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BRIEF COMMUNICATION

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Structural and functional characterisation of the Toll like receptor 9 of *Aotus nancymaae*, a non-human primate model for malaria vaccine development

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Abstract In the absence of suitable rodent animal models for *Plasmodium falciparum* malaria, the efficacy testing of asexual blood-stage vaccine candidates in *Aotus nancymaae* represents a tool to select between different formulations before conducting expensive human clinical trials. CpG oligonucleotides (ODN) specifically promote the production of pro-inflammatory and Th1-type cytokines and they enhance the immunogenicity of co-administered antigens. Toll like receptor 9 (TLR-9) binds directly and sequence-specifically to single-stranded un-methylated CpG-DNA mediating the biological effects of CpG ODN. We cloned and functionally characterised the TLR-9 cDNA of *A. nancymaae*. The cDNA encompassed 3,099 bp predicted to code for 1,032 amino acid residues. Results of homology searches to human TLR-9 suggested that the receptor is 93 and 94% identical at the nucleotide and amino acid sequence levels, respectively. Stimulation of splenocytes of *A. nancymaae* with CpG ODN resulted in proliferative responses in all animals analysed. FACS analysis of cultures incubated with CpG ODN 2006 indicated that the B cell marker CD20 was up-regulated consistent with B cell activation. The high level of sequence conservation of Aona-TLR-9 reinforces the suitability of *A. nancymaae* as animal model for malaria subunit vaccine development.

The nucleotide sequence has been submitted to the GenBank nucleotide sequence database under the accession number AY788894.

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The vertebrate innate immune system recognises conserved, pathogen-associated molecular patterns and responds through the secretion and activation of a number of effector molecules (Klinman 2004). Recognition of un-methylated CpG di-nucleotides within specific flanking bases has evolved as a pattern recognition mechanism used by the innate immune system to detect the DNA of pathogens (Klinman 2004). The central involvement of the Toll like receptor 9 (TLR-9) in responses to un-methylated CpG di-nucleotides has been demonstrated using TLR-9-deficient mice (Hemmi et al. 2000). CpG DNA is bound directly by the TLR-9 in the lysosomal compartment after cellular uptake (Cornelie et al. 2004; Latz et al. 2004; Rutz et al. 2004). B cells and plasmacytoid dendritic cells are the main human cell types that express TLR-9 and respond directly to CpG ODN stimulation. Human memory B cells constitutively express high levels of TLR-9 and proliferate and differentiate to immunoglobulin-secreting cells in response to CpG (Bernasconi et al. 2003). CpG ODN interact with TLR-9 expressed by plasmacytoid dendritic cells, improving antigen presentation and triggering the production of chemokines, Th1-type and pro-inflammatory cytokines (including INF-γ, IL-6, IL-12, IL-18 and TNF-α). Hence, CpG ODN have great potential to be developed as adjuvant for T- and B-cell responses (Klinman 2004).

At least three structurally distinct classes of CpG ODN have been described in primates. K-type CpG ODN induce strong B- and NK-cell activation, D-type ODN provoke high levels of IFN-α secretion by plasmacytoid dendritic cells, whilst C-type ODN combine properties of K- and D-type ODN (Vollmer et al. 2004). Examples of K-type include ODN 2006, an ODN with four "TCGT" motifs separated by poly-T (Hartmann et al. 2000). Addition of K-type ODN to vaccines against influenza virus, measles virus, hepatitis B virus surface antigen or tetanus toxoid increased antigen-specific antibody titres in mice by up to

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H. sapiens      MGFCRSALHPLSLLVQAIMLAMTLALGTLPAFLPCELQPHGLVNCNWLFLKSVPHFSMAA 60
A. nancymaae    MGFCCSALHPLSLLVQAMMLAMTLALGTLPAFLPCELQPHGLVNCNWLFLKSVPHFSVAA 60
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H. sapiens      PRGNVTSLSLSSNRIHHLHDSDFAHLPSLRHLNLKWNCPPVGLSPMHFPCHMTIEPSTFL 120
A. nancymaae    PRGNVTSLSLSSNRIHHLHDSDFAHLPSLRHLDLKWNCPPVGLSPMHFPCHMTIEPNTFL 120
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H. sapiens      AVPTLEELNLSYNNIMTVPALPKSLISLSLSHTNILMLDSASLAGLHALRFLFMDGNCYY 180
A. nancymaae    AVPTLEELNLSYNSITAVPALPKSLVSLTLSRTNILVLDSASLAGLHALRFLFMDGNCYY 180
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H. sapiens      KNPCRQALEVAPGALLGLGNLTHLSLKYNNLTVVPRNLPSSLEYLLLSYNRIVKLAPEDL 240
A. nancymaae    KNPCRRALEVAPGALLGLGNLTHLSLKYNNLTVVPRNLPSSLEYLLLSYNHIIKLAPGDL 240
                *****:*********************************************:*:**** **

