenous peptide repertoire presented and observing in the pool of alloreactive T cells different single peptide specificity [26, 27]. Moreover, structural analysis of pHLA/TCR complexes showed that the bound peptide contributes to this interaction with a significant number of TCR contacts [28, 29].

Cross-reactivity is the molecular basis of allorecognition

As previously mentioned, somatic recombination during thymic development is a completely random process able to virtually generate a huge number of unique TCRs with different specificity. Although this huge repertoire undergoes to selective maturation processes during development in the thymus, a very high number of TCRs is efficiently generated and becomes competent to trigger immune response against several antigenic peptides. However, this is not enough because a careful estimation of the peptide repertoire showed that potentially immunogenic peptides in the environment are much more numerous than the number of different TCRs at any moment [30].

One of the most relevant characteristics of TCR is its cross-reactivity that is the ability to recognize more than one specific pHLA complex. In this way, the TCR repertoire specificity is expanded and provides a broad protection against infective agents. In parallel, this feature makes TCRs prone to be alloreactive.

Thanks to the growing number of studies focused on pHLA/TCR interactions, today five mechanisms of cross-reactivity have been described (Figure 3) [22, 30] and are briefly reported below.

- Induced Fit [31].

  A single TCR can be able to bind different ligands through structural adjustments of its CDR loops. Mazza et al observed this phenomenon for a single TCR, named BM3.3, which was able to recognize three different peptides bound to the same molecule. Comparison of the corresponding crystal structures

showed that TCR changes the conformation of CDR loops, modifying its binding site to best accommodate different ligands.

- Differential TCR Docking [32].
  In this case, binding of the same TCR to different pHLA complexes can be achieved by different docking orientations. Colf et al, reported an example of such cross-reactivity showing the ability of the same TCR to interact with different ligands through different binding modes.

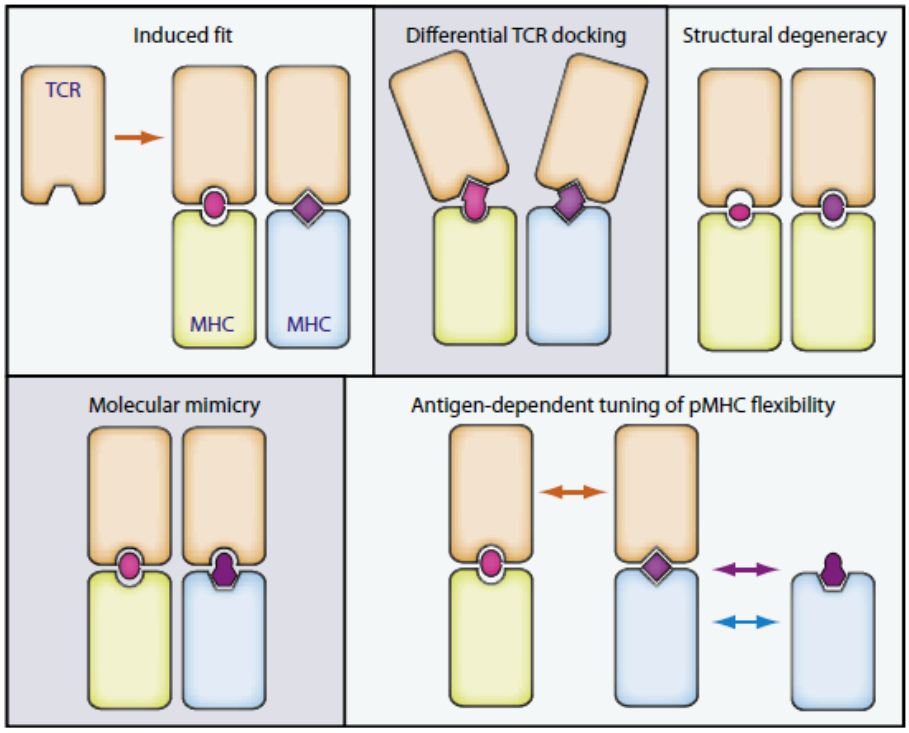
- Structural Degeneracy [33].
  A reduced number of specific interactions can allow the same TCR to recognize in a degenerate way different ligands. Such structural degeneracy confers to TCR a degree of tolerance to some substitutions in TCR contacting positions, resulting in cross-reactivity. For example, Li et al showed that TCR 3A6 is able to recognize a peptide derived by myelin basic protein bound to HLA-DR2a as well as other different and more immunogenic peptides, revealing a very poor contact interface without any hydrogen bond or salt bridges between the TCR and the peptide.

- Molecular Mimicry [34].
  These terms mean that TCR ligands share key structural and chemical features, allowing crow-reactivity. This mechanism

was one of the first proposed to explain cross-reactivity involved in both allorecognition and autoimmune diseases. However, clear structural evidence was elusive until Macdonald et al provided it in 2009. They reported that TCR LC13 was able to recognize an immunodominant epitope of Epstein-Barr virus restricted by HLA-B*08:01. Moreover, it was also able to cross-react with two different allopeptides presented by B*44:02 and B*44:05. The comparison of their crystal structures revealed that LC13 recognizes the three different ligands with the same overall topology despite of differences between allo- and viral- peptides. Key interactions with anchor residues are preserved in this mechanism.

- Antigen-Dependent Tuning of Peptide-HLA Flexibility [35].
  Cross-reactivity can be due to conformational flexibility in the pHLA complex allowing recognition of different ligands by the same TCR. As reported by Borbulovych et al, adjustment of pHLA complex can adapt the ligand for TCR interaction. In this case, TCR selects one of two or more conformations in dynamic equilibrium.

Figure 3: Mechanism for an individual TCR to cross-react with different pHLA ligands [30].



Alloreactivity and immunodominance of HLA antigens

All factors described above affect the allogeneic T cell response determining existence of immunodominance hierarchies between HLA antigens.

The term "immunodominance" was originally referred to the ability of various antigens to induce stronger humoral response respect to other antigens [36]. However, this concept was later extended to anti-viral and anti-bacterial immune response. More recently this was applied to minor Histocompatibility Antigens [37] observing this phenomenon in the context of both anti-tumoral immune response and alloreactivity. Recent studies suggested that some HLA molecules could elicit stronger response than others [38]. In particular, this was observed for some HLA-B molecules respect to HLA-A ones, although this bias was dependent on the self-HLA and gender context.

Mechanisms underlying such hierarchies are still unclear and more efforts are required to be completely disclosed. However, antigen processing and presentation seem to play a major role in this mechanism , so that immunodominant determinants are efficiently processed by proteasome, then efficiently bound by HLA molecules and finally presented to specific T cells, which are very frequent in the T cell repertoire [36]. Processing and presentation of such immunodominant peptides and their association with allogeniec HLA molecules could influence the immunogenicity of these molecules. Therefore, a better understanding of the mechanisms underlying immunodominance will subsequently improve our comprehension of immunogenicity of HLA molecules and their role in alloreactivity.

Allogeneic hematopoietic stem cell transplantation

Today allogeneic HSCT is a common treatment for a variety of diseases although it is primarily applied for hematologic and lymphoid cancers [39]. Moreover, onco-hematological patients, who undergo to HSCT, are treated with myeloablative doses of radiation and/or chemiotherapy in order to increase eradication of tumor cells from bone marrow. However, such high chemio/radiotherapeutic doses also have killing collateral effects on healthy stem cells, consequently destroying the hematopoietic function. For this reason, transplantation of a new hematopoietic system from allogeneic donors is required in order to rescue the hematopoietic function of patients.

The major complication of this procedure is the Graft versus Host Desease (GvHD) [40]. Such clinical event is consequent to an alloreactive ummune response of the donor immune system against the patient tissues recognized as non-self. GvHD occurs in both acute and chronic form, characterized by distinctive clinical and histological features.

Together with risk of GvHD, allogeneic HSCT is also associated with a lower risk of tumor relapse due to an anti-tumor immune reaction mediated by alloreactive T cells. Such phenomenon is referred to as Graft versus Leukemia (GvL) effect [41]. In particular, in haploidentical HSCT this immune pressure on leukemic cells is remarkable due to the high degree of mismatches in the HLA loci, and it becomes evident when relapsing leukemic cells show loss of the mismatched HLA haplotype [42].

Therefore, the challenge in allogeneic HSCT for malignant disease treatments is to prevent GvHD without losing the GvL effect.

Understanding the molecular basis of alloreactivity could be extremely useful to improve the performance of this treatment in such direction.

The best suitable donor

The degree of HLA matching between donor and recipient is one of the major factors affecting the outcome of allogeneic HSCT [43]. In order to reduce the GvHD risk, selection of a compatible donor is one of the most relevant issues in transplantation.

In current opinions, HLA-identical siblings are considered the best donors for HSCT. However, only one-third of patients with indication of HSCT have such a donor available. An alternative option is the employment of unrelated donors [43]. This option was extremely facilitated by the huge growth in the past 20 years of national and international donor registries that allowed to search for HLA-matched unrelated donors all around the world (http://www.bmdw.org/). Current guidelines indicate that the best unrelated donor should be perfectly "allele" matched with recipient at the classical HLA loci, HLA-A, B, C, DRB1 and DQB1 ("10/10") [43]; while DPB1 is poorly considered due to its weak linkage disequilibrium with the other loci.

However, exploitation of mismatched donors to improve the outcome of HSCT performed as curative treatment for onco-hematological patients is particularly promising. HLA mismatches are associated with a reduced relapse risk due to GvL effect [44]. This observation raised interest in a new "functional" matching approach, respect to the classical "allelic" matching [45]. This approach is intended to define

“permissive” and “non-permissive“ mismatches on the basis of their functional impact on alloreactivity, in order to improve the performance of HSCT by reducing GvHD while keeping high GvL effect. To disclose the molecular basis of such permissive and non-permissive mismatches is particularly attractive, and it offers the possibility to take more intelligent and patient-specific decisions during the selection of unrelated donors for HSCT.

Immunogenicity of HLA-DPB1: towards functional definition of HLA permissiveness

Initial definition of “permissible” or “acceptable” was made in the context of solid organ transplantation by observing, in clinical practice, successful outcomes also after treatment in presence of major HLA mismatches [46]. Such mismatches were not able to elicit strong immune responses in a large cohort of single HLA-A, B, or DR antigen matched kidney transplantations. On the contrary, some HLA mismatches were associated with a significant increased of graft rejections and defined as “taboo” mismatches [47, 48].

In parallel, several attempts have been done in the context of HSCT. In some cases, the definition of permissiveness was addressed through definition of which amino acid substitutions or polymorphic regions are important for allorecognition [49-51]. Other groups addressed this problem looking at the whole structure of the HLA molecules. This approach lead to two different algorithms of matching, HLAMatchmaker and HistoCheck, which have been proved to be not predictive of T cell alloreactivity [52, 53].

A particular interest relies on locus DPB1. As mentioned above, this locus is in weak linkage disequilibrium with other HLA loci and, for this reason, it is often found mismatched in HSCT even though other HLA loci are fully matched. This offered the possibility to study alloreactivity directed to this locus independently from mismatches to other HLA loci.

In 2001, Fleischhauer et al showed that a particular DPB1 allele, DPB1*09:01, was target of alloreaction determining a case of rejection after HSCT [54]. Subsequently, the same group isolated two T cell clones from this patient, named 501 and 538, and demonstrated, through cross-reactivity test against a panel of B cell lines bearing different DPB1 alleles, that a DPB1*09:01 encoding alloreactive T cell epitope (TCE) was shared with different DPB1 alleles. On the basis of this cross-reactivity, patterns of DPB1 alleles were divided into three different groups with predicted differential immunogenicity: high (TCE group 1: DPB1*09:01, *10:01, *17:01), intermediate (TCE group 2: DPB1*03:01, *14:01, *45:01), low (TCE group 3: all others) immunogenicity [55].

Starting from the assumption that more immunogenic alleles were able to encode a clinically relevant T cell epitope, Fleischhauer et al proposed a new functional matching algorithm for DPB1 alleles, named TCE3 algorithm. According to this algorithm, allelic mismatches within the same TCE3 group were considered "permissive", while mismatches including alleles in different groups were defined "non-permissive". Application of this algorithm in a retrospective study demonstrated that such matching algorithm is

predictive of the outcome after transplantation in a cohort of 118 HSCT [55].

In a subsequent study, Crocchiolo et al refined TCE3 algorithm by integration with functional data reported in literature thus proposing a new algorithm called TCE4 [56]. They suggested a new classification of DPB1 alleles subdividing the previously mentioned TCE group 3 in two subgroups TCE4 group 3 and 4. Again, a retrospective study performed in a cohort of 621 HSCT, facilitated through the Italian Registry for onco-hematologic adult patients, demonstrated the clinical relevance of such matching approach. Recently, a new retrospective study was performed in a cohort of 8539 HSCT submitted to the International Histocompatibility Working Group [57]. This study definitively proved the impact of "permissive" matching on the outcome after HSCT, thus reducing incidence of GvHD respect to "non-permissive" mismatches while keeping low the risk of relapse respect to DPB1 allele matched.

DPB1 constitutes a good example of how it could be useful to define of HLA permissiveness in the context of HSCT. To achieve this aim it will be necessary to improve our knowledge about HLA immunogenicity in order to exploit HLA mismatches in an intelligent way.

Scope of the thesis

The aim of my thesis is to define the molecular basis underlying the clinical relevance of permissive and non-permissive DPB1 mismatches in order to improve our understanding of the HLA immunogenicity in the context of transplantation.

My results are described in different chapters after the introductive **Chapter 1**. In **chapter 2** we demonstrated the linkage between permissive and non-permissive DPB1 mismatches and the intensity of alloreactive immune response. We observed that alloresponse in non-permissive conditions were significantly higher than in permissive conditions. In **chapter 3**, we characterized DPB1-encoded T cell epitopes at the molecular level. We addressed this question through site directed mutagenesis of DPB1*09:01 (HLA-DP9), a prototype of the most immunogenic alleles, by using homology-modeling approach to drive mutagenesis to the most relevant polymorphic residues. A panel of different DP specific T cell lines and clones was used to test the impact of mutagenesis on allorecognition. In **chapter 4** and **chapter 5**, it is described the identification and homology modeling of 3 new allelic variants of HLA-A. An homology modeling approach was used to investigate the role of amino acid substitution encoded by new alleles. In **chapter 6**, a summary and general discussion describes the conclusions obtained in this thesis illustrating the new molecular insights on HLA immunogenicity achieved in this work.

