Nonhuman primates are relevant models for research in hematology, immunology and virology

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ABSTRACT. Nonhuman primates have been used for biomedical research for several decades. They have proved to be models that are relevant to humans because of the high level of gene homology which underlies physiological and biochemical similarities. The similarity of monkeys to humans has been used to investigate pathophysiological mechanisms in hematology, immunology and virology. New therapeutic procedures can be assessed in primates by using materials, in particular pharmacological reagents, and methods designed for humans. The relevance of these models also relies on the use of species-specific pathogens and the availability of recombinant, homologous cytokines. The introduction of more and more sophisticated cell and gene therapy protocols in hematopoietic cell transplantation and immunotherapy requires the development of preclinical trials similar to clinical settings. For several decades now, baboons and cynomolgus/rhesus monkeys have been the most useful primate models in experimental hematology, and this has contributed to numerous therapeutic advances. Primate models of AIDS have been developed to study the pathogenesis, transmission and immune responses to infection, and to test vaccines and drugs. Primate research should be restricted in quantity, and mainly designed with the aim of removing uncertainty as to the safety and clinical benefit to the patient, of new biomedical protocols.

Keywords: nonhuman primate model, hematopoiesis, stem cell, immunology, virology, cytokines, AIDS, vaccine

FROM BASIC TO PRECLINICAL RESEARCH: BABOONS, MACAQUES AND MARMOSETS ARE MODELS PERTINENT TO HUMANS FOR STUDIES IN HEMATOLOGY, IMMUNOLOGY AND VIROLOGY

Large, nonhuman primates (NHPs) have primarily been used to improve organ transplantation, either as surgical models or as substitutes compensating for the shortage of human organs. Allogeneic and xenogeneic models have been developed to study transplantation immunology (*i.e.* acute and chronic rejection). As an example, a long-term, discordant, porcine-to-primate, bone marrow (BM) engraftment has been reported in a cynomolgus monkey submitted to non-myeloablative conditioning and treated with pig recombinant stem cell factor (SCF) and interleukin-3 (IL-3) [1]. Primates from the *Hominidae* and *Cercopithecidae* families are immunologically concordant to humans and are as such of interest for putative xenografting. However, primate-to-human xenotransplantations are a matter of ethical as well as scientific debate, especially regarding viral safety.

The convention on international trade in endangered species of wild fauna and flora (CITES) distinguishes animals the trade of which is prohibited (in annex I) and animals for which trade is regulated (www.cites.org/eng/disc/text.shtml). According to the CITES, experimental research with *Hominidae* (chimpanzee, gorilla) primates is prohibited except for special, applied research in virology [2].

The two *Cercopithecidae* (baboons and macaques from the Old World) and *Ceboidae* (marmosets and squirrel monkeys from the New World, see *table 1*) families provide most of the primates used for biomedical research, in particular in hematology, immunology and virology. There is a wide diversity in body weight according to genera and species *(table 2)*.

NHPs are models relevant to humans because of the high level of gene homology which underlies physiological and biochemical similarities. Historically, the Rhesus monkey (*Macaca mulatta*) model was used by Wiener to describe the Rh system [3]. A high level of homology has been reported between humans and baboons for MHC class I molecules (90%) [4] and for transcriptosome genes of BM $CD34^+$ cells [5]. Nucleotidic and proteic sequences of numerous cytokines are quite homologous in humans and macaques (93-99%) [6]. The similarities between the sequences of macaque and human antibodies (Abs) are equivalent to the similarities between human Abs originating from different individuals. As for marmosets, which are always natural hematopoietic chimera resulting from a double fertilization associated with placental anastomoses, they provide a highly original graft model due to the tolerance status between twins [7, 8].

