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DR haplotype diversity of the cynomolgus macaque as defined by its transcriptome

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Abstract The *DR* region of particular primate species may display allelic polymorphism and gene copy number variation (region configuration polymorphism). The sum of these distinct types of polymorphism is defined as complexity. To date, however, the *DR* region of cynomolgus macaques (*Macaca fascicularis*) has been poorly defined. Transcriptome analysis of a pedigreed colony, comprising animals from Indonesia and Indochina, revealed a total of 15 *Mafa-DRA* and 57 *DRB* alleles, specifying 28 different region configurations. The *DRA* alleles can be divided into two distinct lineages. One lineage is polymorphic, but the majority of the amino acid replacements map to the leader peptide. The second lineage is at best oligomorphic, and segregates with one specific *Mafa-DRB* allele. The number of *Mafa-DRB* genes ranges from two to five per haplotype. Due to the presence of pseudogenes, however, each haplotype encodes only one to three *bona fide DRB* transcripts. Depending on the region configuration in which the *Mafa-DRB* gene is embedded, identical alleles may display differential transcription levels. Region configurations appear to have been generated by recombination-like events. When genes or gene segments are relocated, it seems plausible that they may be placed in the context of distinct transcription control elements. As such, *DRB* region-related transcription level differences may add an extra layer of polymorphism to this section of the adaptive immune system.

Keywords MHC · Transcriptome · Nonhuman primates · Comparative immunology

Introduction

Traditionally, Indian rhesus monkeys have been a prime species of choice for biomedical research. However, the import embargo on these animals has led to the search for alternatives; as a result, cynomolgus macaques (*Macaca fascicularis*) have become increasingly important as model species in recent years. To date, cynomolgus macaques have been used in studies for a variety of infectious diseases such as HIV/SHIV, tuberculosis, and dengue, as well as in transplantation and autoimmunity research (Aoyama et al. 2009; Benferhat et al. 2009; Capuano et al. 2003; Greene et al. 2010; Guirakhoo et al. 2004; Ma et al. 2009; Mee et al. 2009b; Wiseman et al. 2009). Since gene products of the major histocompatibility complex (MHC) play a crucial role in a variety of immune responses, detailed knowledge of the genetic background of these macaques has received increasing attention. The MHC class II region of Mauritian monkeys has been studied extensively, but due to a founder effect their *DRB* region shows limited levels of polymorphism and diversity (Blancher et al. 2006, 2008; Bonhomme et al. 2008; Mee et al. 2009a; O'Connor et al. 2007; Wojcechowskyj et al. 2007). In animals of other geographic origins, the diversity of the *DR* region is extensive (Aarnink et al. 2010; Doxiadis et al. 2006, 2010; Leuchte et al. 2004; Wei et al.

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2007). For instance, in Philippine and Vietnamese cynomolgus macaque populations, up to 14 *DRA* alleles have been detected (Aarnink et al. 2010). Additionally, animals originating from Indochina and the Indonesian islands, showed a very high degree of *DRB* region polymorphism with 49 different *Mafa-DRB* regions described (Doxiadis et al. 2010).

In the latter study, a highly polymorphic microsatellite, D6S2878, had been used that maps to intron 2 of all *DRB* genes and pseudogenes with an intact exon 2–intron 2 segment. Most contemporary and more detailed information on *Mafa-DRB* genes is based on isolated exon 2 data. In the present report, we were keen to determine which *DRB* genes represent *bona fide* class II transcripts. In addition, we wanted to examine the level of polymorphism of *DRA* transcripts and their linkage to *DRB* haplotypes, in order to extend our knowledge of the *DR* region composition of monkeys of Indonesian and Indochinese origin.

