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Characterisation of MHC haplotypes in a breeding colony of Indonesian cynomolgus macaques reveals a high level of diversity

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Abstract Recent reports have revealed that cynomolgus macaques obtained from different geographic origins may be more or less suitable for particular studies depending on the specific question(s) being addressed, e.g. Mauritian cynomolgus macaques are particularly suitable for detailed immunological studies against a limited genetic background while less conserved populations may be more appropriate to predict breadth of vaccine coverage in the genetically diverse human population. We have characterised MHC haplotypes in 90 Indonesian cynomolgus macaques using microsatellite and reference strand conformational analysis. Thirty unique haplotypes were defined in the cohort, emphasising the high degree of diversity in this population of cynomolgus macaques. The majority of haplotypes were present at a frequency of ≤6%. Transcription profiles indicated that each haplotype was associated with two to eight transcribed class I alleles. The results corroborate previous reports of the extensive MHC diversity of Indonesian cynomolgus macaques and provide additional data to inform colony management decisions. Further, definition of the MHC diversity of the population satisfies one of the prerequisites to MHC association studies and detailed immunological investigations in this outbred non-human primate species.

Keywords MHC · Genotyping · *Macaca fascicularis* · Indonesian · Microsatellite

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Introduction

Macaque monkeys (*Macaca* spp.), particularly cynomolgus, rhesus and pigtail macaques, are widely used in infectious disease pathogenesis models (Feichtinger et al. 1990; Dittmer et al. 1996; Maggiorella et al. 1998; Montgomery et al. 1999; Nalca et al. 2010; Sharpe et al. 2010), vaccine development (Gotch et al. 1991; Negri et al. 2004; Berry et al. 2007; Kawada et al. 2007; Maggiorella et al. 2007; Weiss et al. 2007; Jiang et al. 2009; Mudd et al. 2010) and organ transplant research (reviewed in Hale et al. 2005). Recently, the demand for macaques of defined MHC genotype has increased in light of numerous reports demonstrating differential control of pathogens, most notably SIV, in animals expressing particular MHC class I alleles and/or haplotypes (Muhl et al. 2002; Mothe et al. 2003; Loffredo et al. 2007; Florese et al. 2008; Sauermann et al. 2008; Mee et al. 2009b, 2010; Aarnink et al. 2011).

Cynomolgus macaque breeding facilities have typically been established using animals from a number of locations including Indonesia, Malaysia, China, the Philippines and Mauritius. Mauritian cynomolgus macaques (MCM) have been shown to be particularly valuable for detailed immunological studies due to the remarkably restricted MHC diversity of feral and captive-bred populations (Wiseman et al. 2007; Mee et al. 2009a). While MCM are clearly valuable as a homogenous population for biomedical studies, many primate facilities also maintain non-Mauritian macaque colonies and in certain cases, it may be more appropriate to perform pathogenesis or vaccine studies in a non-human primate species more representative

of the outbred human population. We therefore investigated the degree of MHC diversity in a population of cynomolgus macaques of predominantly Indonesian origin in a UK breeding colony to determine whether the high degree of MHC diversity reported in other colonies of Indonesian macaques (Pendley et al. 2008; Kita et al. 2009; Otting et al. 2009) was reproduced and to provide additional genetic data to inform colony management decisions. Using microsatellite and reference strand conformational analysis (RSCA), we identified 30 distinct haplotype configurations among 90 macaques. The data confirm and extend recent reports of the extensive MHC diversity in this population, facilitate genetics-led colony management decisions and provide a foundation for detailed immunological analyses using Indonesian cynomolgus macaques.

Methods

Animals

Ninety cynomolgus macaques of primarily Indonesian origin housed at a UK breeding facility were sampled. The colony was established from approximately 100 founder animals, and animals in the current study represent the third to fifth generation descendants of the founders. Records indicate that limited mixing of animals of two different geographic origins occurred prior to the establishment of the extant 'Indonesian' breeding colonies. These colonies have subsequently been housed separately from Mauritian macaque colonies. The study cohort included 73 cynomolgus macaques from four newly established, specific pathogen-free colonies (Mee et al. 2009c) each comprising 14–22 macaques. Where available, parental samples were analysed to infer or confirm haplotypes. Animals were housed and maintained in accordance with UK Home Office guidelines for care and maintenance of non-human primates.

DNA extraction

Blood samples from all animals were taken into EDTA. DNA was extracted by phenol–chloroform extraction and isopropanol precipitation, resuspended in molecular-grade water and diluted to 10 ng/μl.