Rhesus/cynomolgus monkeys and baboons have specific advantages in research. Adult baboons are large primates (25 kg on average), allowing repetitive blood and BM sampling and leukapheresis-driven, mobilized hematopoietic stem and progenitor cell (HSPC) collection [9], with-

Table 1
Order of Primates

	Suborders	Genera and species	
		Latin name	Common name
Old World	Catarhini	*Papio anubis*	Anubis baboon
		Papio cynocephalus	Yellow baboon
	Cercopithecoidae	*Papio papio*	Guinea baboon
	Superfamily	*Papio ursinus*	Chacma baboon
		Papio hamadryas	Hamadryas baboon
		Macaca mulatta	Rhesus monkey
		Macaca fascicularis	Cynomolgus monkey
		Chlorocebus aethiops	African green monkey
	Hominidae	*Pan troglodytes*	Chimpanzee
	Superfamily *	*Gorilla gorilla*	Gorilla
		Pongo pygmaeus	Orang-outan
New World	Platyrrhini		
		Callithrix jacchus	Marmoset
	Ceboidae	*Saimiri sciureus*	Squirrel monkey
	Superfamily		

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Table 2
Body weight (kg) of different primates

Genus and species	At birth	Adult age	
		Male	*Female*
Cynomolgus monkey	0.54 - 0.74	5 - 30	5 - 15
Rhesus monkey	0.4 - 0.6	4 - 9	6 - 11
Squirrel monkey	0.045 - 0.065	0.60 - 0.78	0.60 - 0.66
Marmoset	0.025 - 0.035	0.275 - 0.360	0.167 - 0.335
Guinea baboon	0.54 - 0.74	22 - 30	11 - 15
Chacma baboon		- 40	

out the arterial catheterising required for macaques [10]. Owing to their venous system, baboons and macaques are relevant models for studying the infusion and recirculation of hematopoietic cell grafts of variable maturity. The intramedullary route is also easily accessible. The ABO system [3], hemoglobin, coagulation and fibrinolysis physiology [11] are similar in humans and baboons.

Yet these models have several disadvantages: low breeding efficiency, high cost of purchase and cytokine treatments, the need for general anaesthesia with ketamin for almost all manipulations, low availability of umbilical cord blood HSPC due to difficulties in pregnancy follow-up, reduction of animal cohorts related to ethical considerations, etc. The indeterminate viral status is also a major concern. Thus, although baboons as well as cynomolgus macaques from Mauritius are considered to be *Herpes B*- negative, most *Cercopithecidae,* apart from African green monkeys (vervets) from Barbados, host viruses such as spumavirus that are involved in *in vitro* cytopathogenic effects on adherent stromal cells which are potentially useful in cell therapy. Moreover, NHPs exhibit a certain degree of species-specificity relative to humans: it has been shown that only 45% and 63% of murine Abs directed against human cytokine receptors and lymphocyte cell antigens cross-react with rhesus monkey and chimpanzee antigen counterparts respectively [12]. Recombinant macaque cytokines are available to assess ligand-receptor binding in homospecific conditions [13], but these studies must always be completed in monkeys by evaluating the recombinant human (rhu) molecules to assess specific toxicity and pharmacological effects before administration to patients.

Of the teams which have developed NHP models *(table 3)*, Berenson *et al.* showed, 17 years ago, that CD34 antigen was expressed at primate HSPC level, by using an Ab raised against human CD34 [14, 15]. The existence of peripheral blood (PB) HSPC and their engrafting potential had previously been demonstrated by Storb *et al.* in non-irradiated and irradiated parabiotic animals [16]. NHP studies include healthy and myelo- or immunosuppressed (using total body irradiation [TBI], immunosuppressors, chemotherapeutic drugs) animal models. The relevance of the latter is particularly related to the easy use of drugs, materials and protocols derived from human clinical practice *(table 4)* [17, 18].

NONHUMAN PRIMATE MODELS IN HEMATOLOGY

In vitro studies

NHPs are a pertinent substitute for humans in most *in vitro* studies, since both share similar cell growth kinetics. Numerous monoclonal, anti-human CD Abs cross-react with monkey CDs *(table 5)*. Cytokine receptor and lymphoid

Table 3
Main research laboratories using nonhuman primates in experimental immunology, hematology and virology