Materials and methods

Animals and cell lines

The Biomedical Primate Research Centre (BPRC) houses a self-sustaining colony of cynomolgus macaques that have been pedigreed mainly by ethological observations and partially based on the segregation of defined MHC haplotypes (de Groot et al. 2008; Doxiadis et al. 2006). The animals originated from mainland Indochina and the Indonesian islands as proven by mtDNA analysis (de Groot et al. 2008). Two animals with Indonesian mtDNA profile were imported from Mauritius. The cynomolgus macaques analyzed for full-length *DRA* and *DRB* belong to an outbred breeding colony (52 out of 58), and are members of 12 pedigreed families with variable member sizes and generations, ranging from eight to 30 animals and from two to six generations. B-lymphoblastoid cell lines (BLCL) of the other six animals were received in collaboration with other European institutions, according to regulations approved by local ethics committees.

Cloning, sequencing, and genotyping

RNA was isolated from BLCLs (Rneasy kit, Qiagen) and subjected to One-step reverse transcriptase polymerase chain reaction (RT-PCR), as recommended by the supplier (Promega). Full length *Mafa-DRA* sequences were amplified by PCR from DNA using primers specific for human DRA 5′ and 3′ untranslated sequences (Lekutis and Letvin 1995): 5′DRA-*Sal*I, 5′-TCC CGT CGA CCG CCC AAG AAG AAA ATG GCC-3′ and 3′DRA-*Bam*HI, 5′-CAT TGG ATC CGA AGT TTC TTC AGT GAT CTT-3′.

Likewise, *Mafa-DRB* sequences were amplified by PCR using primers specific for human 5′- and 3′ untranslated sequences (Lekutis and Letvin 1995): 5′DRB-*Sal*I, 5′-GCC CGT CGA CCT GTC CTG TTC TCC AGC ATG-3′ and 3′ DRB-*Bam*HI, 5′-GGC GGG ATC CCT TTT CAT CCT GCA AAG CTG-3′. Primers were synthesised by Invitrogen (Paisley, UK). PCR was performed as earlier described by de Groot and co-workers (2004). The RT-PCR products were cloned using the Genejet cloning kit (Fermentas). After transformation, a minimum of 32 colonies were selected for plasmid isolations. Sequencing reactions were performed using the BigDye terminator cycle sequencing kit, and samples were run on an automated capillary sequencing system (ABI Genetic Analyzer 3130*XL*). The sequences were analysed using SeqMan Pro (DNASTAR, Lasergene 8.1.2.), and alleles are based on at least three clones with identical sequences from different monkeys or independent PCRs from one monkey. To define loci and lineages, alignments of the sequences were made using the MacVector™ version 11.1.2 (Oxford Molecular Group). DRB-STR typing was performed according to published methods (de Groot et al. 2008; Doxiadis et al. 2010). In total, 11 *Mafa-DRA* and 56 *Mafa-DRB* sequences have been deposited in the EMBL database (Accession numbers FR717360–FR717426, Table 1) and have been officially designated by the IPD-MHC database (Robinson et al. 2003).

Results and discussion

Definition of *DR* haplotypes

The 52 pedigreed cynomolgus macaques included in this study are members of 12 families comprising two to six generations, which belong to a self-sustaining breeding colony; an example of a pedigreed family has been provided (Fig. 1). Therefore, segregation analyses of the respective alleles within the macaque families allowed the definition of *DR* haplotypes. In some cases, *DR* haplotypes detected in family members have been confirmed by the presence of identical alleles defined by analyses of unrelated animals.

DRA polymorphism

Within the panel of Indonesian and Indochinese cynomolgus macaques, 15 different *DRA* alleles could be defined, seven of which had not been previously described (Table 1, bold). All but one of these alleles belong to one lineage: *Mafa-DRA*01*. In contrast to the *HLA-DRA* gene, of which only three alleles are documented, the *Mafa-DRA* gene is polymorphic, and the degree of its polymorphism