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Chapter 2

Significantly higher frequencies of alloreactive CD4+ T cells responding to nonpermissive than to permissive HLA-DPB1 T-cell epitope disparities

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Letter to the editor:

Increasing evidence suggests that donor-recipient disparities for human leukocyte antigen (HLA)-DPB1 can be of clinical importance in unrelated hematopoietic stem cell transplantation (HSCT) [1]. Two overlapping algorithms for functional T-cell epitope (TCE) matching involving 3 (TCE3) or 4 (TCE4) groups of DPB1 alleles have previously been shown to be significantly predictive of survival after 10/10 and 9/10 matched unrelated HSCT [2, 3]. In both TCE3 and TCE4, nonpermissive mismatches are directed against 2 groups of immunogenic antigens encoded by DPB1*09:01, 10:01, 17:01 (TCE3/4 group 1) and DPB1*03:01, 14:01, 45:01 (TCE3/4 group 2), respectively [2, 3]. In TCE3, all other frequent DPB1 alleles including DPB1*02:01, 04:01, 04:02 and others are classified as poorly immunogenic TCE3 group 3, and DPB1 mismatches against these alleles are predicted to be permissive [2]. In TCE4, TCE3 group 3 is further subdivided into 2 separate groups comprising DPB1*02 (TCE4 group 3) and the other alleles (TCE4 group 4), with intermediate and poor immunogenicity, respectively [3].

Rutten and colleagues have recently shown that T-cell responses could be obtained against DP antigens from all 4 groups [4, 5], thereby confirming the observations that led to the discovery of the DP locus by primed lymphocyte testing [6], as well as those obtained later in mixed lymphocyte reactions (MLRs) [7, 8]. Interestingly, Rutten and colleagues observed high levels of cytokine production by CD4+ T cells in response also to autologous DP molecules presumably

presenting minor histocompatibility antigens [5], suggesting that the HeLa cell transfectants expressing DP but not other class II antigens used in their experiments may not quantify physiologic frequencies of alloreactive T helper cells, which increase substantially with the number of mismatched HLA-DP alloantigens in classical in vitro assays[9, 10].

Here, we have quantified the frequency of alloreactive CD4+ T cells responding to permissive or nonpermissive TCE3 or TCE4 DP mismatches, in classical MLRs between peripheral blood mononuclear cells (PBMCs) of responder (R)–stimulator (S) pairs matched for 10/10 of the non-DPB1 alleles. When S presented both a permissive and a nonpermissive mismatch, the percentage of responding CD4+ T cells was more than 10-fold higher against the nonpermissive (DPB1*09:01, 10.65%) compared with the permissive mismatch (DPB1*04:02, 0.88%; Figure 1A), and this result was highly reproducible in 3 independent experiments (data not shown). Importantly, in 24 MLRs, we found a consistently higher percentage of CD4+ T cells responding to nonpermissive DPB1 mismatches according to TCE3 (n=9; mean 10.13% ± 7.51%; Figure 1B left panel) or TCE4 (n=14; mean 7.72% ± 6.96%; Figure 1B right panel), compared with permissive mismatches according to TCE3 (n=15; mean 2.34% ± 2.82%; Figure 1B left panel) or TCE4 (n=10; mean 1.81% ± 2.82%; Figure 1B right panel). In the Kruskal-Wallis analysis of variance, this difference was significant both for TCE3 ($P < .05$) and TCE4 ($P < .05$). Responses against DPB1*02 (TCE4 group 3), classified as permissive for TCE3 but nonpermissive for TCE4, were significantly lower than those against TCE3/4 groups 1 and 2 (10.13%

± 7.51% and 3.39% ± 2.8%, respectively; $P < .04$, Mann-Whitney test) but higher than those against TCE4 group 4 (1.81% ± 2.82%), resulting in no significant net effect on the predictive value of TCE3 and TCE4.

Our data provide, for the first time, in vitro evidence for differential immunogenicity of DPB1 according to our algorithms. Interestingly, ex vivo evidence was previously reported by Rutten and colleagues [4] who showed that in 2 patients after 10/10 matched HSCT, the number of T cells responding to mismatched DP alloantigens was highest for TCE3/4 group 2 (2.72%), lower for TCE4 group 3 (1.08%), and lowest for TCE4 group 4 (0.41%). Further work is needed to determine the molecular and cellular basis of our algorithms, including the role of the DPα chain.

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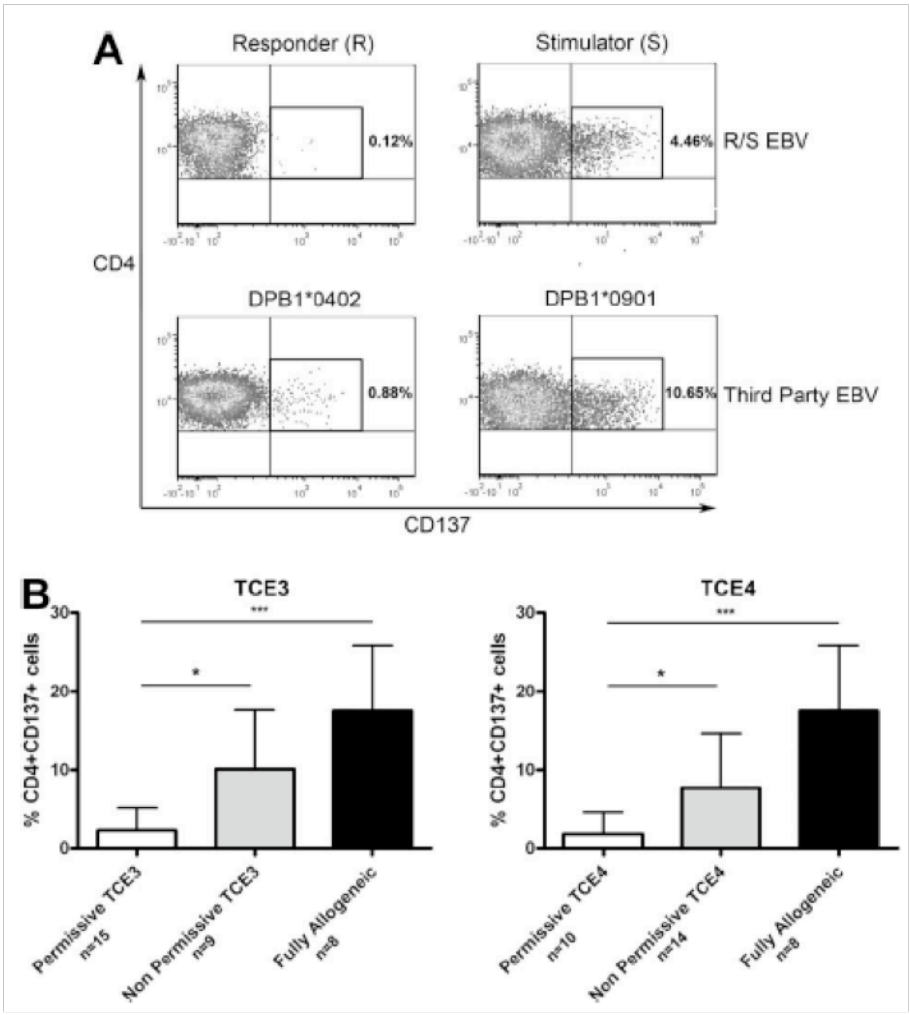
Figure Legends

Figure 1. Quantification of alloreactive CD4+ T cells responding to permissive or nonpermissive DPB1 TCE3 or TCE4 disparities. Classical 1-way MLRs were set up between R-S pairs of unrelated volunteers selected for the same patient and matched between each other for 10/10 of the HLA-A, B, C, DRB, and DQB1 alleles, but mismatched for -DPB1. R cells consisted of peripheral blood mononuclear cells (PBMCs), while S cells in most cases were PBMCs depleted of CD3+ T cells, at a ratio of 1:1. After 2 rounds of stimulation in the presence of 150 IU/mL IL-2, CD4+ T cells were rechallenged overnight with B lymphoblastoid cells (BLCLs) from R, S, or from third-party donors sharing only 1 mismatched DPB1 allele with S. Responding T cells were quantified by FACS analysis for surface expression of the activation marker CD137. In several cases, CD4+ T cells expressing CD137 upon challenge with DPB1 typed third-party BLCLs were FACS-sorted and their specificity for the relevant DP alloantigen was confirmed (not shown). (A) Exemplative analysis of alloreactive CD4+ T cells responding to a permissive or a nonpermissive DPB1 mismatch on the same S cell. R and S cells carried DPB1*02:02, 04:01 and DPB1*04:02, 09:01, respectively, and thus S cells presented 1 TCE3/4 permissive (DPB1*04:02) and 1 TCE3/4 nonpermissive (DPB1*09:01) mismatch. (B) Mean percentage of CD4+ T cells responding to permissive or nonpermissive DPB1 mismatches according to TCE3 (left panel) or TCE4 (right panel), in a series of 24 MLRs. At the moment of testing,

cultures contained a mean of 53.75% ± 24.94% CD4+ T cells. The mean percentage of T cells expressing CD137 in response to autologous R-BLCLs was 2.06% ± 1.62%. The percentage of T cells responding specifically to allogeneic read-out BLCLs was calculated as the total percentage of CD137+ T cells after allogeneic stimulation minus the percentage of CD137+ T cells after autologous stimulation. Fully allogeneic R-S pairs (n=8) were used as positive controls and yielded a mean of 17.53% ± 8.28% specifically responding CD4+ T cells, with a mean of 44.75% ± 25.17% CD4+ T cells. Pairwise comparison of the results obtained in the different groups was performed by the Kruskal-Wallis test followed by the Dunn multiple comparison posttest. (Left panel) In the TCE3 permissive group (n=15), the mismatched DPB1 allele expressed by S was encoded by DPB1*02:01 (n=4), 02:02 (n=1), 04:01 (n=5), 04:02 (n=3), 11:01 (n=1), 13:01 (n=1). In the TCE3 nonpermissive group (n=9), the mismatched DPB1 allele expressed by S was encoded by DPB1*03:01 (n=3), 09:01 (n=2), 10:01 (n=3) or 17:01 (n=1). The frequency of CD4+ T cells specifically responding to TCE3 permissive mismatches was significantly lower compared with TCE3 nonpermissive mismatches (*$P < .05$) and compared with fully mismatched third-party alloantigens (***$P < .001$). (Right panel) In the TCE4 permissive group (n _ 10), the mismatched DPB1 allele expressed by S was encoded by DPB1*04:01 (n=5), 04:02 (n=3), 11:01 (n=1), 13:01 (n=1). In the TCE4 nonpermissive group (n=15), the mismatched DPB1 allele expressed by S was encoded by DPB1*02:01 (n=4), 02:02 (n=1), 03:01 (n=3), 09:01 (n=2), 10:01 (n=3) or 17:01 (n=1). The frequency of CD4+ T cells specifically responding to

TCE4 permissive mismatches was significantly lower compared with TCE4 nonpermissive mismatches (*$P < .05$) and compared with fully mismatched third-party alloantigens (***$P < .001$).

Figure 1


A
Responder (R)
Stimulator (S)
0.12%
4.46%
R/S EBV
CD4
DPB1*0402
DPB1*0901
0.88%
10.65%
Third Party EBV
CD137
B
TCE3
TCE4
% CD4+CD137+ cells
Permissive TCE3 n=15
Non Permissive TCE3 n=9
Fully Allogeneic n=8
Permissive TCE4 n=10
Non Permissive TCE4 n=14
Fully Allogeneic n=8

Chapter 3

Complex and divergent patterns of amino acid residues are involved in T cell alloreactivity to HLA-DP

Running Title: Alloreactive T cell epitopes of HLA-DP

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Abstract

Increasing evidence suggests an important role for immunodominance hierarchies of HLA alloantigens in alloreactivity and clinical transplantation; however, attempts to pinpoint such hierarchies to individual amino acids or hypervariable regions have been disappointing so far. Here we have addressed this question in the context of HLA-DP9, the prototype of an immunogenic HLA-DP molecule, which has been shown to elicit relevant alloreactivity in clinical transplantation. On the basis of homology modeling and multiple sequence alignment, 8 amino acid positions (9,35,55,56,57,69,76,84) likely to be involved in peptide binding and/or T cell receptor interaction in hypervariable regions (HvR) A-F were selected for site directed mutagenesis of HLA-DPB1*09:01, and expressed in homozygous B cell lines using a lentiviral vector expression platform. Alloreactive T cell effectors specific for wild type HLA-DPB1*09:01 (n=6) or for DPB1*10:01 and DPB1*1701, respectively but crossreactive to DPB1*09:01 (n=2), were used to test the impact of mutagenesis on recognition by g-interferon ELISpot or CD107a degranulation assays. Individual point mutations were shown to have a clear-cut effect of either abrogating, enhancing or not affecting recognition by the different effectors. For T cells specifically alloreactive to HLA-DPB1*09:01, recognition was influenced by a complex pattern of residues, distributed among all 6 HvR, which was different for each of the 6 effectors studied. In contrast, for the 2 T cells cross-reactive to HLA-DPB1*09:01, only 2 amino acids (positions 69 and 76) had an influence on allorecognition, suggesting

the presence of a more restricted set of T cell epitopes in the context of cross-reactivity rather than nominal specificity. Our data are consistent with peptide involvement in alloreactivity to HLA-DPB1*09:01, and underline the complexity of allorecognition which may be one of the mechanisms underlying the immunogenicity of this molecule.

Introduction

Human Leukocyte Antigen (HLA)-DP, a member of HLA class II family, was initially described in 1980 to be able to elicit variable T cell responses in mixed lymphocyte reactions (MLRs) [1]. Since then, evidence for the importance of this locus in eliciting humoral and cellular alloresponses relevant for clinical transplantation, both of solid organs and of hematopoietic stem cells, has been obtained [2-4][5, 6]. Like the other HLA class II molecules, HLA-DP are cell ab-chain heterodimers encoded by two polymorphic genes, DPA1 and DPB1. The DPA1 locus is essentially dimorphic with two predominant alleles, DPA1*01:03 and DPA1*02:01, among the 33 alleles described to date. In contrast, the DPB1 locus is highly polymorphic with 152 alleles coding for 131 proteins (www.ebi.ac.uk/imgt/hla) [7]. HLA-DPB1 polymorphism is clustered into 6 hypervariable regions (HvR) A-F encoded by exon 2 and shuffled between different alleles [8-10]. In the context of solid organ transplantation [11], HLA-DP specific alloantibody reactivity can be assigned to some of these HvR, whereas prediction of T cell alloreactivity on their basis has not been reliable so far.

Increasing evidence suggests that, similar to self-HLA-restricted antigen specific responses which are frequently governed by immunodominant epitopes [12-14], immunodominance hierarchies can also be unraveled for T cell alloreactivity. This has been shown for inter- and intra-locus hierarchies for HLA class I antigens, which elicit alloreactivity of different strength according to the mismatched HLA antigen and the self-HLA background of the responder [15, 16].

In line with these observations, our group has shown that HLA-DP antigens can be divided into 3-4 T cell epitope (TCE) groups on the basis of alloreactive T cell cross-reactivity patterns, and that the strength of the *in vitro* alloresponse is significantly higher to HLA-DP mismatches across different TCE groups, compared to those within a single TCE group [17]. The HLA-DPB1 TCE group matching approach has raised interest also in the clinical community because it was shown to significantly correlate with the outcome of hematopoietic stem cell transplantation (HSCT) from volunteer unrelated donors (VUD) [18-20].

In the present study, we set out to elucidate the molecular basis of the HLA-DPB1 TCE group matching model, by performing site-directed mutagenesis (SDM) of HLA-DPB1*09:01, prototype of DPB1 alleles mediating strong T cell alloreactivity from TCE group mismatched responders. In particular, we sought to determine if the immunogenicity of TCE group mismatches involving HLA-DPB1*09:01 reflects the presence of a restricted set of immunodominant epitopes, or on the contrary of a broad number of different independent epitopes.