RNA extraction and cDNA synthesis

RNA was extracted from 1.5 ml fresh EDTA-treated blood using the QIAamp RNA blood mini kit (Qiagen, West Sussex, UK) according to the method recommended by the manufacturer. Approximately 1 μg of RNA was converted to cDNA using the Accuscript High Fidelity 1st Strand cDNA Synthesis Kit (Agilent Technologies, Stockport, UK) and the provided oligo(dT) primer.

Microsatellite analysis

Microsatellite analysis was performed using a previously described method (Wiseman et al. 2007).

Reference strand conformational analysis

Amplicons spanning 305 bp of exons 2–3 of the class I heavy chain gene were prepared from cDNA as described previously (Mee et al. 2009a). A 1.5 μl volume of standard or sample was mixed with 3 μl diluted fluorescent-labelled referenced strand and denatured at 95°C for 5 min. The reaction was then cooled at −1°C/s to 55°C, held for 5 min and cooled at −1°C/s to 15°C. Samples were refrigerated until use. Immediately prior to use, the sample was diluted 1:5 in ultrapure water and 2 μl was added to 8 μl water containing 0.08 μl ROX-ET900 size ladder (GE Healthcare, Buckinghamshire, UK). Samples were separated on an ABI 3130 genetic analyser, using a 36-cm capillary filled with 4% (*w*/*w*) non-denaturing conformational analysis polymer buffered with 1× running buffer containing EDTA (Applied Biosystems). Samples were electrokinetically injected at 15 kV for 15 s and separated at 4 kV for 60 min at a constant temperature of 30°C. Apparent mobility was defined in arbitrary units corresponding to the scan number at which the peaks of the ROX-ET900 ladder were detected. Mobility bins for each FLR/allele combination were defined using Genemapper v4.0 (Applied Biosystems). All samples were re-examined manually to ensure correct calling of peaks.

Data analysis

Haplotypes were initially characterised by identifying microsatellite profiles that extended across all 18 markers and were present in three or more animals. The presence of a number of heterozygote animals carrying Mauritian haplotypes facilitated the definition of the correct phase of a number of haplotypes. For haplotypes where only two macaques displayed identical microsatellite patterns, parental DNA samples were analysed to confirm inheritance and validity of data. Unassigned haplotypes were then re-examined to identify those sharing at least 14 of 18 markers with an established haplotype. RSCA was performed on RNA samples where available (88% of macaques); if identical RSCA profiles were observed, the haplotypes were considered to be identical at the class I allele transcript level. Finally, recombinant haplotypes were defined where three or more consecutive markers corresponding to distinct haplotypes were present on the same chromosome.

Results

Microsatellite analysis defines at least 30 haplotypes in Indonesian cynomolgus macaques

We determined MHC haplotypes in 90 Indonesian cynomolgus macaques using a panel of microsatellite markers previously reported to be highly informative for the genotyping of Mauritian cynomolgus macaque populations. Thirty-five unique microsatellite profiles were identified with each profile present in at least two macaques (Supplemental Fig. 1). Due to the mixing which occurred prior to the establishment of the Indonesian colonies, five of these profiles which accounted for 12% of all chromosomes studied corresponded to MHC haplotypes previously described in Mauritian cynomolgus macaque populations (Wiseman et al. 2007; Mee et al. 2009a). Analysis of heterozygote animals carrying Mauritian haplotypes allowed for the initial determination of the phase of a number of Indonesian haplotypes. Of the 30 Indonesian haplotypes identified (Table 1), most were found at low frequency (≤6% of chromosomes), emphasising the high level of diversity among this population of macaques (Fig. 1). Twenty animals possessed one haplotype to which a unique microsatellite profile could not be assigned due to the lack of additional animals with a matching profile. Thirty-four chromosomes (19% of all chromosomes) appeared to carry recombinant haplotypes as evidenced by the combination of microsatellite profiles corresponding to two or more of the major haplotypes and/or unassigned microsatellite patterns. Only four of these recombinants appeared to be derived from recombination of Mauritian and Indonesian haplotypes, the remainder were Mauritian/Mauritian, Indonesian/Indonesian or Indonesian/unassigned (Supplemental Fig. 1). In two cases, inheritance of recombinant haplotypes was confirmed by pedigree analysis [animals 548FBG (dam), 548FBGA (offspring) and 980ABAE (sire), Supplemental Fig. 1].