H. sapiens      ANLTALRVLDVGGNCRRCDHAPNPCMECPRHFPQLHPDTFSHLSRLEGLVLKDSSLSWLN 300
A. nancymaae    ANLTALRVLDVGGNCRRCDHAPNPCMECPRHFPQLHPNTFSHLSRLEGLVLKDSSLSWLN 300
                ************************************:**********************

H. sapiens      ASWFRGLGNLRVLDLSENFLYKCITKTKAFQGLTQLRKLNLSFNYQKRVSFAHLSLAPSF 360
A. nancymaae    ASWFHGLGNLRVLDLSENFLYECITKTKAFQGLTQLRKLNLSFNYQKRVSFAHLSLAPSF 360
                ****:*****************:************************************

H. sapiens      GSLVALKELDMHGIFFRSLDETTLRPLARLPMLQTLRLQMNFINQAQLGIFRAFPGLRYV 420
A. nancymaae    GSLFSLEELDMHGIFFRSLDETTLRPLARLSRLQTLHLQMNFINQAQLGIFGAFPGLRHV 420
                **.:*:***********************. ****:************** ******:*

H. sapiens      DLSDNRISGASELTATMGEADGGEKVWLQPGDLAPAPVDTPSSEDFRPNCSTLNFTLDLS 480
A. nancymaae    DLSDNRISGASELAATTGEADGGGRVWLQPGDLAPAPVDAPSSEDFTANCSTLNFTLDLS 480
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H. sapiens      RNNLVTVQPEMFAQLSHLQCLRLSHNCISQAVNGSQFLPLTGLQVLDLSHNKLDLYHEHS 540
A. nancymaae    RNNLVTVRPEMFARLSHLQCLRLSHNCIMQAVNGSQFLPLTDLRVLDLSHNKLDLYHEHS 540
                *******:*****:************** ************.*:*****************

H. sapiens      FTELPRLEALDLSYNSQPFGMQGVGHNFSFVAHLRTLRHLSLAHNNIHSQVSQQLCSTSL 600
A. nancymaae    FTELPRLEALDLSYNSQPFGMQGVGHNFSFVAHLRTLRYLSLAHNNIHSRVSPQLRSTSL 600
                ************************************:**********:** ** ****

H. sapiens      RALDFSGNALGHMWAEGDLYLHFFQGLSGLIWLDLSQNRLHTLLPQTLRNLPKSLQVLRL 660
A. nancymaae    RALDFSGNALGRMWAEGDLYLHFFQGLSGLIWLDLSQNSLHTLLPRTLGNLPKTLQVLRL 660
                ***********:**************************** ******:** ****:******

H. sapiens      RDNYLAFFKWWSLHFLPKLEVLDLAGNQLKALTNGSLPAGTRLRRLDVSCNSISFVAPGF 720
A. nancymaae    RDNKLAFFKWRSLALLRKLEALDLAGNQLKALTNGSLPAMTRLRKLDVSCNSISFVAPGF 720
                *** ****** ** :* ***.****************** ****:***************

H. sapiens      FSKAKELRELNLSANALKTVDHSWFGPLASALQILDVSANPLHCACGAAFMDFLLEVQAA 780
A. nancymaae    FSKAKKLRELNLSANALKTVDPSWFGPLVGALKILDVSTNPLHCACGAAFVDFLLEVQAA 780
                *****:*************** ******.**:*****:***********:*********

H. sapiens      VPGLPSRVKCGSPGQLQGLSIFAQDLRLCLDEALSWDCFALSLLAVALGLGVPMLHHLCG 840
A. nancymaae    VPGLPSRVRCGSPAQLQGQSIFAQDLRLCLDEALSRDCFSFSLLAVALGLGVPLLHQLCG 840
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H. sapiens      WDLWYCFHLCLAWLPWRGRQSGRDEDALPYDAFVVFDKTQSAVADWVYNELRGQLEECRG 900
A. nancymaae    WDLWYCFYLGLAWLPRWGRRGGRREDALPYDAFVVFDKAQSAVADWVYNELRRQLEECRG 900
                *******:* *****  **:.** **************:************* *******