Materials and methods

B Lymphoblastoid cell lines

HLA homozygous Epstein-Barr virus-transformed B Cell Lines (BLCLs) were purchased from the European Collection of Animal Cell cultures (ECACC). Other HLA-typed BLCLs were locally established and cultured using standard procedures. The panel of BLCLs used in this study and their respective HLA typings is listed in Table 1.

HLA typing

Genomic low resolution HLA-A,B,C,DRB,DQB1 typing was performed by PCR-Sequence Specific Oligonucleotide Probing (SSOP) using the Dynal Reli^{TM} kits (Lifetechnologies, Paisley, UK) according to the manufacturer's recommendations. Genomic high resolution HLA-A,B,C,DRB,DQA1,DQB1,DPB1 typing was performed by PCR-Sequence Specific Priming (SSP) using the Olerup^{TM} kits (Olerup GmbH, Vienna, Austria) according to the manufacturer's recommendations.

Monoclonal Antibodies and Flow Cytometry

The following panel of directly conjugated monoclonal antibodies (mAbs) was used in this work: anti-CD4 allophycocyanin (APC) [clone RPA-T4], anti-CD137 phycoerythrin (PE) [clone 4B4-1], anti-CD107a fluorescein isothiocyanate (FITC) [clone H4A3] (Becton Dickinson, BD, Milan, Italy), anti-low-affinity nerve growth factor

receptor (LNGFR)-APC [clone ME20.4] (Miltenyi Biotec, Bologna, Italy). The following panel of unconjugated mAbs was used in inhibition studies: anti-pan HLA-DP [clone B7/21], anti-HLA-DR [clone L243] (Biolegend, Uithoorn, The Netherlands). An unconjugated human anti-HLA-DP antibody [clone TL-3B6], specific for the epitope 84-87 DEAV shared by different HLA-DP antigens including HLA-DP9, was used for cell surface staining in combination with a secondary goat anti-human IgG-FITC antibody (Jackson ImmunoResearch, Newmarket, UK). A panel of 24 mAbs specific for T cell receptor (TCR)-variable region beta (TCR-Vb) families were contained in the IOTest Beta Mark TCR-Vb repertoire Kit (Beckman Coulter, Milan, Italy), namely the following clones were used: 3D11 (Vb 5.3), ZOE (Vb 7.1), CH92 (Vb 3), FIN9 (Vb 9), E17.5F3 (Vb 17), TAMAYA1.2 (Vb 16), BA62.6 (Vb 18), IMMU157 (Vb 5.1), ELL1.4 (Vb 20), IMMU222 (Vb 13.1), JU74.3 (Vb 13.6), 56C5.2 (Vb 8), 36213 (Vb 5.2), MPB2D5 (Vb 2), VER2.32 (Vb 12), AF23 (Vb 23), BL37.2 (Vb 1), IG125 (Vb 21.3), C21 (Vb 11), IMMU546 (Vb 22), CAS1.1.3 (Vb 14), H132 (Vb 13.2), WJF24 (Vb 4), ZIZOU4 (Vb 7.2). Flow cytometry data acquisition and analysis was performed on a fluorescence activated cell sorter (FACS) Canto II (BD) using both FACSDiva (BD) and FCS Express 3.0 software programs (De Novo Software, Los Angeles CA, USA). Cell sorting was performed using the FACS VANTAGE system with DIVA option (BD).

Isolation of T cell effectors alloreactive to HLA-DP

Peripheral blood mononuclear cells (PBMCs) were isolated by standard Ficoll gradient from different pairs of HLA-typed healthy VUD selected for the same patient and hence high-resolution matched for 9-10/10 of the HLA-A,B,C,DRB,DQB1 alleles, but HLA-DPB1 mismatched, or from a patient in complete remission from chronic myeloid leukemia (CML) prior to allogeneic HSCT and her family donor [5]. BLCL were locally established from all pairs, and the relevant HLA typings are listed in Table 1.

For isolation of T cell effectors alloreactive to HLA-DP, PBMCs of the different pairs were used as responders (R) or stimulators (S) in one-way mixed lymphocyte reactions (MLR). S were irradiated at 60Gy and used in a 1:1 ration with R, in the presence of 150U/ml exogenous interleukin-2 (IL-2; Novartis, Varese, Italy), for 14 days with one intermittent re-stimulation, prior to functional testing. Some of the cultures were cloned after 14 days by limiting dilution analysis (LDA) in the presence of 300U/ml of IL-2, 100Gy irradiated allogeneic S BLCL and 60Gy irradiated third-party PBMCs, according to previously published methods [5]. Alternatively, T cell cloning was performed by FACS single cell sorting of alloreactive CD4+/CD137+ T cells specifically responding to the S BLCL after 24-hour stimulation. Long-term maintenance of alloreactive T cell lines and clones was performed by bimonthly stimulation with a feeder mixture consisting in 100 Gy irradiated donor S BLCL and 60 Gy irradiated third-party PBMCs in the presence of 300 U/ml IL-2, according to previously published protocols [5].

Functional characterization of alloreactive T cell effectors

Gamma Interferon (g-IFN) ELISpot, CD137 T cell activation and CD107a degranulation assays were used for functional characterization of alloreactive T cell effectors. For the ELISpot assay, T cells were incubated overnight with target BLCLs at a 1.5:1 ratio, and g-IFN-producing spots were visualized on 96-well MultiScreen HTS IP Filter Plates (Millipore, Milan, Italy) using the AEC staining kit (Sigma-Aldrich, Milan, Italy), according to the manufacturer's recommendations. Spot counting was performed using the A.EL.VIS 4-Plate ELISpot Reader system (AELVIS, Hannover, Germany). For the CD137 assay, T cells were incubated overnight with target BLCLs at a 1:3 ratio, and CD4+ subsets were stained for expression of the activation marker CD137 using appropriately labeled relevant mAbs. For the CD107a assay, T cells were incubated for 1 hour with target BLCLs at a 1.5:1 ratio, followed by addition of Monensin-A (Sigma-Aldrich, Milan, Italy) for an additional 3 hours. CD4+ subsets were stained for expression of the degranulation marker CD107a using appropriately labeled relevant mAbs.

For mAb inhibition studies, target BLCLs were pre-incubated with the relevant mAb at a final concentration of 1 and 10μg/ml for ELISpot and CD107a assays, respectively, prior to addition of effector T cells.

Recognition levels of BLCLs expressing mutant variants of HLA-DPB1*09:01 by alloreactive T cells are reported as relative response with respect to recognition of wild type HLA-DPB1*09:01 and calculated as follows: (T cell response $_{[\text{Mutant variant}]}$ − T cell response$_{[\text{Negative control}]}$)/(T cell response$_{[\text{wild type}]}$ − T cell response$_{[\text{Negative control}]}$).

TCR-Vb analysis

TCR-Vb families expressed by alloreactive T cell effectors were determined by cell surface staining of CD4+ T cells subsets with specific mAbs cocktails.

Homology Modeling of HLA-DP9

Homology modeling of HLA-DP9 (DPB1*09:01-DPA1*02:01) was performed using the Swiss Model Workspace [21], a fully automated protein structure homology-modeling server, on the basis of the recently reported crystal structure of HLA-DP2 (DPB1*02:01-DPA1*01:03) [22]. HLA-DP Amino acid sequences were obtained from the ImMunoGeneTics/HLA (IMGT/HLA) database (http://www.ebi.ac.uk/imgt/hla/) [7]. An HLA-DP9 presented peptide derived from streptococcal M12 protein, MP-10R13 [23], was modeled by aligning with the pDRA peptide from the HLA-DP2 crystal. The QMEAN Z-score was used to estimate the quality of the HLA-DP9 model [24]. Model analysis and pictures were performed using the Swiss-PDB Viewer software (DeepView 4.0.4, http://www.expasy.org/spdbv/) [25].

HLA-DPB1 cloning and site-directed mutagenesis

The full-length cDNA of HLA-DPB1*09:01, *10:01, *35:01, *17:01, *01:01 and *04:01 was amplified by RT-PCR from BLCLs (Table 1) using primers previously described in the literature [5]. The 6.9 kb PCR products were digested by *EcoRI/XhoI* and cloned into a modified pCR2.1-TOPO (Life technologies, Monza, Italy) containing

the multi-cloning site of pBluscript II SK- (Agilent Technologies, Milan, Italy). Site-directed mutagenesis (SDM) of HLA-DPB1*09:01 was performed using the Quick change II Site-directed mutagenesis kit (Agilent Technologies) following the manufacturer's instructions. Coding sequences of wild type and mutant variants were confirmed by automated DNA sequencing (Primm, Milan, Italy).

Lentiviral vector mediated gene transfer of HLA-DPB1

Wild type and mutant HLA-DPB1 coding sequences were subcloned into a bidirectional promoter lentiviral expression vector (LV) previously described by others [26], under the control of the human phosphoglycerate kinase (hPGK) promoter, and with a truncated version of the low-affinity NGF receptor (ΔLNGFR) under the control of a minimal core promoter derived from cytomegalovirus (minCMV) coordinately expressed as cell surface marker. LV production and target cell infection was performed according to standard protocols.

Quantitative assessment of cell surface marker expression levels

Quantitative assessment of transduced cell surface molecules (ΔNGFR or HLA-DP carrying the 84-87 DEAV motif) was performed by determination of Molecules of Equivalent Soluble Fluorochrome (MESF) [27]. Standardization was performed by use of SPHERO Rainbow Calibration Particles (Spherotech, Lake Forest, USA) made up of 8 calibrated populations of beads with known MESF. Median Fluorescence Intensity (MFI) for each fluorochrome was converted into the respective MESF value by means of linear regression analysis on a standard curve generated by SPHERO Rainbow Particles

according to the manufacturer's instructions. MESF values were corrected by subtracting the MESF value of unstained cells or cells stained with secondary antibody alone.

Statistical analyses

Expression levels of transduced cell surface molecules (ΔNGFR or HLA-DP carrying the 84-87 DEAV motif) are reported as mean±standard error (SE) of independent experiments. Statistical comparisons of cell surface expression levels between different transduced BLCL, and of BLCL target recognition levels by alloreactive T cell effectors, were performed using the unpaired t-test, with $p<0.05$ as threshold for significance.

Results

Homology modeling and site-directed mutagenesis of HLA-DPB1*09:01

HLA-DPB1 variability is clustered into 6 HvR A-F around the peptide antigen binding groove, with polymorphic amino acid residues shuffled between different alleles (Table 2). In order to select the most informative amino acids for pinpointing by SDM the molecular basis of the observed immunodominance hierarchies in alloreactivity to HLA-DP, we performed homology modeling of HLA-DPB1*09:01, prototype of DPB1 alleles mediating strong T cell alloreactivity from TCE group mismatched responders, on the basis of the previously published crystal structure of the DPA1*01:03-DPB1*02:01 heterodimer HLA-DP2 [22]. A number of amino acids in all 6 HvR were identified as likely to interact with bound peptide, or with the TCR on responder T cells (Figure 1). On this basis, a total of 8 amino acid residues at positions 9, 35, 55, 56, 57, 69, 76, and 84, respectively, in the HLA-DP9 backbone were selected for SDM (Table 3). Amino acid substitutions were chosen in such a way to cover the most frequent naturally occurring variations at these positions (Table 2), for a total of 10 SDM (Table 3).

LV-mediated expression of HLA-DP in BLCL

Wild type and mutant HLA-DPB1*09:01 were expressed in 2 read-out BLCLs, MGAR and VAVY, homozygous for HLA-DPB1*04:01-DPA1*01:03 and DPB1*01:01-DPA1*02:01, respectively (Table 1).

HLA-DP04 and HLA-DP01 encoded by these alleles belong to the same distinct TCE group, and have been shown not to be cross-recognized by T cells alloreactive to HLA-DP9 [17, 20]; Table 2). Importantly, HLA-DPB1*04:01 does not encode the 84-87 DEAV motif which is instead carried by DPB1*09:01, thereby enabling us to discriminate transduced from endogenous HLA-DPB1 alleles expressed on the cell surface of MGAR by use of the DEAV-specific mAb TL-3B6. This was not possible for VAVY due to the presence of the 84-87 DEAV motif on endogenous HLA-DP01 (Table 2).

A bi-directional LV vector expression platform was used for expression of wild type and mutant HLA-DPB1*09:01 together with the truncated form of the NGFR as reporter gene [26] (Figure 2A). Both transgenes were coordinately expressed on the cell surface of MGAR (Figure 2B). Comparative quantitative evaluation of cell-surface expression of the panel of HLA-DPB1*09:01 mutants by MGAR showed no significant differences with those of wild type transfectants, with the exception of mutants F35Y, D57E and D84G which showed lower expression levels (Figure 2C). These observations, made by direct staining of the transduced HLA-DP antigens with the TL-3B6 mAb specific for the 84-87 DEAV motif, were confirmed also by staining for the reporter surface molecule DNGFR (Figure 2D), suggesting that DNGFR expression is a valid surrogate for HLA-DP transgene expression for this vector. Cell surface expression levels of DNGFR on VAVY showed no significant variations between the different transfectants, suggesting homogenous expression levels also of the HLA-DPB1*09:01 transgenes on these cells (Figure 2E).

Isolation and characterization of T cell effectors alloreactive to HLA-DP9

T cell clones and lines specifically alloreactive to wild type HLA-DPB1*09:01 (6 effectors; Table 4), or to DPB1*10:01 or DPB1*17:01 but cross-reactive to DPB1*09:01 (2 effectors; Table 4), were raised by MLR between 5 different unrelated R/S pairs matched for all HLA loci except for HLA-DPB1. HLA-DP restricted allorecognition of stimulator BLCL was demonstrated for all effectors by mAb inhibition studies (Figure 3A,B). Moreover, all T cells were able to specifically recognize wild-type HLA-DPB1*09:01 after LV-mediated gene transfer into MGAR or VAVY, or both (Figure 3C). Interestingly, dependency of HLA-DPB1*09:01 allorecognition on DPa chain polymorphism, evidenced by selective recognition of transfected MGAR (DPA1*01:03) or VAVY (DPA1*02:01), was observed for 5 out of 8 T cells studied (Figure 3C). However, the relevance of DPa chain polymorphism in allorecognition of HLA-DP9 was not dependent on mismatching for the relevant DPA1 allele on the stimulator cell, as demonstrated by DPA1 typing of several R/S pairs (Table 1), and by variable DPA1 preference in different alloreactive T cells raised form the same individual (Figure 3C). In line with previous reports [2, 3, 28], the TCR-Vb usage of T cells specifically alloreactive to wild type HLA-DPB1*09:01 was highly variable (Table 4). Interestingly, the 2 T cell effectors raised against HLA-DP10 or DP17 but cross-reactive with HLA-DP9, obtained from 2 unrelated individuals, showed predominant usage of the same TCR-Vb4 family (Table 4).