Table 1 *Mafa* full-length *DRA* and *DRB* alleles detected in 58 animals

Allele	Animal	Accession number
Mafa-DRA		
*DRA*01:01:01*	Bilboa	EF208826
DRA*01:01:09	Kraa	FR717418
*DRA*01:02:01:01*	Yukka	EF208827cx
*DRA*01:02:05*[a]	Yabaa	FR717422
DRA*01:02:20	Trespa	FR717419
DRA*01:02:21	Kraa	FR717420
*DRA*01:03:01*	Vivaa	AM943638
*DRA*01:03:02*[a]	Riva	FR717425
*DRA*01:03:03*[a]	Bilboa	FR717421
DRA*01:03:07	Cyn83	FR717417
DRA*01:03:08	Hoeba	FR717416
*DRA*01:09*[a]	Blo	FR717423
DRA*01:10:01	Kippa	FR717424
DRA*01:10:02	Joshua	FR717426
*DRA*02:01:01:01*	Clint	EF208828
Mafa-DRB		
*DRB1*03:06:01*[a]	Zazaa	FR717409
*DRB1*03:08:01*	Kippa	FR717406
DRB1*03:08:02	Alfa	FR717373
*DRB1*03:09*[a]	Zola	FR717393
*DRB1*03:12:01*[a]	Bufo	FR717381
*DRB1*03:14*	Cyn83	FR717369
*DRB1*03:15*	Cyn83	FR717368
*DRB1*03:16*[a]	Friko	FR717400
*DRB1*03:17*[a]	Roza	FR717396
*DRB1*03:21*[a]	Yukka	FR717399
*DRB1*04:03*[a]	Yabaa	FR717380
*DRB1*04:11*	Vivaa	FR717374
*DRB1*10:02*[a]	Indy	FR717386
*DRB1*10:04*	Kippa	AF492283
*DRB1*10:10*[a]	Yukka	FR717398
*DRB3*04:01*[a]	Kraa	FR717372
*DRB4*01:01*	Clint	FR717382
*DRB4*01:02*[a]	Cyn81	FR717363
*DRB4*01:03*[a]	Joshua	FR717414
*DRB5*03:01:01*[a]	Gayo	FR717383
*DRB5*03:01:02*[a]	Cyn80	FR717384
*DRB5*03:04*[a]	Joshua	FR717415
*DRB5*03:05*[a]	Vip	FR717385
*DRB5*03:06*[a]	Just-So	FR717371
*DRB5*03:09*[a]	Zazaa	FR717408
DRB5*03:15	Cyn81	FR717387
DRB5*03:16	Tabasco	FR717413
DRB*W1:07	Cyn82	FR717365
DRB*W1:08	Juanita	FR717403
*DRB*W3:03:01*	Hippo	FR717370
*DRB*W3:04:01*[a]	Cyn80	FR717361
*DRB*W3:05*	Cyn80	FR717362
*DRB*W3:06*	Cyn82	FR717366
*DRB*W4:05*[a]	Dojo	FR717391
*DRB*W5:01*	Indy	FR717376
DRB*W6:06	Cyn80	FR717360
DRB*W6:07	Kippa	FR717405
*DRB*W7:02*	Geisha	FR717404
*DRB*W7:07*	Cyn82	FR717367
*DRB*W21:01*[a]	Zola	FR717394
*DRB*W21:01*	Clint	FR717375
*DRB*W20:02*[a]	Canada	FR717410
*DRB*W20:02*[a]	Bufo	FR717364
*DRB*W25:04*[a]	Dojo	FR717390
DRB*W25:05	Nanaea	FR717389
DRB*W25:06	Canada	FR717411
*DRB*W26:02:01*	Cyn82	FR717388
*DRB*W36:01*[a]	Dojo	FR717395
DRB*W36:04	Alfa	FR717378
*DRB*W37:01*[a]	Yabaa	FR717379
*DRB*W40:01*	Friko	FR717401
*DRB*W49:01*	Hippo	FR717377
DRB*W49:01:02	Jura	FR717412
*DRB*W53:01*[a]	Jena	FR717407
*DRB*W66:01*	Rassoa	FR717402
*DRB*W67:01*[a]	Roza	FR717397
*DRB*W68:01*[a]	Nanaea	FR717392

[a] Extension of existing alleles. Previously unreported alleles are depicted in bold

appears also to be higher than in a thoroughly studied Indian rhesus macaque population (de Groot et al. 2004). However, the variations between these alleles are mainly due to synonymous substitutions, thus indicating a strong purifying selection operating on the gene and the exons specifying the antigen biding site (Hughes and Nei 1989). The *DRA*01* alleles detected in our cohort give rise to only five different amino acids replacements, which are either situated in the leader sequence or within the transmembrane part of the molecule. These results are comparable to analyses of cynomolgus monkeys of other origins (Aarnink et al. 2010; O'Connor et al. 2007).