H. sapiens      RWALRLCLEERDWLPGKTLFENLWASVYGSRKTLFVLAHTDRVSGLLRASFLLAQQRLLE 960
A. nancymaae    RWALRLCLEERDWLPGKTLFENLWASVYGSRKTLFVLAHTDRVSGLLRASFLLAQQRLLE 960
                ************************************************************

H. sapiens      DRKDVVVLVILSPDGRRSRYVRLRQRLCRQSVLLWPHQPSGQRSFWAQLGMALTRDNHHF 1020
A. nancymaae    DRKDVVVLVILSPEGCRSRYVRLRQRLCRQSVLLWPHQPSGQRSFWAQLGIALTGDNHHF 1020
                *************:* *********************************:*** *****

H. sapiens      YNRNFCQGPTAE 1032
A. nancymaae    YNRNFCQGPTAE 1032
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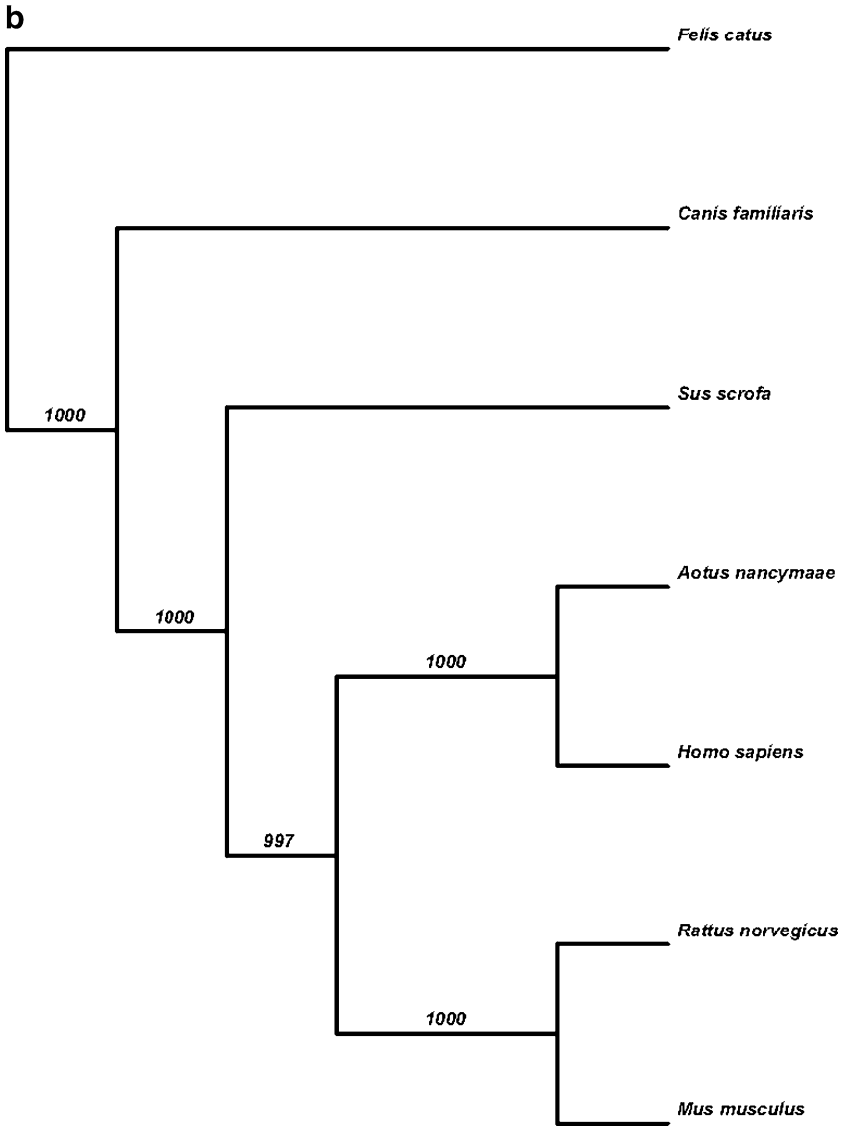
Fig. 1 **a** Amino acid sequence comparison of TLR-9 of *A. nancymaae* and *H. sapiens* using the ClustalW program available at http://www.ebi.ac.uk/clustalw/. Symbols "*","." and "." indicate identical, conserved and semi-conserved amino acid residues, respectively. Both sequences were analysed for their domain structure using the SMART program available at http://www.smart.embl-heidelberg.de/. The predicted signal sequence is given in *italics*, the leucine-rich repeat region (*LRR*) is *underlined* and the *boxed sequences* indicate the Toll-interleukin 1-resistance domain (*TIR*). **b** Unrooted phylogenetic tree established from deduced TLR-9 amino acid sequences of *Canis familiaris* (NP_001002998), *Sus scrofa* (NP_999123), *Rattus norvegicus* (NP_937764), *Homo sapiens* (NP_059138), *Felis catus* (AAN15751), *Mus musculus* (NP_112455) and *A. nancymaae* AY788894 using the neighbour-joining algorithm of the PHYLIP 3.572 program package available at http://www.bioweb.pasteur.fr. The *numbers at the nodes* indicate the percentage of recovery of that node in 1,000 bootstrap replications