Effects of single amino acid substitutions in HLA-DP9 on T cell allorecognition

The effect of single amino acid substitutions at different putative key positions for peptide binding and/or TCR interaction on allorecognition of HLA-DP9 was tested by challenging the 8 alloreactive effector T cells with MGAR or VAVY transfectants, according to the established DPa chain preference of each effector. Recognition levels were analyzed relative to those of wild type HLA-DP9, and showed clear-cut results throughout, i.e. as being either significantly reduced, significantly enhanced or not affected by the different amino acid substitutions (Figure 4). It should be noted that the 3 MGAR transfectants with significantly lower exogenous HLA-DP surface expression levels compared to wild type (Figure 2C), F35Y, D57E and D84G, showed similar or even enhanced recognition levels by at least some of the T cell effectors studied, suggesting that mutant HLA-DP was present at sufficient amounts on the cell surface to allow for efficient recognition (Figure 4).

Only two mutants, F35L and E56D, did not interfere with recognition by any of the alloreactive T cell effectors studied (Figure 4 and 5). In contrast, all other mutants significantly reduced recognition levels by some but not all alloreactive T cells. The most striking effect was displayed by mutants H9Y and F35Y which significantly impaired or abrogated recognition by 5 out of 6 T cells specifically alloreactive to wild type HLA-DP9, followed by mutants D55A, E69K, V76M and D84G which significantly reduced recognition by 4 out of 6 of such T cells. In contrast, for the T cells specifically alloreactive to HLA-

DP10 or DP17, only two mutants, E69K and V76M, significantly interfered with crossreactivity to HLA-DP9 (Figure 4 and 5).

Strikingly, none of the 8 alloreactive T cells studied displayed the same pattern of sensitivity to single amino acid substitutions in HLA-DP9. The greatest variability was seen between T cells specifically alloreactive to wild-type HLA-DP9, with 6/6 entirely different sensitivity patterns (Figure 5). In contrast, T cells specifically alloreactive to HLA-DP10 and 17 but cross-reactive to HLA-DP9 showed more restricted though not identical sensitivity patterns (Figure 5).

Discussion

In the present study, we sought to elucidate the molecular basis of the immunologically and clinically relevant HLA-DPB1 TCE group matching model [18-20, 29], by studying the role of individual amino acids for the alloresponse to HLA-DPB1*09:01, prototype of DPB1 alleles mediating strong T cell alloreactivity from TCE group mismatched responders. Our results show that immunogenicity of this alloantigen is reflected by a wide spectrum of T cell responses, with different epitopes recognized by 6/6 of the effectors specifically alloreactive to HLA-DP9 (Figure 4 and 5). On the other hand, the more restricted epitope-specificity of 2 effectors nominally specific for other HLA-DP antigens but cross-reactive with HLA-DP9 suggests that the repertoire of alloreactive T cells reacting to different alleles of the same TCE group may be more limited. This provides an attractive explanation for the observed stronger immunogenicity of inter- versus intra-TCE group mismatches at HLA-DP. According to this model, inter-TCE group mismatches are target of a wide spectrum of T cell responses, leading to the observed high frequency of T cells specifically activated in response to such mismatches [17]. In contrast, inter-TCE group mismatches might be target of a more restricted panel of T cell effectors, thereby reducing the number of responder T cells. TCR-Vb family usage, which was highly divergent for the 6 nominally HLA-DP9 specific but identical for the 2 HLA-DP9 cross-reactive T cells (Table 4), provides an additional hint in this direction,

although a larger number of HLA-DP9 cross-reactive T cells needs to be studied to further strengthen this hypothesis.

In this study, we focused our attention on polymorphic amino acids predicted to be crucially involved in peptide binding and/or TCR contact, based on homology modeling (Figure 1). It is interesting to note that virtually all positions selected on the basis of their relevance for peptide binding (9,35,55,69,76,84) were found to be crucial for allorecognition by at least some of the alloreactive T cells (Figures 4 and 5). This observation is compatible with increasing evidence from the literature in favor of peptide involvement in most if not all alloreactions, including those with clinical relevance after allogeneic HSCT [30-33]. Interestingly, introduction of tyrosine at positions 9 and 35 had a strikingly more marked effect on allorecognition by most T cells than introduction of phenylalanine or leucine at these positions, respectively, showing that the biochemical nature of the substitution, and not its position alone, are relevant determinants (Figure 4).

Most amino acids found here to be relevant for allorecognition of HLA-DP9 have been previously reported to play a role for alloreactivity to HLA-DP in MLR [8, 34] or more specifically to HLA-DP2 [35, 36]. The novelty of our findings relys in the demonstration of the above discussed strong heterogeneity of the alloresponse across TCE group mismatches. This observation could also be at the basis for the difficulty to pinpoint T cell alloreactivity against HLA-DP to defined amino acid stretches in HvR, an approach that has led to inconclusive results when applied to retrospective analysis of the clinical outcome of HLA-DP mismatched HSCT ([37]

[9]; our unpublished observations). In contrast, serological epitopes of HLA-DP can be more readily assigned to defined sequence motifs, including dimorphic epitopes at positions 65/69, 56 and 85-87, respectively [11, 38]. While amino acid 69 was found to be an important player for T cell alloreactivity also in this and previous reports [34, 36], position 56 was one of the few residues that did not show interference with any of the alloreactive T cells in the present study (Figure 4 and 5). Moreover, although not specifically tested here, the predictions from our homology models did not argue in favor of an important role of positions 85-87 for T cell allorecognition via peptide binding or TCR contacting (Figure 1). These observations underscore important differences between the molecular mechanisms underlying humoral and cellular alloreactivity. This is in line with established evidence that humoral, but not cellular alloreactivity can be predicted on the basis of conformational considerations that rely strongly on the primary amino acid sequence of the HLA backbone [39]. Attempts to apply similar approaches to T cell alloreactivity have been disappointing [40, 41], further supporting the notion that peptide involvement may account for much of the limitations in predicting T cell allorecognition on the basis of HLA backbone structure or conformation alone.

In conclusion, the present study provides new evidence in support of the concept that the clinically relevant stronger immunogenicity of HLA-DPB1 TCE group mismatches, compared to TCE group matches, is reflected by a highly divergent and complex TCE repertoire against the former which might be more limited against the latter. Identification of specific allopeptides associated with these

responses is warranted to allow us to further dissect their molecular basis.

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Figure Legends

Figure 1: Homology modeling of HLA-DP9. The model was constructed on the basis of the previously published crystal structure of HLA-DP2 [22], by aligning the amino acid sequences of the DPA1*01:03-DPB1*02:01 and the DPA1*02:01-DPB1*09:01 heterodimers, using the Swiss Model Workspace. The DRA-derived peptide from the HLA-DP2 crystal structure was substituted by the HLA-DP9 presented MP-10R13 peptide from the M12 protein. Striped ribbons represent the DPa and DPb chain in yellow and blue, respectively; the peptide backbone and sticky residues are shown in grey and black, respectively. A) Overall view of the peptide-binding groove. Shown are the locations of the 6 HvR A-F, with relevant polymorphic amino acid residues in different colors. B-E): Magnification of polymorphic amino acid residues in the different HvRs, and their relationship with bound peptide amino acid side chains. DPab amino acid residues with or without predicted relevance for interaction with peptide and/or TCR are indicated in black and grey font, respectively. Only amino acids in black font were chosen for subsequent SDM (Table 3). B) HvR A, B, C (amino acid residue 55); C) HvR C (amino acid residues 55-57); D) HvR D-E; E) HvR F.

Figure 2: LV-mediated expression of HLA-DP. A) Schematic representation of the bidirectional LV vector used for expression of the ΔLNGFR reporter and HLA-DPB1 alleles, driven by a minimal CMV promoter fused to hPGK, or by the hPGK promoter, respectively, in opposite directions. SVpA: Symian Virus 40

polyAdenilation signal sequence; WPRE: Woodchuck hepatitis virus Post-transcription Regulatory Element. B) Cell surface expression of wild type HLA-DP9 and ΔLNGFR on the BLCL MGAR before (MGAR UTD) or after (MGAR DP9) LV-vector mediated gene transfer, assessed by FACS-staining with mAb TL-3B6 and ME20.4, respectively. Endogenous HLA-DP expression is not revealed by mAb TL-3B6 which picks up the 84-87 DEAV motif encoded by HLA-DPB1*09:01 but not by endogenous DPB1*04:01 on MGAR BLCL. C-E: Quantification of cell surface expression levels of HLA-DP using the mAb TL-3B6 (C) or ΔLNGFR using the mAb ME20.4 (D,E) after LV-mediated gene transfer into the BLCL MGAR (C,D) or VAVY (E). Expression of exogenous HLA-DP9 on VAVY could not be tested because this BLCL carries endogenous HLA-DPB1*01:01 which encodes the 84-87 DEAV motif picked up my mAb TL-3B6. For each BLCL, a panel of transfectants with wild type or mutants HLA-DPB1*09:01 was analyzed, along with a negative control transfectant represented by HLA-DPB1*01:01 and DPB1*04:01 for MGAR and VAVY, respectively. Cell surface expression levels are reported as molecules of equivalent soluble fluorochrome (mean±SEM of 5 independent experiments). Statistical comparisons of cell surface expression levels, using BLCL transfected with wild type HLA-DPB1*09:01 as reference, were performed by the two-tailed unpaired t-test: *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$.

Figure 3: Functional characterization of T cells alloreactive to HLA-DP. A) Raw data of a representative g-IFN ELISpot assay. T cell clone C301-TO/MI-9 was stimulated overnight with responder

(Auto) or stimulator (Allo) BLCLs, in the absence (2 left panels) or presence (2 right panels) of 1mg/ml mAb B7/21 (anti-HLA-DP) or L243 (anti-HLA-DR). B) Specificity of T cell effectors alloreactive to HLA-DP used in this study (see also Table 4). All effectors were tested against responder (Auto) or stimulator (Allo) BLCLs, in the absence or presence of of 1mg/ml mAb B7/21 (anti-HLA-DP) or L243 (anti-HLA-DR). Results are the mean±SD of duplicate experiments. Left panel: Number of spots produced in g-IFN ELISpot assays in response to overnight target cell incubation. Right panel: percentage of CD4+ T cells up-regulating the activation marker CD137 in response to overnight target cell incubation. C) DPα-chain preference of T cell effectors alloreactive to HLA-DP used in this study (see also Table 4). All effectors were tested against the MGAR (DPA1*01:03) or VAVY (DPA1*02:01) after LV-mediated gene transfer of wild type HLA-DPB1*09:01 (DP9) or DPB1*01:01 (DP01) and DPB1*04:01 (DP04) as negative controls for MGAR and VAVY, respectively. Left panel: Number of spots produced in g-IFN ELISpot assays in response to overnight target cell incubation. Right panel: percentage of CD4+ T cells up-regulating the activation marker CD107a in response to 4-hour target cell incubation. [a] indicates responder/stimulator (R/S) pairs used to isolate alloreactive T cell clones and lines (Table 1).

Figure 4: Impact of site-directed mutagenesis on T cell allorecognition of HLA-DP9. T cell effectors alloreactive to wild type HLA-DP9 (see Table 4 and Figure 3) were tested in g-IFN ELISpot (7 effectors) or CD107a assay (M4-DE/PD-17) for

recognition of a panel of 10 site-directed HLA-DP9 mutants after LV-mediated gene transfer into BLCL MGAR (black bars) or VAVY (grey bars). Results are expressed as relative response with respect to recognition of wild type HLA-DP9, and are the means±SD of 3 independent experiments. Statistical comparisons of recognition levels of mutant versus wild type HLA-DP9 was performed on raw data by the two tailed unpaired t-test: *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$.

Figure 5: Synthetic representation of the impact of site-directed mutagenesis on T cell allorecognition of HLA-DP9 by different effectors. Red, amino acid substitutions significantly reducing T cell recognition; green, amino acid substitutions significantly increasing T cell recognition; grey, amino acid substitutions with no significant impact on T cell allorecognition. Data were extrapolated from the results shown in Figure 4.

Table 1: HLA typing of BLCLs in this study.

BLCL	Origin[a]	HLA						
		A*	B*	C*	DRB1*	DQB1*	DPA1*	DPB1*
MGAR[b]	Commercial	26:01	08:01	07:01	15:01	06:02	01:03	**04:01**
VAVY[b]	Commercial	01:01	08:01	07:01	03:01	02:01	02:01	**01:01**
BM21[b]	Commercial	01:01	41:01	17:01	11:01	03:01	02:01	**10:01**
AQ01TON[b]	Local	02:01	13:02, 35:01	04:01, 06:02	07:01, 14:01	02:02, 05:03:01	nd	04:01, **17:01**
DKM345ALT[b]	Local	02:01, 24:02	35:01, 51:01	04:01, 14:02	01:01	05:01	nd	04:02, **35:01**
DV[c]	Local	30:01	39:01, 49:01	07:01, 12:03	07:01, 16:01	02:02, 05:02	nd	02:01, 04:02
DG[c]	Local	30:01, 02	39:01, 49:01	07:01, 12:03	07:01, 16:01	02:02, 05:02	nd	02:01, **09:01**
TO[d]	Local	30:02, 01:01	08:01, 18:01	05:01, 07:01	03:01	02:01	01:03	02:02, 04:01
MI[d]	Local	30:02, 01:01	08:01, 18:01	05:01, 07:01	03:01	02:01	01:03, 02:01	04:02, **09:01**
DU[e]	Local	01:01, 02:01	57:01, 45:01	06:02, 16:04	07:01, 11:01	03:03, 03:01	nd	04:01, 13:01
FF[e]	Local	01:01, 02:01	57:01, 45:01	06:02	07:01, 11:01	03:03, 03:01	nd	04:02, **09:01**
UD[f]	Local	02:01, 11	08, 51:01	01, 07:01	03:01, 11:01	02:01, 03:01	nd	01:01, 04:02
VR[f]	Local	02:01, 11	08, 51:01	01, 07:01	03:01, 11:01	02:01, 03:01	nd	02:01, **10:01**
DE[g]	Local	02:01, 03:01	07:02, 13:02	07:02, 06:02	07:01, 15:01	02:02, 06:02	nd	02:01, 04:01
PD[g]	Local	02:01, 03:01	07:02, 13:02	07:02, 06:02	07:01, 15:01	02:02, 06:02	nd	03:01, **17:01**

[a] Origin: Commercial BLCL were purchased from the ECACC. Locally established BLCLs were derived from healthy VUD pairs selected for the same patient and hence 9-10/10 high-resolution matched for HLA-A,B,C,DRB,DQB1, but mismatched for HLA-DPB1, with the exception of DV and DG which were from a patient in complete remission of CML and her related stem cell donor, respectively [5]. [b] indicates BLCLs used for molecular cloning of HLA-DPB1 coding sequences of the alleles marked in bold text. [c-g] Pairs marked with identical letters were used for isolation of alloreactive T cell effectors used in further functional experiments of the study.

Table 2: Amino acid sequence alignment of HvR A-F encoded by HLA-DPB1 alleles. Dashes indicate amino acid identity to the consensus encoded by HLA-DPB1*09:01.