In contrast to the polymorphic *DRA*01* lineage, only one allele of the second lineage, *DRA*02*, has been detected in our cohort. In contrast to the *Mafa-DRA*01* allotypes, the *DRA*02* lineage is typified by five amino acid replacements, two in the leader section and three that map to the alpha 1 domain that defines the scaffolding of the antigen-binding site (Table 2). An identical or similar allele has been detected in Chinese rhesus macaques (Doxiadis et al. 2008) and in the pigtailed macaque

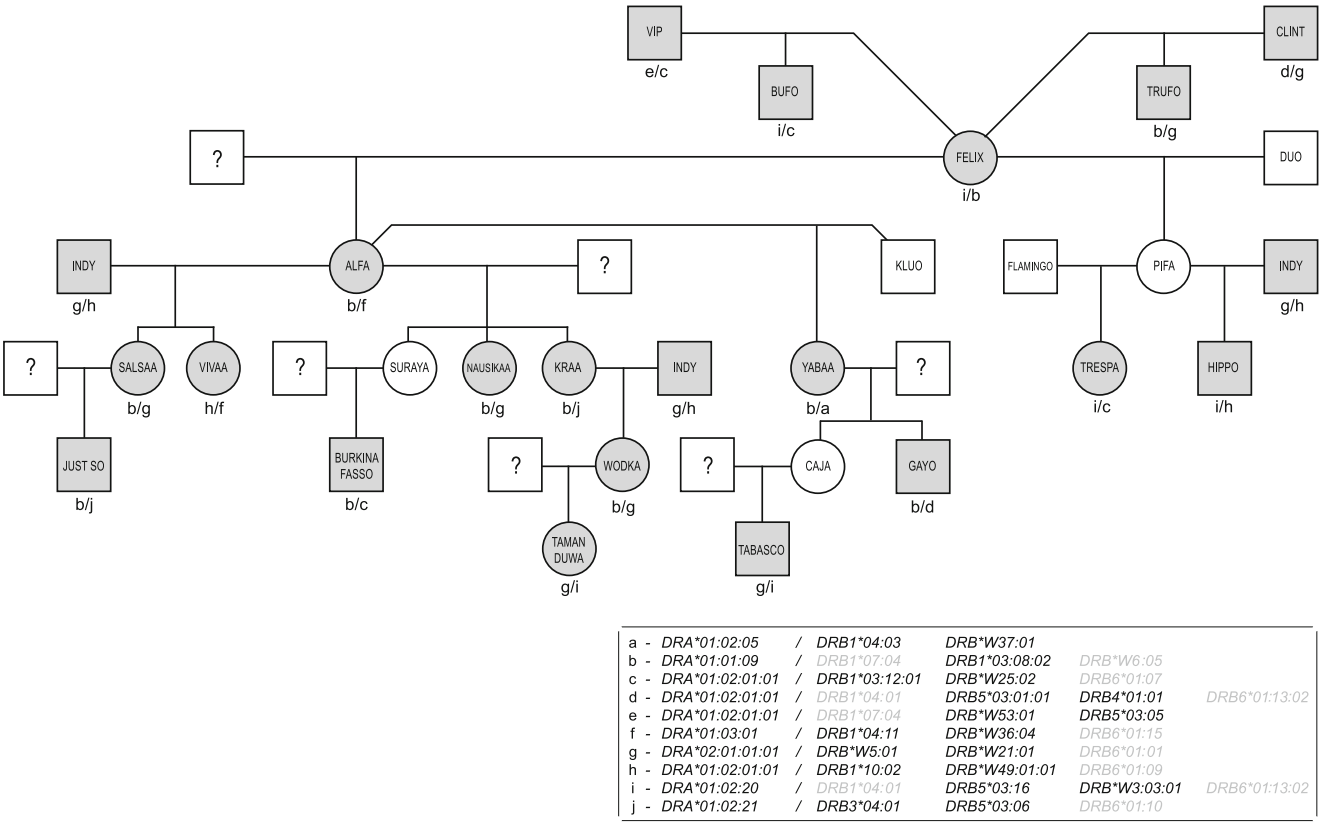

Fig. 1 Pedigree of one cynomolgus family with the segregation of *DRA/DRB* haplotypes indicated. The analyzed animals are marked by *shading*; a *question mark* indicates that the sire could not be identified. Transcribed genes are depicted in bold