3 orders of magnitude (Klinman 2004). Optimal immunostimulatory CpG DNA motifs differ between mouse and human based on amino acid sequence variations between the extracellular regions of TLR-9 (Bauer et al. 2001). Therefore, experiments to determine whether a particular CpG ODN may be of therapeutic benefit in humans are initiated in rodents, followed by evaluations in non-human primates finally leading to phase I clinical trials (Cooper et al. 2004; Krieg et al. 2004).

The development of a safe and effective malaria vaccine remains an urgent medical need for human populations living in malaria-endemic regions (Malaney et al. 2004; Richie and Saul 2002). *Aotus nancymaae* is one of the few permissive hosts for the asexual forms of two major human malaria parasites, *Plasmodium falciparum* and *P. vivax* (Gysin 1998). Immunisation and parasite challenge studies in *A. nancymaae* can rationally support decisions concerning further development of subunit malaria vaccines based on

Fig. 1 (continued)



distinct antigens, antigen combinations and antigen delivery systems (Chang et al. 1996). Aluminium compounds, the only adjuvant used widely in humans, do not support the development of Th1-type cell-mediated immune responses. Since this arm of the cellular immune response seems to play a central role in protection against a number of infectious diseases including malaria (Gupta 1998), novel approaches to develop vaccine formulations that induce Th1-type and cytotoxic T cell responses are needed.

We carried out immunogenetic studies to characterise the adaptive immune system of *A. nancymaae* in order to define at the molecular level its suitability as an experimental model in asexual blood-stage malaria vaccine development (Daubenberger et al. 2001; Diaz et al. 2000a,b, 2002; Favre et al. 1998; Nino-Vasquez et al. 2000; Vecino et al. 1999). In the current study, we characterised the cDNA sequence of the TLR-9 of *A. nancymaae* (Aona-TLR-9), followed by the description of in vitro stimulation of *A. nancymaae* spleen cells by distinct CpG ODN. This study was motivated by the hypothesis that CpG ODN might have suitable adjuvant activity for the induction of Th1-mediated immune responses in malaria vaccine development (Jones et al. 1999). Additionally, a recent report demonstrated that schizont extracts of *P. falciparum* may contain a novel non-CpG ODN ligand binding to human TLR-9. Investigations of this novel TLR-9 malaria parasite interaction in a suitable animal model may lead to better insights into early events in host–parasite interactions in malaria (Pichyangkul et al. 2004).

The analysed animals were captured in the Colombian Amazon area close to Leticia and kept at the experimental primate station of the Fundacion Instituto de Immunologia de Colombia (FIDIC) in accordance with the recommendations of the Committee on Care and Use of Laboratory Animals, US and the Colombian National Institute of Health guidelines for use of laboratory animals and supervised by the Colombian Wildlife Corporation (CORPOAMAZONIA). Mononuclear cells from three healthy monkeys numbered as 23964, 23967 and 23968 were obtained by splenectomy followed by density gradient separation. Total RNA was