		Hypervariable Regions (HvRs)																
TCE		A			B			C			D		E	F				
group[a]	DPB1*	8	9	11	33	35	36	55	56	57	65	69	76	84	85	86	87
1	09:01	V	H	L	E	F	V	D	E	D	I	E	V	D	E	A	V
	10:01	-	-	-	-	-	-	-	-	E	-	-	-	-	-	-	-
	17:01	-	-	-	-	-	-	-	-	-	-	-	M	-	-	-	-
2	03:01	-	Y	-	-	-	-	-	-	-	L	K	-	-	-	-	-
	14:01	-	-	-	-	-	-	-	-	-	L	K	-	-	-	-	-
	45:01	-	-	-	-	-	-	-	-	E	L	K	-	-	-	-	-
	86:01	-	-	-	-	-	-	-	-	-	-	-	M	G	G	P	M
3	02:01	L	F	G	-	-	-	-	-	E	-	-	M	G	G	P	M
	02:02	L	F	G	-	L	-	-	-	E	-	-	M	G	G	P	M
	02:03	L	F	G	-	L	-	E	A	E	-	-	M	G	G	P	M
4	01:01	-	Y	G	-	Y	A	A	A	E	-	K	-	-	-	-	-
	04:01	L	F	G	-	-	A	A	A	E	-	K	M	G	G	P	M
	04:02	L	F	G	-	-	-	-	-	E	-	K	M	G	G	P	M
	05:01	L	F	G	-	L	-	E	A	E	-	K	M	-	-	-	-
	06:01	-	Y	-	-	-	-	-	-	-	L	-	M	-	-	-	-
	11:01	-	Y	-	Q	Y	A	A	A	E	L	R	M	-	-	-	-
	13:01	-	Y	-	-	Y	A	A	A	E	-	-	I	-	-	-	-
	15:01	-	Y	G	Q	Y	A	A	A	E	L	R	M	V	G	P	M
	16:01	L	F	G	-	-	-	-	-	E	-	-	M	-	-	-	-
	19:01	L	F	G	-	-	-	E	A	E	-	-	I	-	-	-	-
	20:01	-	Y	-	-	-	-	-	-	-	L	K	M	-	-	-	-
	23:01	L	F	G	-	-	-	A	A	E	-	K	M	G	G	P	M
	46:01	L	F	G	-	-	-	-	-	-	-	-	M	G	G	P	M

[a] HLA-DPB1 alleles are classified into 4 TCE groups according to a previously described algorithm for HSCT donor-recipient matching [18, 20, 29].

Table 3: Amino acid residues targeted by SDM of HLA-DPB1*09:01.

HvR	Position	Pocket[a]	Wild type	Amino acid substitution[b]	Construct[b]
A	9	9	H	Y	H9Y
A	9	9	H	F	H9F
B	35	9	F	Y	F35Y
B	35	9	F	L	F35L
C	55	9	D	A	D55A
C	56	out	E	A	E56A
C	57	out	D	E	D57E
D	69	4 and 7	E	K	E69K
E	76	4	V	M	V76M
F	84	1	D	G	D84G

[a] Pocket numbers correspond to the peptide amino acid residues they accommodate; "out" indicates amino acids located outside the peptide binding groove [22]. [b] Amino acid substitutions, reflected by the names of the resulting constructs, were selected on the basis of homology modeling (Figure 1).

Table 4: Alloreactive T cell effectors used in this study.

Effectors[a]	Isolation[b]	Origin[c]	DPB1*		Nominal Specificity	TCR-Vβ[d]
			Responder	Stimulator		
C538-DV/DG-9	LDA	HSCT Rejection [5]	02:01, 04:02	02:01, **09:01**	DP9	**8** (100%)
C508-DV/DG-9	LDA	HSCT Rejection [5]	02:01, 04:02	02:01, **09:01**	DP9	**2** (100%)
M1-DV/DG-9	MLR	HSCT Rejection [5]	02:01, 04:02	02:01, **09:01**	DP9	**13.6** (40%), **nd** (60%)
C4-TO/MI-9	LDA	Healthy VUD	02:02, 04:01	04:02, **09:01**	DP9	**13.1** (100%)
C301-TO/MI-9	LDA	Healthy VUD	02:02, 04:01	04:02, **09:01**	DP9	**23** (100%)
M2-DU/FF-9	MLR	Healthy VUD	04:01, 13:01	04:02, **09:01**	DP9	**20** (100%)
C70-UD/VR-10	LDA	Healthy VUD	01:01, 04:02	02:01, **10:01**	DP10	**4** (100%)
M4-DE/PD-17	MLR	Healthy VUD	02:01, 04:01	03:01, **17:01**	DP17	**4** (85%), **8** (3%), **17** (3%)

[a] the name of each T cell effector is composed as follows: C or M indicating the presence of a clone obtained by LDA or a T cell line obtained by MLR is followed by an arbitrary number and acronyms of the R/S pair from which the effector was derived (see Table 1); the final number after the hyphen indicates nominal specificity for HLA-DP9, 10 or 17, respectively.
[b] indicates method used for isolate of alloreactive effector T cells. LDA; Limiting Dilution Assay, MLR; Mixed Lymphocyte Reaction.
[c] indicates the clinical condition of the R/S pairs from which each effector was derived.
[d] TCR-Vβ usage as determined by flow cytometry. For T cell lines obtained by MLR, the percentage of the observed TCR-Vb family in the total CD4+ T cells is indicated in brackets. Nd; not determined.

Figure 1

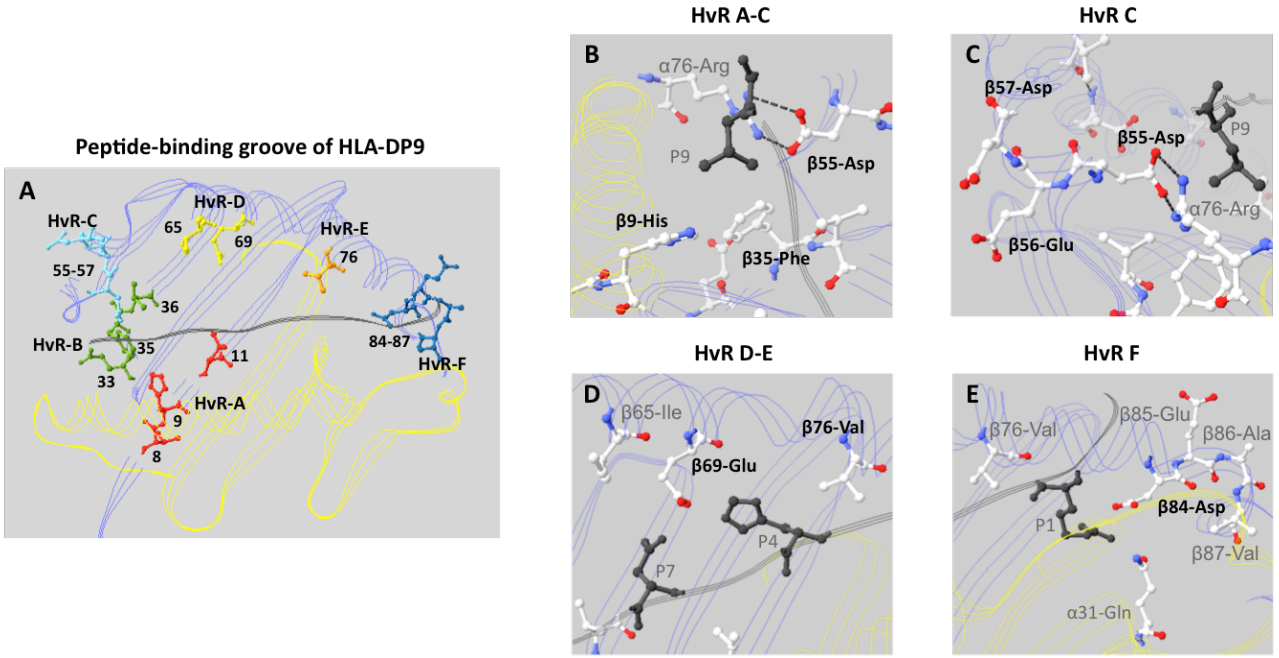

Peptide-binding groove of HLA-DP9
A
HvR-C
HvR-D
HvR-E
HvR-B
HvR-A
HvR-F
55-57
65
69
76
36
35
33
11
84-87
9
8
HvR A-C
B
α76-Arg
P9
β55-Asp
β9-His
β35-Phe
HvR C
C
β57-Asp
β55-Asp
P9
α76-Arg
β56-Glu
HvR D-E
D
β65-Ile
β76-Val
β69-Glu
P4
P7
HvR F
E
β76-Val
β85-Glu
β86-Ala
β84-Asp
P1
β87-Val
α31-Gln

Figure 2

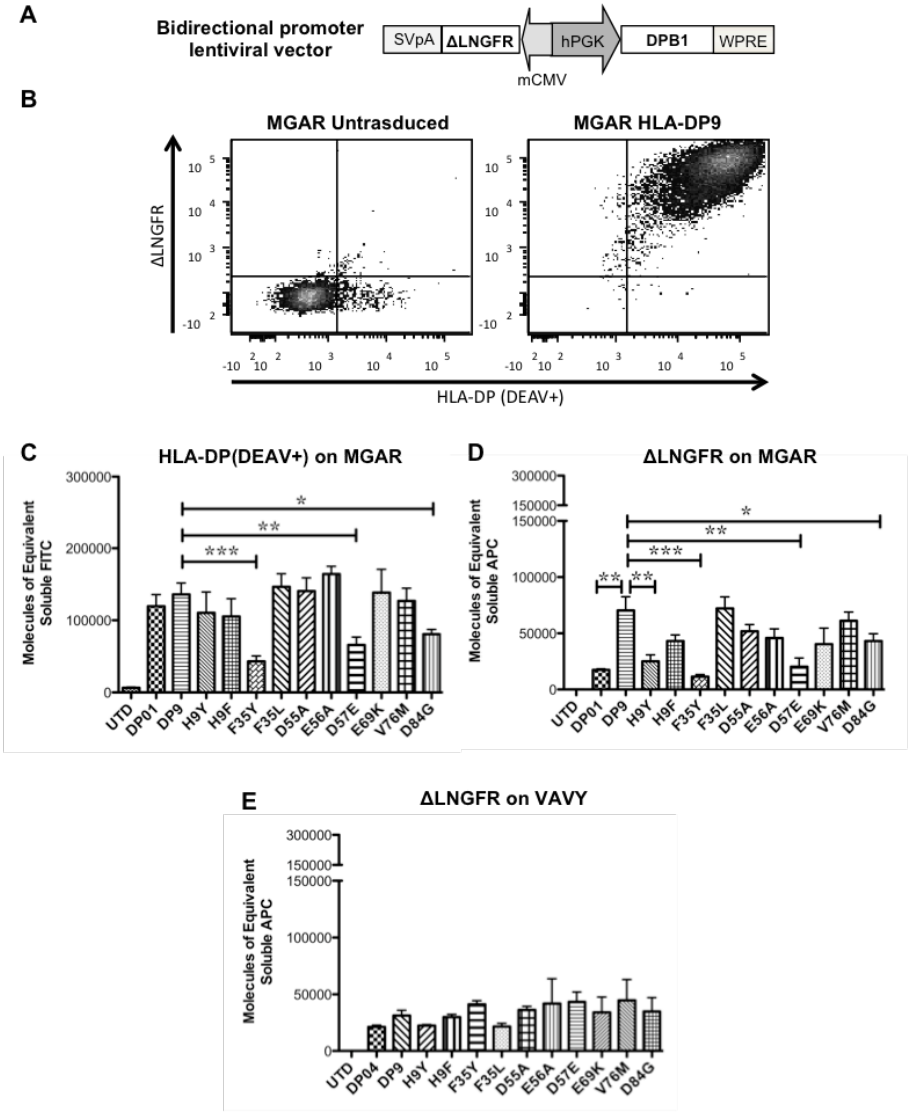

A
Bidirectional promoter lentiviral vector
SVpA
ΔLNGFR
hPGK
mCMV
DPB1
WPRE
B
MGAR Untrasduced
MGAR HLA-DP9
ΔLNGFR
HLA-DP (DEAV+)
C
HLA-DP(DEAV+) on MGAR
Molecules of Equivalent Soluble FITC
UTD
DP01
DP9
H9Y
H9F
F35Y
F35L
D55A
E56A
D57E
E69K
V76M
D84G
D
ΔLNGFR on MGAR
Molecules of Equivalent Soluble APC
E
ΔLNGFR on VAVY
DP04

Figure 3

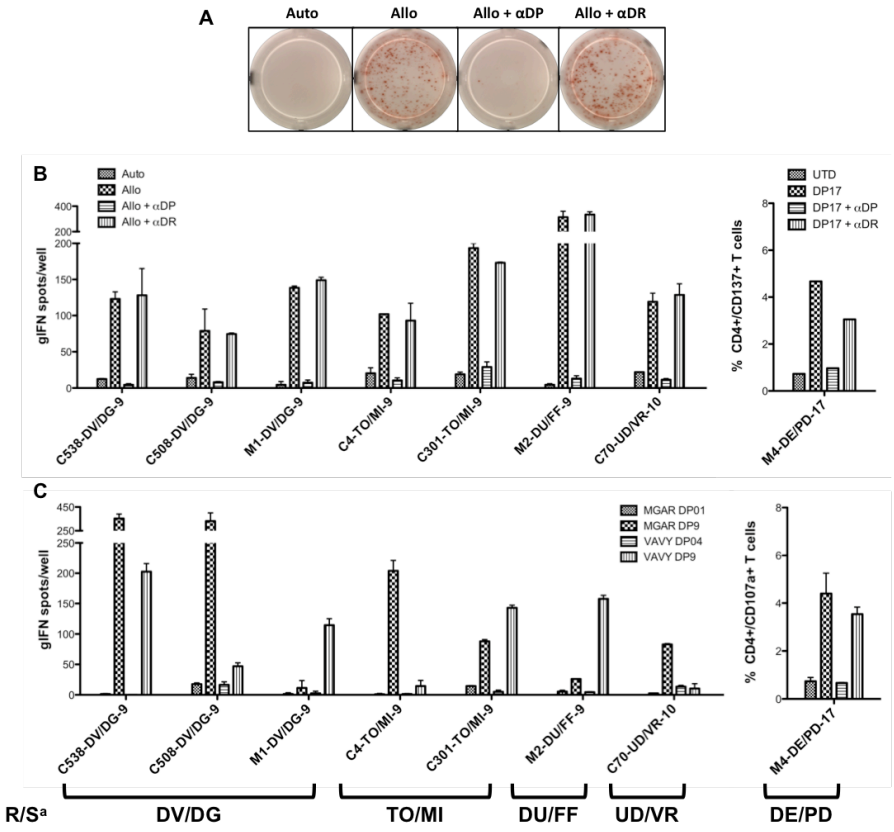

A
Auto
Allo
Allo + αDP
Allo + αDR
B
Auto
Allo
Allo + αDP
Allo + αDR
gIFN spots/well
C538-DV/DG-9
C508-DV/DG-9
M1-DV/DG-9
C4-TO/MI-9
C301-TO/MI-9
M2-DU/FF-9
C70-UD/VR-10
UTD
DP17
DP17 + αDP
DP17 + αDR
% CD4+/CD137+ T cells
M4-DE/PD-17
C
MGAR DP01
MGAR DP9
VAVY DP04
VAVY DP9
gIFN spots/well
% CD4+/CD107a+ T cells
R/Sa
DV/DG
TO/MI
DU/FF
UD/VR
DE/PD