(Aarnink et al. 2010; O'Connor et al. 2007). Thus, *DRA*02* seems to be an evolutionarily old entity. Within our cohort, the *Mafa-DRA*02:01:01:01* allele is always present in *cis* configuration with a certain *DRB* haplotype (Table 3, #28)

Table 2 Polymorphic amino acid sites of Mafa-DRA

	LP			α1			TC 2
	-2	-1	-1		2	3	0
	3	2	0	4	2	1	8
Mafa-DRA*01:01:01	E	I	V	E	F	I	V
Mafa-DRA*01:01:09	-	-	-	-	-	-	-
Mafa-DRA*01:02:01:01	I	-	-	-	-	-	-
Mafa-DRA*01:02:05	I	-	-	-	-	-	-
Mafa-DRA*01:02:20	I	-	-	-	-	-	-
Mafa-DRA*01:02:21	I	-	-	-	-	-	-
Mafa-DRA*01:03:01	V	-	M	-	-	-	-
Mafa-DRA*01:03:02	V	-	M	-	-	-	-
Mafa-DRA*01:03:03	V	-	M	-	-	-	-
Mafa-DRA*01:03:07	V	-	M	-	-	-	-
Mafa-DRA*01:03:08	V	-	M	-	-	-	-
Mafa-DRA*01:10:01	V	-	-	-	-	-	-
Mafa-DRA*01:10:02	V	-	-	-	-	-	-
Mafa-DRA*01:09	E	-	-	-	-	-	I
Mafa-DRA*02:01:01	A	T	-	D	Y	L	-

encoding a *DRB*W21:01* and *DRB*W5:01* allotype. This haplotype is present in monkeys from Indochina as well as in animals from the Indonesian islands (Doxiadis et al. 2010) and Mauritius (Aarnink et al. 2010). Furthermore, the *Mafa-DRA*02* allele has been observed in animals originating from the Philippines together with another *DRB* configuration that also harbours a *DRB*W5:01* allele (Aarnink et al. 2010). Therefore, a steric preference of the *Mafa-DRA*02*-encoded α chain for the *DRB*W5:01*-encoded β chain to form a stable molecule seems to be plausible. Although the amino acid changes within the alpha-1 domain of the DRA*02 chain are conservative, an additional possibility that the resulting DR molecule is able to present a set of peptides, which are advantageous in controlling certain pathogens, cannot be excluded.

Mafa-DR haplotypes

As has been shown recently by means of microsatellite and exon 2 typing, cynomolgus macaques show abundant levels of *DRB* region configuration polymorphisms: that is, haplotypes that vary in the number and content of *DRB* genes (de Groot et al. 2008; Doxiadis et al. 2010). Most of the haplotypes encode three *DRB* genes or pseudogenes