Analysis of RSCA profiles confirms distinct repertoires of class I*A* and *B* alleles

In several cases different microsatellite allele sizes were associated with the same haplotype, e.g. two IND 3 profiles were identified (Table 1), each of which shared 15 marker sizes but differed at three. We confirmed that such haplotypes carried common MHC class I genes by analysing the class I transcript profile using RSCA (representative RSCA profiles are shown in Fig. 2). Haplotypes with divergent microsatellite profiles were classed as identical if no more than four microsatellites exhibited different allele sizes and similar RSCA profiles could be identified in all animals carrying that haplotype. Where only two macaques were shown to display the same microsatellite pattern and RSCA profile, microsatellite analysis was performed on parental animals to confirm inheritance. Inheritance was confirmed for 17 haplotypes in this way; the remaining 13 haplotypes were all detected in at least three unrelated animals. RSCA identified two to eight distinct peaks per haplotype, consistent with the numbers of class I alleles previously reported in Indonesian populations of cynomolgus macaques (two to eight transcribed alleles per animal, Pendley et al. 2008; Otting et al. 2009). Within the 30 novel haplotypes, none shared more than two class I alleles as determined by RSCA. Thus, despite a small number of shared alleles, each haplotype appears to carry a largely distinct repertoire of class I alleles.

Discussion

We have investigated MHC diversity in a large cohort of Indonesian macaques housed in a UK breeding facility and identified at least 30 distinct ‘Indonesian’ MHC haplotype configurations among 90 macaques. The actual number of haplotypes likely exceeds this number since a further 20 microsatellite patterns could not be confirmed due to the absence of samples from related animals. Several putative additional haplotypes could be identified in animals carrying one assigned haplotype (Supplemental Fig. 1), however for consistency we did not consider these to be authentic haplotypes unless they met the criteria described in the “Methods” section, i.e. present in at least three animals or confirmed by inheritance. Since the animals in this study were selected from active breeding colonies, it will be possible to confirm these patterns in the future using samples from offspring. As the colony is self-sustaining, the entire genetic diversity of the breeding population can be described and subsequently manipulated by selective breeding of animals with the most appropriate genotypes. The results confirm previous reports (Pendley et al. 2008; Kita et al. 2009; Otting et al. 2009) that Indonesian macaques exhibit a high degree of MHC diversity and are more outbred than the previously characterised MCM. Notably, the data also indicate that a high degree of diversity is maintained within our colonies despite the recent establishment of SPF colonies by selective regrouping of animals (Mee et al. 2009c). RSCA profiles suggested that between two and eight class I alleles are transcribed from each haplotype, though the actual number may be higher since some alleles are likely transcribed at a level below the detection limit of RSCA and different alleles may co-migrate when hybridised to the fluorescent reference strand. More sensitive technology, such as high-throughput pyrosequencing, would provide a more complete profile of transcribed class I alleles, but such an approach was outside the scope of the present study.

Table 1 Microsatellite profiles of 30 MHC haplotypes identified in Indonesian cynomolgus macaques

Marker	IND 1	IND 2	IND 3	IND 4	IND 5	IND 6	IND 7	IND 8	IND 9	IND 10	IND 11	IND 12	IND 13	IND 14	IND 15
D6S2972	117	119	117	117	119	119	129	127	121	119	123	117	117	127	121
D6S2970	289	300	304, 308	300	300	352	300, 304	300	300	308	352	304	300	308	ND
D6S2854	191, 195	191	195	191/195	191/195	195	191	195	195	195	191	195	195	Null	195
D6S2704	159	143	145	165	134	152	163	148	155	159	163	134	152	141	163
D6S2847	317	317	319	317/319	317/319	317	319	317	319	317	317	317	319	319	317
C4_2_25	237	235	235	235	235	235	235	235	235	235	235	235	235	235	235
D6S2691	231	239	274	286, 281, 283	295, 300	258	263, 265	274	260	270	274/288/281	235	323	286	286
9268	236	234/236	227/235	236	231, 236	234	234/220	236	234	229/236	220	233	233, 236	233	234, 236
MICA	202	205	202	202	202	202	195	205	202	202	202	205	202	202	205
D6S2793	269	246	260	269	246	246	260, 263	246	254	246	246	254	277	246	244
D6S2782	339	335	335	339	335	335	335	339	339	335	339	335	335	335	339
D6S2669	108	133	135	129	108	123	127	106	96	96	129	108	123	106	108
D6S2892	199	200	202, 204	202	202	204	204	206	Null	199	202	202	202	202	199
DRA-CA	271	269	239	237	237	269	271	268	258	239	239	237	239	237/268	265
D6S2876	217	209	209	214	214	209	214	209	226	214	195	195	217	209	217
D6S2747	209	194	205	Null	194	ND	205	205	Null	205	201	205/207	207	205	207
D6S2745	309	300	296	300	300	300	296/304	296	300	296	309	296	309	296	311
D6S2771	411	394	394	394, 399	394	394	393	393	397	394	399	393	393	393	393