Figure 4

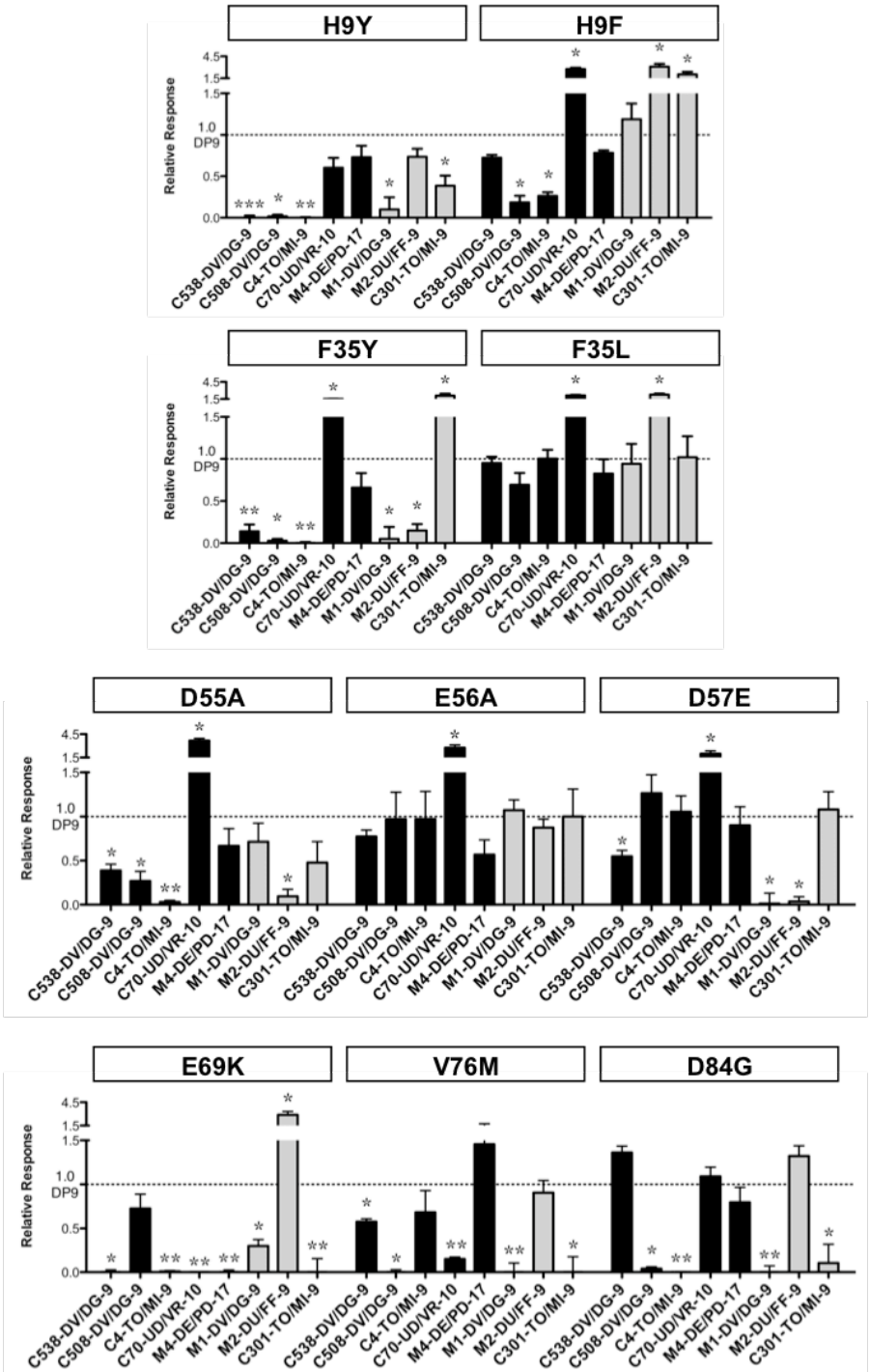
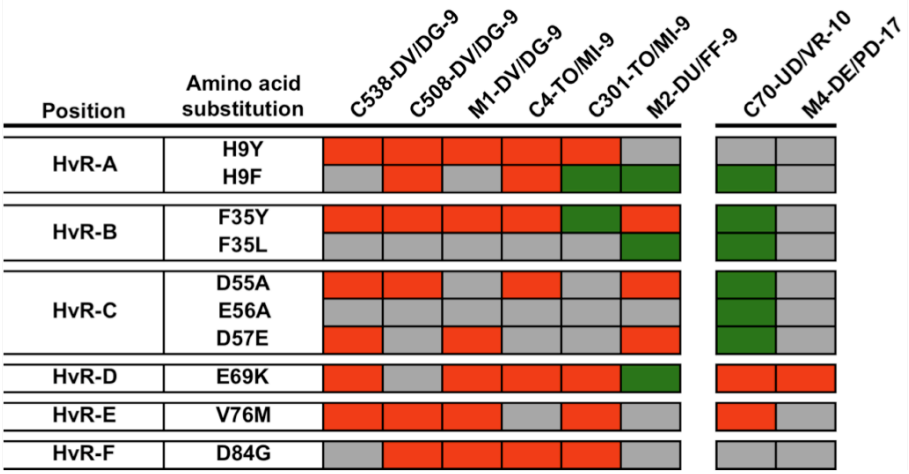

H9Y
H9F
F35Y
F35L
D55A
E56A
D57E
E69K
V76M
D84G
Relative Response
DP9
C538-DV/DG-9
C508-DV/DG-9
C4-TO/MI-9
C70-UD/VR-10
M4-DE/PD-17
M1-DV/DG-9
M2-DU/FF-9
C301-TO/MI-9

Figure 5


Position
Amino acid substitution
C538-DV/DG-9
C508-DV/DG-9
M1-DV/DG-9
C4-TO/MI-9
C301-TO/MI-9
M2-DU/FF-9
C70-UD/VR-10
M4-DE/PD-17
HvR-A
H9Y
H9F
HvR-B
F35Y
F35L
HvR-C
D55A
E56A
D57E
HvR-D
E69K
HvR-E
V76M
HvR-F
D84G

Chapter 4

Description and molecular modeling of two novel HLA alleles: HLA-A*0343 and A*0345

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Abstract

We report the identification of two novel human leucocyte antigen (HLA) in two Caucasian individuals. HLA-A*0343 differs from A*03010101 by four changes at nucleotides 411–414 (CCGG>TGAA) and by a point mutation at position 418 (G>C). These differences lead to two amino acid substitutions at codon 114, where arginine has changed into negatively charged glutamic acid, and at codon 116, where aspartic acid has changed into positively charged histidine. Molecular modeling showed that these changes have a profound influence on the overall charge of the F pocket of the groove, resulting in potentially important changes in the peptide repertoire. HLA-A*0345 was found in a hematological female patient candidate to bone marrow transplantation. This new variant differs from HLA-A*03010101 at position 845 (C>A) encoding an amino acid change of threonine to asparagine at codon 258 located in the α3 domain. Molecular modeling does not suggest a substantial role of this substitutions on the interaction with β2-microglobulin or CD8.

Article

In this study, we describe the identification of two novel human leucocyte antigen (HLA)–A*03 alleles, officially named A*0343 and A*0345 [1, 2]. These two variants were found in two Caucasian individuals.

1. HLA-A*0343

The HLA-A*0343 allele was identified in a cord blood unit (MICB#0120061308), the HLA profile of which resulted in HLA-A*03,*3011; B35,*4102; DRB1*010101,*130301.

Low-resolution HLA-A typing was performed with a microarray bead-based technique (polymerase chain reaction–sequence-specific oligonucleotide) (Lambda Array Beads Multi-Analyte System LABMAS, One Lambda, Canoga Park, CA) [3]. In our laboratory practice, the presence of a rare allele is usually confirmed with a different technique; therefore, to verify the presence of A*3011, high-resolution typing of HLA-A*30 was carried out with polymerase chain reaction using sequence- specific primers (PCR-SSP) (Olerup SSP, Saltsjoebaden, Sweden) [4], and no specific amplification was obtained. Direct sequencing was then performed to clarify these results.

HLA-A alleles were first amplified with locus specific primers, then forward and reverse sequencing of exons 2, 3, and 4 were performed (Atria Genetics, San Francisco, CA). Sequences were processed with a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) and

analyzed with Assign software (Conexio Genomics, Australia). The sequence obtained did not match any known allele combination.

To sequence the two HLA-A alleles in isolation, the amplification product was ligated into vector pCRII-TOPO (TOPO-TA cloning kit; Invitrogen, Leek, The Netherlands) and cloned according to the manufacturer's recommendations. Both forward and reverse DNA sequencing reactions were performed. Various bacterial colonies were used to obtain amplicons from independent PCR reactions to eliminate any possible artifacts.

Exon 2, 3, and 4 sequencing of the two segregated alleles was repeated, and the presence of a new HLA-A*03 variant, together with HLA-A*03010101, was confirmed.

The new HLA-A allele differs from HLA-A*03010101 for five nucleotide changes in exon 3; namely, the nucleotides CCGG at positions 411–414 were replaced by TGAA, and a single base G was substituted by C at position 418 (Fig. 1a). These differences led to three codon changes, as shown in Fig. 1a, that are responsible for two amino acid substitutions at codons 114 and 116, where Arg has changed into Glu and Asp into His, respectively.

These five polymorphisms are present in nearly all HLA-A*30 alleles, except for A*3026. Furthermore, HLA-A*3011 shows some other similarities with the HLA-A*03010101 allele in exon 2, explaining the result obtained with SSO typing.

The side chains of amino acids at positions 114 and 116 of the HLA class I α-chain contribute to form the floor of the peptide-binding groove. Analysis of the high-resolution crystal structure of HLA-A*1101 (PDB ID. 1X7Q), a member of the HLA-A3 supertype, has

shown the contribution of residues at these positions on the shape and charge geometry of the groove, determining a putative impact on the bound peptides [5]. On the basis of this crystal structure, HLA-A*0301 and A*0343 molecular models were constructed using the SWISS-MODEL workspace and analyzed by Swiss-PDB Viewer [6].
In A*0301, Arg 114, a positively charged residue, and Asp 116, a negatively charged residue, appear to interact with each other, forming a spur that protrudes toward the peptide (Figs. 2a, 2c). In A*0343, these residues are substituted by a negatively charged Glu and a positively charged His, respectively, inverting the charge horizon on the floor of the peptide binding groove (Figs. 2b, 2d). Comparison between the two models indicates that the amino acid substitutions encoded by the A*0343 allele are likely to play a relevant role in altering the peptide binding affinity and/or repertoire. As the two residues are located in the F pocket, which accommodates the anchor residue P9/P10 of bound peptide, the described amino acids may have a profound effect on the overall repertoire of peptides presented by the two alleles.

2. HLA-A*0345

This new variant was found in a peripheral blood sample from a hematologic patient affected by monoblastic acute myeloid leukosis candidate for a bone marrow transplantation who was typed as HLA-A*03new, *260101; B*350201,*4901; C*04,*07; DRB1*1104; DRB3*0202; DQB1*0301.
According to the manufacturer's protocol a 2-kb fragment was amplified (exons 1–5 of HLA-A locus) (Atria Genetics, San

Francisco, CA) and then exons 2, 3, and 4 were sequenced in both directions. The analysis of these sequences revealed the presence of a new allele that could be either a variant of HLA- A*03 or A*26 due to a new polymorphism in exon 4 that has not been observed before in any other HLA-A alleles.

The two HLA-A alleles were then separated using a commercial kit for the single group-specific sequencing strategy (S3 HLA-A* Group Specific Sequencing Set; Protrans, Ketsch, Germany).

The sequence data analysis confirmed the presence of a novel HLA-A*03 allele, officially named HLA-A*0345 by the World Health Organization (WHO) Nomenclature Committee [2], which differs from HLA-A*03010101 for a single nucleotide substitution at position 845 in exon 4. This point mutation is responsible for a coding change from ACC to AAC at codon 258 with a consequent amino acid change from Thr to Asn.

The exon 4 nucleotide sequence of HLA-A*0345 is shown in Fig. 1b, aligned with that of HLA-A*03010101.

Amino acid residue 258 is located in the α3 domain and is likely relevant to the interaction with either β2-microglobulin or the T cell coreceptor CD8. To investigate the putative role of the amino acid substitution between HLA-A*0301 and HLA-A*0345, two models were constructed based on the crystal structure of the murine MHC H-2Dd complexed with the murine CD8αβ heterodimer (PDB ID. 3DMM), the only MHC/CD8αβ crystal structure available in the Protein Data Bank to date [7]. Both A*0301 and A*0345 were modeled using the SWISS-MODEL workspace and analyzed by Swiss-PDB Viewer [6].

In both models, residue 258 is located on the surface of the α3 domain, opposite to the surface contacted by β2-microglobulin and adjacent to the surface interacting with the CD8αβ heterodimer (Fig. 3). This suggests that residue 258 does not play a relevant role in the interaction of the α-chain with either β2-microglobulin or CD8, suggesting a limited functional relevance of the amino acid substitution encoded by A*0345.

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Figure Legends

Fig. 1. (a) Exon 3 nucleotide sequence for HLA-A*0343 is compared with that of A*03010101. Dashes indicate identity with A*03010101. Allele A*0343 has EMBL Nucleotide Sequence Database Accession Number FM210536. (b) HLA-A*0345 exon 4 nucleotide sequence is compared with that of A*03010101. Dashes indicate identity with A*03010101 and A*0345. The allele A*0345 has EMBL Nucleotide Sequence Database Accession Number FM253686. The names A*0343 and A*0345 were officially assigned by the WHO Nomenclature Committee in September and November 2008, respectively. (This follows the agreed policy that, subject to the conditions stated in the Nomenclature Report [1], names will be assigned to new sequences as they are identified. Lists of such new names will be published in a forthcoming WHO Nomenclature Report.)

Fig. 2. Comparison of the peptide binding groove in the HLA-A*0301 and HLA-A*0343 models. The molecular surface is colored on the basis of amino acid types: hydrophobic amino acids are shown in white; polar amino acids in yellow; and negatively and positively charged amino acids in red and blue, respectively. (a, b) TCR view of the peptide binding groove: peptide is represented with a green ribbon. (c, d) Slab view of peptide binding groove through residues 114 and 116.

Fig. 3. Interaction of α3 domain with CD8 and β2-microglobulin in HLA-A*0301 (A) and HLA-A*0345 (B) models. Models are represented with colored ribbon: CD8 α-chain and β-chain, respectively, in red and green; α3 domain in magenta; and β2-microglobulin in blue. White arrows indicate the 258 residue; the molecular surface contacted by the CD8 heterodimer is indicated by different colors: red, contacted by CD8 α-chain; green, contacted by CD8 β-chain; yellow, contacted by both.