Table 3 *Mafa-DR* haplotypes defined by exon 2 and full-length sequencing

hapl #	*DRA* locus	1st *DRB* locus	2nd *DRB* locus	3rd *DRB* locus	4th *DRB* locus	5th *DRB* locus
1	***DRA*01:02:01:01***	***DRB1*03:06:01***	***DRB5*03:09***	*DRB*W65:01*	*DRB6*01:12*	
2	***DRA*01:10:01***	***DRB1*03:08:01***	***DRB1*10:04***	*DRB6*01:09*		
3	***DRA*01:02:05***	***DRB1*03:09***	***DRB*W20:01***	*DRB6*01:07*		
4 (c^a)	***DRA*01:02:01:01***	***DRB1*03:12:01***	***DRB*W25:02***	*DRB6*01:07*		
5	***DRA*01:03:01***	*DRB1*03:12*	***DRB*W26:02:01***	***DRB4*01:01***		
6	***DRA*01:02:01:01***	*DRB1*03:13*	***DRB*W36:01***	***DRB*W1:08***	*DRB6*01:05*	
7	***DRA*01:03:07***	***DRB1*03:14***	***DRB1*03:15***	*DRB6*01:12*		
8	***DRA*01:03:02***	***DRB1*03:16***	***DRB*W40:01***			
9a	***DRA*01:03:01***	***DRB1*03:17***	***DRB*W6:07***	***DRB*W67:01***	*DRB6*01:06*	*DRB6*01:12*
9b	***DRA*01:03:03***	***DRB1*03:17***	***DRB*W6:07***	***DRB*W67:01***	*DRB6*01:06*	*DRB6*01:12*
10	***DRA*01:02:01:01***	***DRB1*03:21***	***DRB1*10:10***	*DRB6*01:24*		
11 (i^a)	***DRA*01:02:20***	*DRB1*04:01*	***DRB5*03:16***	***DRB*W3:03:01***	*DRB6*01:13:02*	
12a	***DRA*01:01:01***	*DRB1*04:01*	***DRB5*03:15***	***DRB4*01:02***	*DRB6*01:13:02*	
12b (d^a)	***DRA*01:02:01:01***	*DRB1*04:01*	***DRB5*03:01:01***	***DRB4*01:01***	*DRB6*01:13:02*	
12c	***DRA*01:01:01***	*DRB1*04:01*	***DRB5*03:01:01***	***DRB4*01:02***	*DRB6*01:13:02*	
13a	***DRA*01:02:01:01***	***DRB1*04:03***	***DRB*W37:01***	*DRB6*01:13:01*	*DRB6*01:13:02?*	
13b (a^a)	***DRA*01:02:05***	***DRB1*04:03***	***DRB*W37:01***			
14 (f^a)	***DRA*01:03:01***	***DRB1*04:11***	***DRB*W36:04***	*DRB6*01:15*		
15 (b^a)	***DRA*01:01:09***	*DRB1*07:04*	***DRB1*03:08:02***	*DRB*W6:05*		
16 (e^a)	***DRA*01:02:01:01***	*DRB1*07:04*	***DRB*W53:01***	***DRB5*03:05***		
17a (h^a)	***DRA*01:02:01:01***	***DRB1*10:02***	***DRB*W49:01:01***	*DRB6*01:09*		
17b	***DRA*01:02:01:01***	***DRB1*10:02***	***DRB*W49:01:02***	*DRB6*01:09*		
18 (j^a)	***DRA*01:02:21***	***DRB3*04:01***	***DRB5*03:06***	*DRB6*01:10*		
19	***DRA*01:02:01:01***	***DRB4*01:01***	***DRB5*03:01:02***	*DRB*W1:02*	***DRB*W6:06***	*DRB6*01:13:02*
20	***DRA*01:10:02***	***DRB4*01:03***	***DRB5*03:04***			
21	***DRA*01:01:01***	***DRB*W1:07***	***DRB*W3:06***	***DRB*W7:07***	*DRB6*01:13:01*	
22	***DRA*01:03:08***	***DRB*W3:03:01***	***DRB*W7:02***	*DRB6*01:13:01*		
23	***DRA*01:03:08***	***DRB*W3:04:01***	***DRB*W3:05***			
24	***DRA*01:03:01***	***DRB*W4:05***	***DRB*W25:04***	*DRB6*01:14*		
25	***DRA*01:03:01***	***DRB*W20:01***	***DRB*W66:01***	*DRB6*01:08*		
26	***DRA*01:09***	***DRB*W20:02***	***DRB*W25:06***	*DRB6*01:11*		
27	***DRA*01:03:03***	***DRB*W68:01***	***DRB*W25:05***	*DRB6*01:11*		
28 (g^a)	***DRA*02:01:01:01***	***DRB*W5:01***	***DRB*W21:01***	*DRB6*01:01*		