Marker	IND 16	IND 17	IND 18	IND 19	IND 20	IND 21	IND 22	IND 23	IND 24	IND 25	IND 26	IND 27	IND 28	IND 29	IND 30
D6S2972	119	119	117	119	121	121	117	117	125	123	117	121	119	117	121
D6S2970	300	300	286	ND	300	300	304	300	304	300	300	300	304	286	300
D6S2854	195	195	195	195	195	191	191	191	191/195	195	191	ND	191	195	195
D6S2704	155	152	161	148	145	170	150	145	155	152	155	134	155	165	157
D6S2847	319	319	317	319	317	319	317	317	317	319	319	317	319	319	319
C4_2_25	235	235	235	235	235	235	235	235	235	235	235	235	235	235	235
D6S2691	218	233	293	235, 236	260	300	238, 239	263	290	266	286	327	290	250	290
9268	Null	226	234, 236	ND	ND	234, 236	239	231/236	244	ND	221	ND	ND	231	ND
MICA	205	205	202	205	205	186	195	205	202	202	195	188	202	202	202
D6S2793	277	246	246	244	260	246	271	260	277	283	281	246	273	273	273
D6S2782	335	335	339	321	339	335	310/335	339	335	335	339	335	335	339	339
D6S2669	123, 129	108, 112	127	106	108	114	125	108	96	96	106	108	131	106	94
D6S2892	206	202	204	206	202	202	ND	202	200/202	199	204	197	200	206	206
DRA-CA	258	239	271	237	273	265	265, 269	281	268	269	287	265	237	247	256
D6S2876	195	209	209	214	195	214	210	214	217	214	ND	202	207	209	214
D6S2747	205	205	ND	209	207	191/207	205	185/202	191	207	191	207	ND	194	191
D6S2745	296	296	311	309	309	311	296	311	296/304	309	300	296	300	300	ND
D6S2771	394	394	390	411	393	393	394	393	390	394	394	390	390	399	394

Figures separated by commas indicate different allele sizes associated with a single haplotype. Figures separated by a forward slash indicate allele sizes that could not be definitively assigned to a single haplotype

ND not determined

This extensive diversity makes Indonesian cynomolgus macaques valuable for biomedical research where the use of an outbred population is desirable. Pathogen association studies at the haplotype level, similar to those performed in MCM, are unlikely to be feasible in cohorts of Indonesian cynomolgus macaques due to the low frequency of shared haplotypes. It is likely, however, that several of the haplotypes share MHC class I alleles. The use of allele-specific PCR in conjunction with recently developed pyrosequencing methods for detailed identification of transcribed class I alleles (Wiseman et al. 2009; Budde et al. 2010) will permit the assignment of individual alleles to each haplotype and facilitate association studies to identify those alleles relevant to immune responses against pathogens. The utility of microsatellite analysis for rapid genotyping of animals will enable the identification of animals carrying rare haplotypes, within this and other cohorts, that represent useful candidates for the identification of novel MHC alleles.

In addition to the novel Indonesian MHC haplotypes, five haplotypes identical to those previously reported in MCM were identified. Breeding records indicate that