Fig. 1

Fig. 2

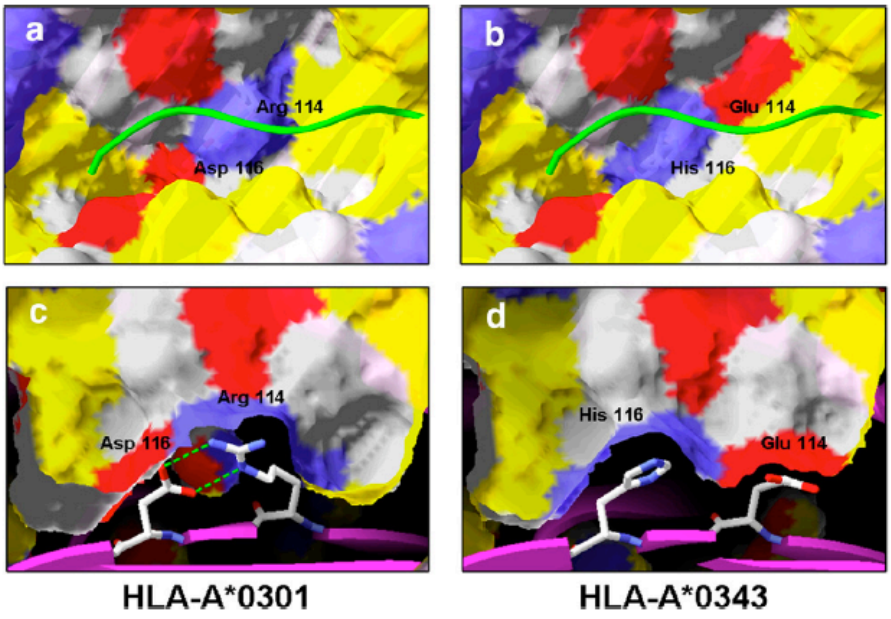
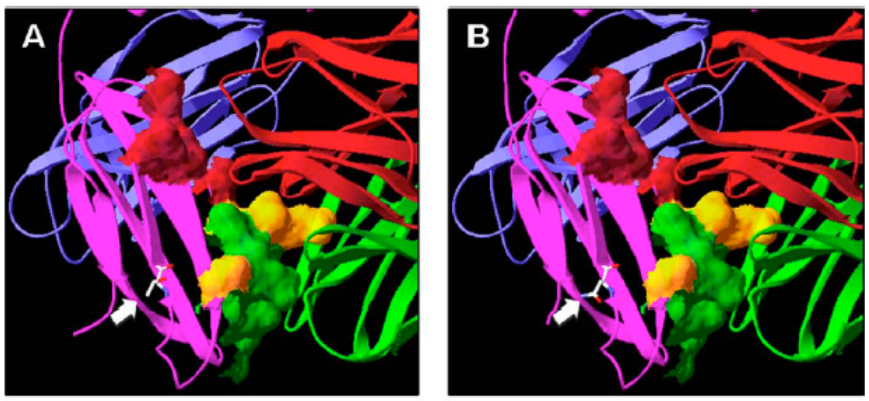

a
Arg 114
Asp 116
b
Glu 114
His 116
c
Arg 114
Asp 116
d
His 116
Glu 114
HLA-A*0301
HLA-A*0343

Fig. 3



HLA-A*0301 HLA-A*0345

Chapter 5

Description and molecular modeling of a novel HLA allele: A*32:22

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Abstract

We describe here the sequence and the molecular modeling of a new variant of HLA-A*32 allele officially named A*32:22. This novel allele has been detected in an Italian cord blood sample by sequence-based typing (SBT). The mutation (CAT→CGT), which has occurred at codon 151, at position 524, implies an amino acidic change from Histidine to Arginine. Residue 151 is located on top of the molecule inside the region contacted by T cell receptor (TCR) and it is possibly involved in docking TCR. A positively charged residue is maintained on this position determining a slight change of electrostatic potential on the molecular surface. This suggests a limited functional relevance of the amino acid substitution encoded by A*32:22.

Article

Improvements of HLA typing methods have contributed to the identification of new alleles and therefore to the expansion of the knowledge about HLA diversity. So far, more than 1729 HLA-A alleles have been reported to the WHO Nomenclature Committee for Factors of the HLA System [1, 2]. Such an extremely high polymorphism, makes HLA system the most important in Hematopoietic Stem Cell Transplantation (HSCT).

In this report, we describe the identification of a new HLA-A* allele, detected in an Italian cord blood sample.

This sample was initially typed at A, B, and DRB1 loci using intermediate resolution sequence-specific oligonucleotide –probe (SSO) and sequence specific primers (Lambda Array Beads Multi-Analyte System®-LABMAS, One Lambda, Inc, Canoga Park CA,USA) [3]. The sample was typed as HLA-B*51,*52; DRB1*03:01:01, *15:02:01 but no typing at A locus was assigned by the interpretation software in use in our laboratory (HLA Vision 2.2.0, One Lambda Inc.). However, polymerase chain reaction–sequence-specific primers (PCR-SSP) [4] (Biotest HLA-A SSP kit, Biotest 63303 Dreieich, Germany) testing gave a result of HLA-A*26, A*32. Thus, the discrepancy between SSP and SSO results supported the hypothesis of the presence of a novel HLA-A allele.

Further analyses by high-resolution direct sequencing (SBT) in both direction of exons 2,3,4 of HLA-A locus were carried out. We used group specific primers (S3 HLA-A* Group Specific Sequencing Set;

Protrans, Ketsch, Germany). DNA sequences were obtained after processing with 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) and analysis with Assign 3.5+ software (Conexio Genomics, Applecross, WA, Australia).

With SBT the HLA-A typing result was as follows: A*26:01:01, *32 new. The allele A*32 new shows one mismatch with A*32:02 at codon 151 (CAT→CGT) (Fig. 1), a mutation which implies an amino acidic change from Histidine to Arginine.

In order to investigate the functional relevance of this polymorphism, we performed Homology Modelling of A*32:02 and A*32:22 encoded proteins using the Swiss-Model workspace [5]. Models were generated on the basis of the crystal structure of A*24:02 in association with β2-microglobulin and a telomerase peptide (PDB id: 2BCK) [6]. Their quality was estimated determining QMEAN Z-score of each model: A*32:02 Z-score: -1.037; A*32:22 Z-score: -1.192 [7]. Analysis of models and crystal structures was performed using the software Swiss PDB Viewer (DeepView 4.0.4) [8].

The polymorphism is located at residue 151 in α2 domain of HLA-A molecule on top of the α-helix forming a sidewall of the peptide binding groove (Fig. 2 A and B). The amino acid Arginine is not involved in interaction with peptide, thus excluding any functional impact of its substitution on peptide repertoire presented to TCR. However, its position is very superficial leading this amino acid to point outside its side chain and making it available to be contacted directly.

We mapped the footprint of TCRs on molecular surface of the HLA-A molecule comparing TCR-HLA contacts (atomic distance less than 5

Å) in crystal structures of 10 unique TCR/pHLA-A complexes (Table 1). We used different colours to mark areas frequently or occasionally contacted (Figure 2 C). Residue 151 is located in proximity of two conserved anchor residues, 154 and 155 (RF: 1); and it itself is often contacted by TCR (RF: 0,7).

The substitution occurring in A*32:22 exchanges a positively charged residue with another one. To verify if this substitution can change electrostatic potential of the molecule, we solved Poisson-Boltzmann equation simulating an aqueous solution with ionic strength of 0.145 mol/l and mapped resulting electrostatic map on surface of the two models (Figure 2 D and E). A slight change is visible on surface of the two models but a positively charged area is conserved and available for docking of TCR.

In conclusion, polymorphism of the new HLA-A allele encodes an amino acid substitution that, on the basis of its nature and location, does not seem to have a functional impact on immunogenicity.

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Figure Legends

Figure 1. Sequence of A*32:22 is compared to that of A*32:02 exon 3. The allele A*32:22 has EMBL Nucleotide Sequence Database Accession Number FN663944.
The name A*32:22 was officially assigned by the WHO Nomenclature Committee for factors of HLA System in January 2010. This follows the agreed policy that, subject to the conditions stated in the most recent Nomenclature Report (Marsh at al., 2010), names will be assigned to new sequences as they are identified.

Figure 2. Homology Modelling of A*32:02 and A*32:22. A-B: Pictures of binding groove of the two models: in red and blue are, respectively, α-helix and β-sheet; in green the peptide in ball and stick representation. Residue 151 is showed in ball and stick representation coloured using CPK colour scheme. C: Footprint of TCR on HLA-A molecule. Molecular surface is coloured regarding to relative frequencies (RF) of TCR contacts in a pool of 10 different TCR/pHLA-A crystal structures: grey (RF=0); yellow (RF=0.1-0.5); orange (RF=0.6-0.8); red (RF=0.9-1) Arrow indicates position of the residue 151. D-E: Representation of electrostatic potential on molecular surface of the two models: red and blue represents, respectively, negative and positive charge ranging from -0.5 to 0.5 kT/e. Arrows indicate position of residue 151 in the two models.

Table 1

List of crystal structures of TCR/pHLA-A used to determine footprint of TCR on HLA-A. All structures are archived in the IMGT/3Dstructure-DB (http://www.imgt.org/3Dstructure-DB) [9].

PDB-ID	TCR	HLA	Peptide	TCR contacts on HLA (residues at D<5 Å)
1ao7	A6	HLA-A*0201	Tax peptide 11-19 (Human T Lymphotropic Virus Type 1)	58 62 65 66 68 69 72 73 149 150 151 152 154 155 158 159 163 166 167 170
1bd2	B7	HLA-A*0201	Tax peptide 11-19 (Human T Lymphotropic Virus Type 1)	58 59 62 63 65 66 69 72 150 152 54 155 157 158 159 161 162 163 166 167
2bnr	1G4	HLA-A*0201	NY-ESO-1 tumor-associated antigen	62 65 66 68 69 72 73 75 76 146 149 150 151 154 155 163
2p5e	TR	HLA-A*0201	Cancer/testis antigen 1B peptide 157-165	19 62 65 66 68 69 72 73 75 76 146 150 151 154 155 158 163
1oga	JM22	HLA-A*0201	Matrix protein M1 peptide 58-66 (Influenza A virus)	65 66 68 69 72 73 75 76 146 149 150 151 152 154 155
3gsn	RA14	HLA-A*0201	HCMV pp65 fragment 495-503 (Cytomegalovirus)	62 65 66 69 70 72 73 75 76 80 146 149 150 151 152 154 155 156 158
3hg1	MEL5	HLA-A*0201	Mart-1 (27-35) peptide	58 61 62 65 66 68 69 70 72 73 76 151 154 155 158 159 163 166 167
3o4l	AS01	HLA-A*0201	BSLF2/BMLF1 peptide	62 65 66 68 69 72 75 76 146 147 150 151 152 154 155 156 157 158 159 161 162 163 166 167
3qdj	DMF5	HLA-A*0201	Mart-1 (27-35) peptide	58 62 65 66 69 70 72 73 76 150 154 155 158 159 163 166 167 170
3qeq	DMF4	HLA-A*0201	Mart-1 (27-35) peptide	58 62 65 66 68 69 70 72 73 75 76 80 154 155 157 158 163

Figure 1

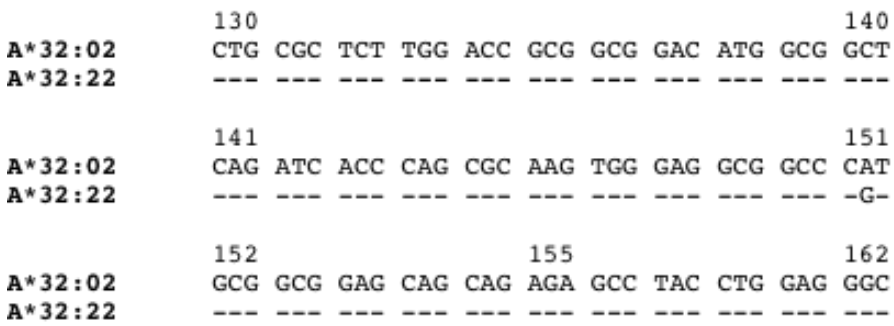
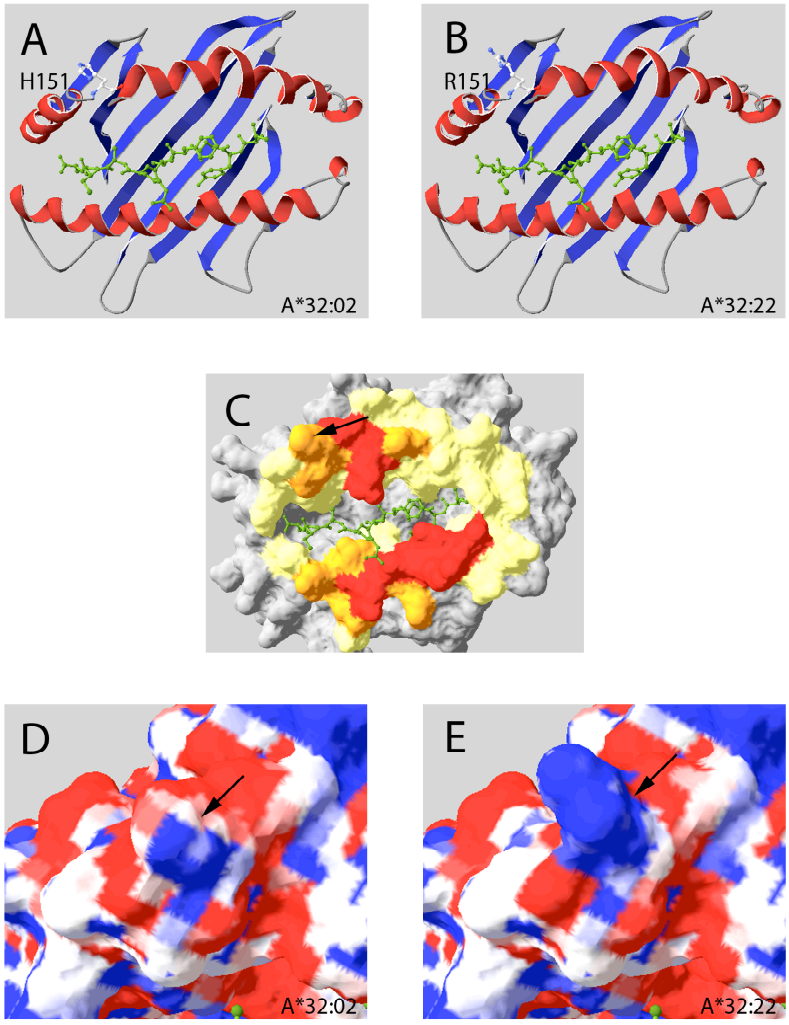

130 140
A*32:02 CTG CGC TCT TGG ACC GCG GCG GAC ATG GCG GCT
A*32:22 --- --- --- --- --- --- --- --- --- --- ---
141 151
A*32:02 CAG ATC ACC CAG CGC AAG TGG GAG GCG GCC CAT
A*32:22 --- --- --- --- --- --- --- --- --- --- -G-
152 155 162
A*32:02 GCG GCG GAG CAG CAG AGA GCC TAC CTG GAG GGC
A*32:22 --- --- --- --- --- --- --- --- --- --- ---

Figure 2



Chapter 6

Summary

Alloreactivity is the major barrier to solid organ and hematopoietic stem cell transplantation (HSCT), determining important clinical events including graft rejection and graft versus host disease. This is due to recognition of non-self Human Leukocyte Antigens (HLA) by the T cell receptor (TCR) on alloreactive T cells educated to recognize foreign peptides in a self-HLA-restricted manner. In the hosting laboratory, alloreactive T cell cross-reactivity patterns suggested the presence of a shared T cell epitope (TCE) encoded by a subset of HLA-DPB1 alleles, thereby defining DPB1 TCE groups which differ in their predicted ability to elicit alloreactive T cell responses. Donor-recipient mismatches across different DPB1 TCE groups were shown to be significantly associated with adverse clinical outcome of unrelated HSCT. However, the molecular basis for this observation was unclear **(Chapter 1)**.