[a] Designation of Fig. 1. Transcribed genes are depicted in bold

belonging to different loci/lineages, but haplotypes with two, four, and five loci are also observed. However, until now it had been unclear which of these alleles are transcribed and, as such, encode potential *bona fide* gene products. The subsequent full-length *DRB* sequencing of RT-PCR products of our cynomolgus macaque panel revealed a total of 57 *DRB* alleles. As can be expected, most of the alleles that were discovered are extensions of *DRB* alleles defined by exon 2 typing (Table 1). Additionally, 11 previously unreported alleles have been detected during the course of this study (Table 1, bold). Some alleles differ in exons other than exon 2 — e.g., *DRB*W7:07* and *DRB*W7:02* — demonstrating that exon 2 typing may not always be sufficient for an unambiguous allele definition. At this stage, it is not understood to what extent polymorphisms in exon 3 may affect actual peptide binding.

In family studies, the segregation of alleles on one chromosome has been determined, and 28 *DR* region configurations have been defined (Table 3; letters in brackets refer to the respective haplotype of Fig. 1). As observed in rhesus macaques, only a few region configurations show limited allelic variation for their *DRA* and/or *DRB* genes (Table 3, #9a/9b; #12a/12b/12c; #13a/13b; #17a/17b). In humans, HLA class II-mediated immune responses may

differ between individuals due to allelic polymorphism. In macaque populations, the strategy is fundamentally different, as allelic variation within a region configuration is virtually absent. The actual outcome is more or less the same, as macaques display abundant region configuration polymorphism at the population level.

With one exception, all *Mafa-DRB* haplotypes are linked to alleles of the *DRA*01* lineage, and only one *DRB* region configuration is associated with the *DRA*02* lineage (Table 3, #28). Per haplotype, one to three *DRB* genes are transcribed, resembling the situation observed in rhesus macaques (de Groot et al. 2004). The *DRB* transcription products of a certain haplotype belong mostly to alleles of different loci/lineages. There are, however, region configurations (e.g., Table 3, #7), which encode two allelic transcripts of the same *DRB* lineage and are therefore probably the result of a recombination process.

As in other primate species such as rhesus macaques, humans, and chimpanzees, *DRB6* always remains untranscribed, and thus is confirmed to be a pseudogene. However, alleles from various other loci/lineages are also not detected at the transcription level (Table 3, grey). As has been shown in previous studies of the rhesus macaque, some alleles that group in the same lineage as, for example, *DRB1*03* (Table 3, #1–10), may be transcribed, whereas others are not detected at the transcription level. Notably, however, is the observation that *Mafa-DRB* alleles, which are identical for exon 2, may be either transcribed (Table 3, #6, *DRB*W1:08*) or untranscribed (Table 3, #19, *DRB*W1:02*). Additionally, a certain allele may be observed as a transcript in the context of one region configuration (Table 3, #4, *DRB1*03:12:01*), whereas it remains untranscribed as a member of another configuration (Table 3, #5, *DRB1*03:12*). A further example is provided by allele *DRB1*04:01*, which is detected in two different region configurations (Table 3, #11 and 12) in our cohort and appears to be untranscribed. In another study (Blancher et al. 2006), the same allele is defined on a cDNA level; here too, the region configuration, in which the authors detected the *DRB1*04:01* allele, is different from configurations #11 and 12 (a, b, c) (Table 3) of our cohort. In configuration #12, we cannot exclude the possibility that the discussed alleles may have mutations outside exon 2. However, the fact that the same or closely related alleles are either pseudogenes or encode *bona fide* transcripts appears to be dependent on the region configuration in which they are situated: for instance, their surroundings on the genome. In cynomolgus macaques, an undocumented high level of *DRB* region configuration-associated diversity has been described (Doxiadis et al. 2010). Since these region configurations appear to be generated by recombination-like events, it seems plausible that genes may be placed next to or far away from a promotor/enhancer region so that transcription may be switched on or off. Future studies relating to the whole genome sequencing of several macaque MHC haplotypes will help to answer these questions.

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