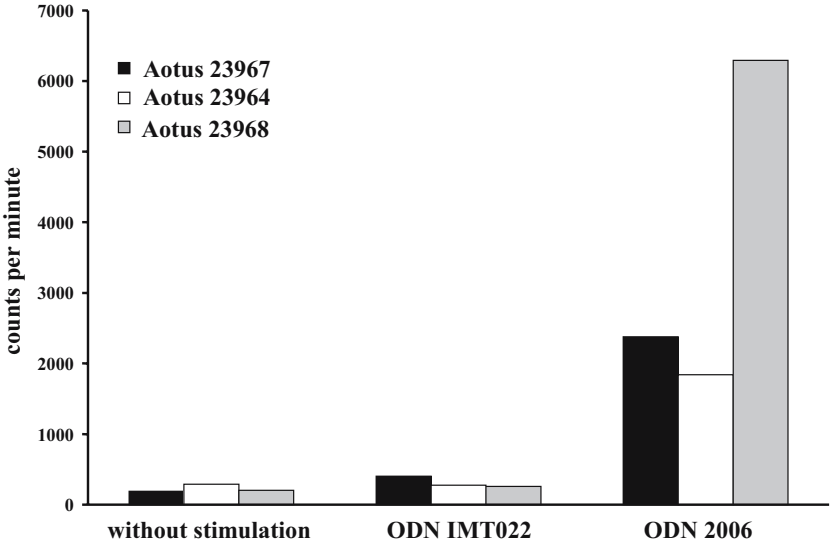


Fig. 2 Proliferative responses of spleen cells of *A. nancymaae* to CpG ODN 2006. *A. nancymaae* spleen cells of three animals were cultured in the absence or presence of 6 µg/ml CpG ODN for 72 h, pulsed during the last 16 h with [^{3}H]thymidine and incorporated radioactivity was measured. Data represent mean counts per minute (cpm) of triplicate cultures and the standard error of means of triplicate cultures was always <15%

isolated from spleen cells of monkey 23968 and cDNA synthesised as described (Daubenberger et al. 2001). Using primer pairs designed according to the human TLR-9 sequence (GenBank accession number NM017442) and located either outside (N- and C-terminal ends) or inside the coding sequence, a set of overlapping RT-PCR products were obtained which comprised the entire coding sequence of *A. nancymaae* TLR-9 (Aona-TLR-9) cDNA. To exclude possible PCR errors, all products were amplified from two independently obtained cDNA, and at least four independent plasmid preparations of each PCR amplification reaction were sequenced. The total length of the Aona-TLR-9 open reading frame (ORF) was 3,099 bp coding for a predicted protein of 1,032 amino acids. The nucleotide and the amino acid sequences displayed 93 and 94% identity, respectively, to the human TLR-9. Alignment of the deduced amino acid sequences of Aona-TLR-9 and human TLR-9 is shown in Fig. 1a. A comparison of structural features using the SMART architecture research programme http://www.smart.embl-heidelberg.de) demonstrated that the structure of Aona-TLR-9 is highly conserved to human TLR-9. Eighteen leucine-rich repeats (LRR) were identified by the programme, followed by one leucine-rich repeat C-terminal domain and the toll interleukin 1-resistance domain (TIR) encompassing residues 869 to 1,016 (Fig. 1a). A single-nucleotide polymorphism of TLR-9 in human populations has been described (Lazarus et al. 2003). One of the allelic variants in humans carried at amino acid position 5 a cysteine instead of arginine (R5C). Alignment of human and Aotus TLR-9 amino acid sequences demonstrated the same sequence polymorphism (R5C) between these two species (Fig. 1a). TLR-9 is a type I membrane receptor displaying an N-terminal extracellular region abundant in leucine-rich repeats, a cysteine-rich domain to the membrane, a single transmembrane region and a cytoplasmic region comprising the conserved TIR domain. A potential CpG-DNA binding domain of TLR-9 located between amino acid residues 523 and 541 has been defined (Rutz et al. 2004). Introduction of double alanine mutations (D535A and Y537A) strongly diminished the CpG-sequence-specific DNA binding, supporting the notion that the region containing D535 and Y537 is involved in DNA binding. These two amino acid residues are conserved between all full-length TLR-9 sequences available in the GeneBank including Aona-TLR-9.

Then we conducted a phylogenetic analysis incorporating all full-length TLR-9 sequences available at GenBank. The analysis was performed employing the PHYLIP 3.572 software package available at http://www.bioweb.pasteur.fr and the phylogenetic tree was constructed according to the neighbour-joining method based on Kimura two-parameter distances estimates (Saitou and Nei 1987). The results of this analysis, shown in Fig. 1b, confirm that the TLR-9 of human and Aotus are closely related, clustering together on a separate branch and separated from rodent and carnivora TLR-9 sequences.