The aim of my thesis was to answer this question by first, investigating if the clinical effect of DPB1 TCE group mismatches was reflected by the strength of the *in vitro* alloreactive T cell response, and second, by site-directed mutagenesis (SDM) of HLA-DP9, prototype antigen mediating strong T cell alloreactivity from HLA-DPB1 TCE group mismatched responders.

The strength of the alloreactive T cell response to HLA-DP was assessed in Mixed Lymphocyte Reactions between healthy responder/stimulator pairs mismatched only at the DPB1 locus, followed by quantitative assessment of responder T cells upregulating

the cell surface activation marker CD137. This allowed us to demonstrate that HLA-DP mismatches across different TCE groups indeed induced significantly higher frequencies of alloreactive responder T cells (n=9; mean 10.13% ± 7.51%) compared to mismatches within TCE groups (n=15; mean 2.34% ± 2.82% $p<0.05$) (Sizzano, Zito, **Crivello et al**., Blood 2010; **Chapter 2)**.

SDM of HLA-DP9, driven by homology-modeling, was performed on 6 polymorphic amino acid residues predicted to be crucial for interaction with bound peptide (positions 9, 35, 55, 69, 76 and 84), and on 2 amino acid residues putatively involved in direct interaction with the TCR (positions 56 and 57). These residues were mutated into amino acids naturally occurring in other HLA-DP variants. A lentiviral vector expression platform was used to express the wild type or mutant HLA-DPB1 in 2 HLA homozygous reporter B lymphoblastoid Cell lines. Alloreactive T cell effectors specific for wild type HLA-DPB1*09:01 (n=6) or for DPB1*10:01 and DPB1*1701, respectively but crossreactive to DPB1*09:01 (n=2), were used to test the impact of mutagenesis on recognition by g-interferon ELISpot or CD107a degranulation assays. Individual point mutations were shown to have a clear-cut effect of either abrogating, enhancing or not affecting recognition by the different effectors. For T cells specifically alloreactive to HLA-DPB1*09:01, recognition was influenced by a complex pattern of residues, distributed among all 6 HvR, which was different for each of the 6 effectors studied. In contrast, for the 2 T cells cross-reactive to HLA-DPB1*09:01, only 2 amino acids (positions 69 and 76) had an influence on allorecognition, suggesting the presence of a more restricted set of T cell epitopes in

the context of cross-reactivity rather than nominal specificity. Our data are consistent with peptide involvement in alloreactivity to HLA-DPB1*09:01, and underline the complexity of allorecognition which may be one of the mechanisms underlying the immunogenicity of this molecule (**Crivello et al.,** manuscript to be submitted; **Chapter 3**).

An additional collaborative study with the North Italian Program for Solid Organ Transplantation was aimed at predicting the functional role of amino acid point mutations in newly described naturally occurring variants of HLA-A3 and A32, respectively, by homology modeling. These studies confirmed data from the literature suggesting a relevant putative role of amino acid substitutions at positions 114 and 116, present in a variant of HLA-A3, on the molecular shape and charge of the peptide binding groove. In contrast, point mutations at position 151 and 258 found in two variants of HLA-A32 and A3, respectively, were not predicted to have important functional impacts, due to limited structural or biochemical changes of the groove (Frison, **Crivello et al.,** Hum Immunol 2010 and 2011; **Chapter 4-5**).

In conclusion, the results from my thesis shed new light on the molecular basis of T cell alloreactivity. In particular, they provide evidence in support of the concept that the clinically relevant stronger immunogenicity of HLA-DPB1 TCE group mismatches, compared to TCE group matches, is reflected by a highly divergent and complex TCE repertoire against the former which might be more limited against the latter. Identification of specific allopeptides associated with these responses is warranted to allow us to further dissect their molecular basis.

General discussion

Alloreactivity is an important biological phenomenon well known to be responsible for several clinical events occurring after solid organ or hematopietic stem cell transplantation (HSCT) such as graft failure, graft versus host disease (GvHD) and graft versus leukemia (GvL) activity. The molecular basis of such phenomenon resides on immunogenicity of HLA molecules, which act as targets for immune response mediated by alloreactive T cells [1]. Therefore, a clear comprehension of the molecular nature of HLA immunogenicity is extremely important to improve performance of transplantation.

In this thesis, locus DPB1 was used as experimental model to study immunogenicity of HLA molecules; this locus offers some advantages respect to other HLA loci. First of all, polymorphism at this locus is simpler than others because it is mainly clustered in 6 Hypervariable Regions (HvR) located around codon 9, 35, 55, 69, 76 and 84 [2-4] and can be described as strings of amino acids shuffled between alleles.

Moreover, locus DPB1 is in weak linkage disequilibrium with other HLA loci and, for this reason, it is poorly considered in matching criteria currently used for selection of unrelated donors for transplantation [5]. Particularly, although matched for all other HLA loci, about 80% of HSCT transplants are performed across DPB1 mismatches [6] offering the unique possibility to study DPB1 immunogenicity without any confounding effects deriving from mismatches at other loci.

Finally, interest on this locus is particularly increased by evidence that DPB1 is a useful target of GvL activity [7], so that understanding of its immunogenicity has important relevance in improving treatment of oncohematological patients.

The results obtained in these three years of PhD program shed new light on immunogenicity of HLA molecules encoded by locus DPB1 leading to two major finding: the demonstration of immunodominance hierarchy among DPB1 alleles, and the description of a complex T cell epitope (TCE) repertoire target of heterogeneous alloreactive response. Moreover, our results are consistent with the involvement of bound peptides on DPB1 specific T cell alloreactivity.

The immunodominance hierarchy among DPB1 alleles

In the last decade, a new functional approach for the definition of DPB1 mismatches between donor and recipient in HSCT has attracted increasing interest in the scientific community [8-10]. DPB1 alleles were subdivided in 4 groups with predicted differential immunogenicity, from very low (TCE group 4) to very high (TCE group 1), on the basis of cross-reactivity patterns of alloreactive T cells and subsequent integration with functional evidence reported in literature [8, 9]. According to this classification, a new matching algorithm was proposed to define “permissive” and “non-permissive” DPB1 disparities. This algorithm was shown to be significantly predictive of the outcome after HSCT [6, 8] although differential immunogenicity among DPB1 alleles was not demonstrated. Indeed, alleles of each group are equally able to elicit alloreactive response as extensively reported in literature [2, 11-15]. However, in chapter 2

we showed that alloreactive response to “non-permissive” mismatches is significantly higher than to “permissive” ones, indicating that DPB1 mismatches have different immunogenic power. Moreover, we observed that when stimulated concomitantly with both TCE group 1 and TCE group 4 alleles, alloreactive response to TCE group 1 (CD4+/CD137+ 10,65%) was much stronger than response to TCE group 4 (CD4+/CD137+ 0,88%). Rutten *et al* further supported these data reporting different percentage of CD4+/CD137+ alloreactive T cells responding to alleles belonging to TCE group 2 (2,72%), 3 (1,08%) or 4 (0,41%) in ex vivo testing of T cells obtained from patients who underwent to DPB1 mismatched HSCT matched at all other HLA loci [15].

These data showed the existence of immunodominance hierarchy among DPB1 alleles reflecting their differential immunogenicity. Such a phenomenon was mainly observed in antiviral and antibacterial immune response, describing the ability of some antigenic determinants to induce stronger immune response respect to others [16]. However, evidence was reported also for alloreactivity to minor Histocompatibility Antigens and HLA class I molecules [17, 18]. The biological and molecular mechanism underlying such phenomena is still unclear and object of research. We addressed this question in the context of locus DPB1 through molecular investigation of its immunogenicity.

A complex repertoire of TCEs activates heterogeneous alloreactive T cell response to HLA-DP

In chapter 3, we investigated the molecular nature of alloreactive TCEs encoded by HLA-DP. We generated site directed mutants of a prototype of the most immunogenic alleles, HLA-DP9, and tested the impact of mutagenesis on alloreactive T cell recognition. We used a panel of 8 DP specific T cell effectors, derived from 5 different individuals. These effectors showed wide heterogeneity in terms of T cell receptor (TCR) expression, as indicated by the wide variety of Variable regions used for their β-chains. Moreover, they showed different preference for the two most frequent variants of DPα chain, DPA1*01:03 or *02:01, and sensitivity to different patterns of amino acid substitutions in DPβ chain. All together these results revealed a heterogeneous alloreactive response directed to HLA-DP9 that implies the existence of a wide T cell epitope repertoire encoded by this antigen.

This finding modified the original basis of the TCE algorithm proposed by our group for functional matching of DPB1. Originally this algorithm was based on one immunogenic epitope encoded by some DPB1 alleles and predictive of their differential immunogenicity [9] while our results indicated the existence of a complex repertoire of TCEs which is target of alloreactive response.

We should remind that alloreactive T cell repertoire depends on two important phenomena: the thymic selection of pre-immune T cell repertoire and the training of T cell compartment of immune system during infections. Indeed, T lymphocytes undergo to a complex maturation process mainly consisting in a positive and negative

selection originating immune-competent cells [19]. Self-HLA molecules and their TCEs play a key role during positive and negative selection in such a way that all TCRs are selected to avoid immune reaction to autologous TCEs. Subsequently, this repertoire is further shaped during immune response to pathogens, which stimulate proliferation and differentiation of specific T cell clones reactive to self-HLA molecules presenting foreign peptides. These two processes cooperate to determine the final composition of circulating T cell repertoire. Alloreactivity can originate from both Naïve and Memory T cells respectively responding to never-encountered epitopes or epitopes mimicking pathogen's infections.

In such context, differential immunogenicity among DPB1 alleles can be explained in two not exclusive ways. The most immunogenic alleles can be able to present a wider and different repertoire of TCEs respect to the less immunogenic ones mainly activating Naïve T cells. Recently, Distler *et al* showed that alloreactivity derives preferentially from naïve precursors supporting this hypothesis [20].

Moreover, some TCEs encoded by the most immunogenic alleles can be able to mimic foreign antigens expressed by diffuse infective pathogens, such as cytomegalovirus and Epstein-Barr virus, activating stronger response from Memory T cells expanded during previous infections. In support of this hypothesis, Amir *et al* recently reported that recognition of allogeneic HLA molecules is a common feature of viral specific Memory T cells [21]. A better understanding of the molecular basis underlying immunodominance hierarchy among DPB1 alleles requires unveiling of the cellular compartment mainly involved on allorecognition of these molecules.

Role of bound peptides on HLA-DP immunogenicity

Alloreactive T cell response to HLA-DP9 showed a strong dependency on different and complex patterns of polymorphic residues residing in the peptide-binding groove. T cell allorecognition was affected by substitutions at position 9, 35 and 55 in the pocket 9, 69 and 76 in the pocket 4 and 84 in the pocket 1. These results are in line with previously reported data on HLA-DP2 [22, 23] and similar results were obtained for corresponding positions in other HLA class II molecules [24-27]. Moreover, analysis of structural models of HLA-A variants further supported a major role on immunogenicity of residue 114 and 116 located into the peptide-binding groove. Functional evidence of the role of such amino acids, as well as of other amino acids located in the peptide-binding groove, has been reported in literature [28, 29]. Finally, a recently published study demonstrated that allorecognition is limited by requirement of presented peptides and takes place with a mode of interaction similar to classical recognition of foreign peptides presented by self-molecules [30].

All these findings are compatible with the involvement of endogenous peptides on immunogenicity of HLA-DP antigens. Polymorphic residues determining the peptide binding affinity of the molecule can play a crucial role influencing both repertoire and conformation of peptides presented to TCR originating the complex T cell epitope repertoire previously described and determining the differential immunogenicity of these molecules.

Conclusions

Our results provide new molecular insights on immunogenicity of HLA-DP molecules allowing a better understanding of alloreactive phenomena directed against these antigens. The most innovative finding is the demonstration that HLA-DP molecules express a complex TCE repertoire target of allorecognition. Differential immunogenicity of DPB1 alleles would be dependent on their ability to encode different repertoires, in terms of number and quality of TCEs presented. Such difference could be related to a different ability of each allele to bind and present a different peptide repertoire.

Alloreactivity could be ascribed as consequence of the difference between TCE repertoires presented by allogeneic and autologous molecules. Therefore, if TCE repertoires between autologous and allogeneic molecules are similar, all alloreactive T cells are eliminated during thymic maturation and no alloreactive response can be initiated; the opposite occurs if these TCE repertoires are too much different.

In the context of transplantation, this assumes a particular importance because it constitutes the molecular basis for functional definition of "permissive" and "non-permissive" mismatches. Donor/recipient pairs could be matched in a more intelligent and patient-specific way minimizing differences in the TCE repertoires and consequently reducing the risk of alloreactions. Indeed, this approach has been shown to be useful for mismatches at locus DPB1 and a more comprehensive understanding of HLA immunogenicity will allow us to extend such approach also to other HLA loci.

Future perspectives

These studies allowed us to improve our comprehension of HLA immunogenicity, in particular of locus DPB1. However, some questions remain still unanswered, therefore more efforts will be required to completely solve this puzzle.

The first important issue concerns which subset of T cells, memory or naïve, mediates the alloreactive response to DPB1. This finding would be important to understand if alloreactivity to DPB1 alleles is directed to new encountered epitopes or epitopes mimicking pathogen's antigens. This second option is particularly intriguing and will lead us to investigate the pathogen involved.

Another important issue is the peptide dependency of alloresponse to DPB1, which implies that one or more allopeptides are required by the most immunogenic alleles to induce their strong immune response. Identification of such peptides would be important for generation of HLA-DP tetramers useful to detect alloreactive T cells in vivo and, moreover, for generation of crystal structures of the complex HLA-DP/peptide/TCR in order to study the real interaction between alloreactive T cell receptors and HLA-DP.

Finally, our major concern is to apply our findings on other HLA loci in order to give a comprehensive understanding of alloreactive phenomena. This knowledge will improve the performance of clinical transplantation through a better exploitation of each advantage that this procedure can offer while keeping control of adverse consequences.

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