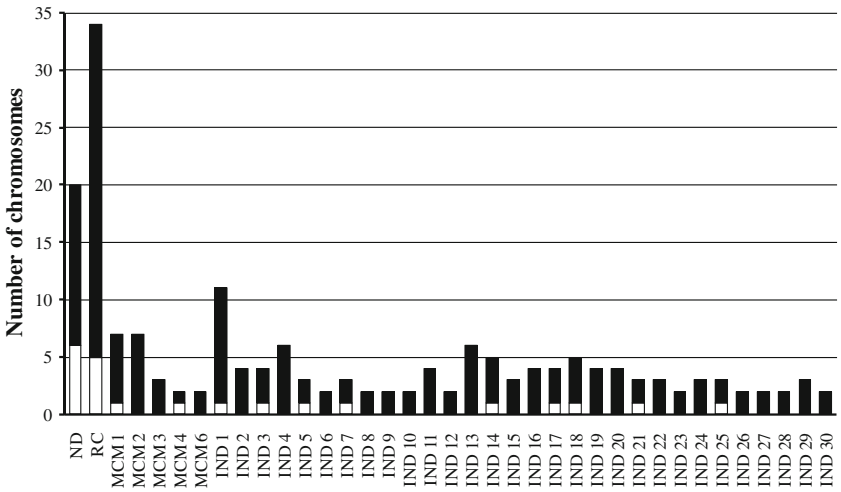

Fig. 1 Frequency of novel MHC haplotypes among a cohort of 90 Indonesian cynomolgus macaques. *Bars* indicate the number of animals carrying each haplotype. *White fill* indicates animals where the haplotype was identified by microsatellite analysis alone. *Black fill* indicates animals where haplotype was identified by microsatellite analysis and confirmed by RSCA. *ND* not determined, haplotype could not be confirmed in two or more animals; *RC* recombinant haplotype; *MCM* previously described Mauritian cynomolgus macaque haplotypes; *IND* novel Indonesian cynomolgus macaque haplotypes

animals of unknown geographic origin, but likely MCM, were mixed with our Indonesian breeding colonies in the past, therefore these MCM haplotypes most likely reflect genetic contamination rather than shared ancestral haplotypes. Though the latter is a possibility, previous reports have suggested only distantly related MHC haplotypes between cynomolgus macaques from Mauritius and Indonesia or the Philippines (Campbell et al. 2008; Pendley et al. 2008). Furthermore, analysis of a separate cohort of animals derived from the same breeding colony confirmed the presence of mitochondrial DNA sequences characteristic of MCM in addition to those from Indonesia (Rose et al., unpublished data). The characterisation of the colony MHC diversity presented herein and the relative ease of microsatellite-based genotyping will facilitate the exclusion of these contaminating MCM MHC haplotypes by selective breeding if required.

In summary, we have characterised MHC haplotypes in a cohort of 90 Indonesian cynomolgus macaques. The results are consistent with previous reports of a high degree of genetic diversity in this population of macaques and add to the growing corpus of data on the MHC genetics of this species. Our findings provide a foundation for improved genetic management of macaque breeding colonies

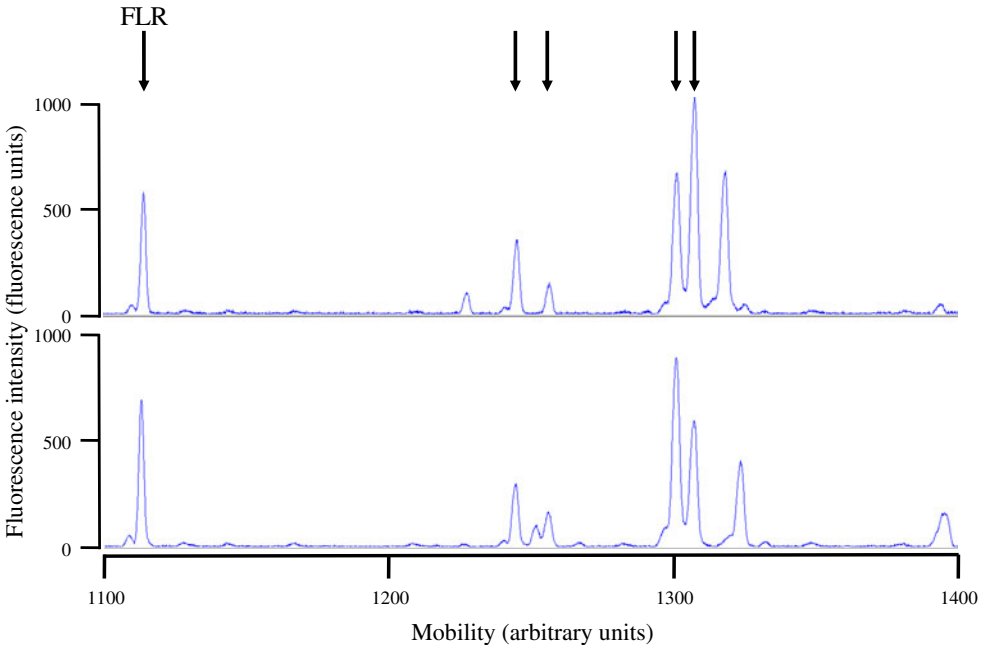

Fig. 2 Comparison of RSCA profiles in selected animals 019GG (*top*) and 019GGB (*bottom*). *Arrows* denote alleles shared between both animals, which also share one MHC haplotype as defined by microsatellite analysis. Residual FLR homoduplex is present in both samples at an apparent mobility of approximately 1,110

and pathogen association studies in an outbred non-human primate species.

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