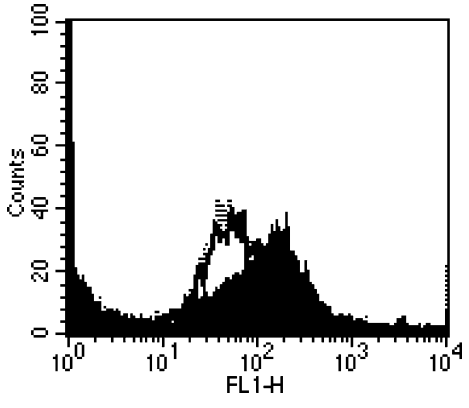


Fig. 3 FACS analysis of *A. nancymaae* splenocytes after CpG ODN incubation. Spleen cells were incubated for 96 h with medium alone (*dotted line*), control CpG ODN IMT022 (*black line*) and ODN 2006 (*filled graph*) at 6 µg/ml. Cells were removed from culture and stained with anti-CD20 mAb B-Ly1 followed by incubation with FITC-labelled anti-mouse IgG antibodies and analysed by FACS analysis

Next, we wanted to demonstrate that CpG ODN activate the spleen cells of *A. nancymaae*. It is well established that K-type CpG ODN induce proliferative responses via TLR-9 binding in B cells (Krieg et al. 1995). Therefore, we measured the proliferative responses of splenocytes upon incubation with ODN 2006 and control ODN IMT022. The K-type CpG ODN 2006 was chosen because of its previously described stimulatory activities in cells derived from human, chimpanzee and rhesus monkey (Hartmann et al. 2000). As control, we included ODN IMT022 that has also been described previously (Elias et al. 2003). Desalted phosphoro-thioate oligonucleotides (ODN), HPLC-purified, were purchased from Metabion GmbH (Germany). Purity was assessed by HPLC assays. ODN were suspended in HBSS buffer (Sigma) and kept at −20°C until use. *A. nancymaee* spleen cells (1×10^5 cells/well) were stimulated for 72 h with ODN 2006 and control ODN IMT022 at 6 μg/ml in 96-well round-bottomed plates or with medium alone. Sixteen hours before harvest, [^{3}H]thymidine was added (1 μCi/well) into the cultures and the incorporated radioactivity measured by scintillation counting. In cultures of splenocytes of all three Aotus animals tested, ODN 2006 induced significant proliferative responses. The [^{3}H]thymidine uptake in cultures incubated with control ODN IMT022 was comparable to un-stimulated cultures, demonstrating that the effect of ODN 2006 was dependent on a distinct nucleotide sequence (Fig. 2).

In order to confirm that Aotus B cells were activated by CpG ODN 2006, we analysed the changes in CD20 expression level. The CD20 molecule, also known as Bp35, is expressed from the pre-B to mature B cell stages, and whilst its precise functional role remains unknown, a role in B cell activation, proliferation and differentiation is suggested (Clark et al. 1985). Briefly, spleen cells of *A. nancymaae* were cultivated at 3×10^5 cells/ml in cell culture medium in 48-well plates (Nunc) for 96 h in the presence of medium alone or ODN 2006 or IMT022. Cells were recovered, transferred to FACS tubes and stained for flow cytometry with the anti-CD20 mAb B-Ly1 (Pharmingen) (Daubenberger et al. 2001). Cells were gated using forward and side scatter parameters for dead cell exclusion and in each sample, 10,000 events were measured and data analysed using the CellQuest software (BD Biosciences). As demonstrated in Fig. 3, incubation of spleen cells with ODN 2006 resulted in a shift of the cell population to the right of the FL1-H axis. In cell cultures incubated with medium and control ODN IMT022, the mean fluorescence intensities were 180 and 141, respectively, whilst in ODN 2006 treated cultures a value of 202 could be measured. Provided that mAb B-Ly1 stains the CD20 homologue in *A. nancymaae*, these results indicate, to our knowledge, for the first time that CD20 might be up-regulated upon incubation of splenocytes with CpG ODN 2006.

In summary, the first complete TLR-9 cDNA sequence of a non-human primate is described in this work. Aotus monkeys immunised with a synthetic malaria peptide formulated in CpG ODN 2006 responded with significantly higher antibody levels compared to animals treated with control ODN (Jones et al. 1999). In combination with our molecular and functional characterisation of the Aona-TLR-9, these results support the suitability of Aotus monkeys in the analysis of CpG ODN as an adjuvant compound for subunit-based vaccines against *P. falciparum* and *P. vivax*.

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