Laboratory	Genera and species of primates used
Fred Hutchinson Cancer Research Center & University of Washington (Seattle)	Baboon; Nemestrina monkey
New England Regional Primate Research Center (Harvard)	Rhesus monkey
University of Maryland Cancer Center (Baltimore)	Rhesus monkey
Emory University School of Medicine (Atlanta)	Rhesus monkey
University of Illinois, College of Medicine (Chicago)	Anubis baboon
Transplantation Biology Research Center Massachusetts (Boston)	Baboon
Hematology Branch NIH (Bethesda)	Rhesus monkey
Erasmus Universiteit (Rotterdam)	Rhesus monkey
University Hospital (Leiden)	Rhesus monkey
CEA (Fontenay-aux-Roses)	Cynomolgus monkey
Institut de Radioprotection et de Sûreté Nucléaire (Fontenay)	Rhesus and cynomolgus monkey
Centre de Recherches du Service de Santé des Armées (Grenoble)	Anubis and chacma baboon; Cynomolgus and rhesus monkey

Table 4
Relevance of nonhuman primates to humans in hematology and immunology research in comparison with mice

	Mouse	Monkey
Physiological parameters evaluated		
Hematopoietic tissues	Bone marrow and spleen	Bone marrow
Differential white blood cell count	Mostly lymphocytic	Mostly granulocytic
CD34+ cells and subsets	Relevance to human?	Yes
Hematopoietic stem cell quiescence	Relative	High [17]
Hematopoiesis clonality	Mono or oligoclonal	Polyclonal
Total body radiosensitivity	< human	Similar
Hematopoietic potential of embryonic stem cells	Yes	Limited development
Hematopoietic potential of extramedullary pluripotent stem cells	Liver, muscle, brain Relevance to human?	In progress: likely to be different from the mouse model
***In vitro* evaluation of hematopoiesis**		
LTC-IC, CAFC, clonogenic progenitors	Yes + CFU_{spleen}	Yes
Progenitor cell radio sensitivity	Similar to human	
Pharmacology and cell therapy		
Hematopoietic responsiveness to recombinant human cytokines	Variable	Yes[1]
PBSC mobilization (a) Apheresis-driven PBSC collection (b)	Yes (a) No (b)	Yes
Autologous HSCT	Syngeneic	Yes
Allogeneic HSCT with non-myeloablative conditioning	Yes	ND[2]
HSPC gene marking	Yes	
Potential of mesenchymal stem cells: ex vivo expansion support (c), immunoregulatory activity (d)	ND Relevance to human?	Yes (c), in progress (d)
Production of prophylactic or therapeutic antibodies	Yes: chimerization or humanization required	Yes: primatization

ND: not yet determined. 1: effective doses generally higher than doses active in human. 2: approach restricted by the low availability of matched donors [18]; clear superiority of the dog model. HSCT: hematopoietic stem cell transplantation.

differentiation marker expression [19, 20] as well as cytokine efficacy upon apoptosis [21] have already been evaluated using monkey cells and reagents designed for humans. Baboon $CD34^+$ cells have been commercially available for a few years. Mesenchymal stem cell cultures of spumavirus-free baboons have been produced and their characteristics maintained for several passages [22]. Dendritic cells can also be obtained from baboon BM $CD34^+$ cells, as well as from the blood and lymphoid tissue of rhesus monkeys following mobilization by FLT-3 ligand [23].

Pathophysiological studies

Physiological hematopoiesis has been studied in monkeys, including *in vivo* platelet tracking, using lipophilic fluorescent dye [24] or radioisotopic indium 111 [25], erythropoiesis and erythropoietin (Epo) production at the kidney level [26, 27]. The demonstration of HSPC quiescence and polyclonality in primates contrasts with rodent hematopoiesis [17, 28].

Myelosuppression following TBI or chemotherapy has been well documented and has proved to be similar in monkeys and humans. The pharmacokinetics of chemotherapy agents has been studied in NHPs [29]. As a model of a reduced intensity conditioning regimen, it has recently been shown that low dose TBI causes clonal fluctuation of NHP HSPC, without impairing long-term recapitulation of stem cell polyclonality [30]. Studies regarding the efficacy of hematopoietic growth factor (HGF) in healthy monkeys provide good models of medullar hyperactivity. The

Table 5
Cross-reactivity of monoclonal Abs raised against different human leukocyte antigens

Cluster of differentiation	Clones	Chimpanzee	Baboon	Cynomolgus monkey	Rhesus monkey
CD1a	MT-102	ND	+	+	+
CD2	RPA-2.10	+	+	+	+
CD4	M-T477	ND	+	+	+
CD8	RPA-T8	+	+	+	+
CD11a	HI111	+	+	+	+
CD11b	ICRF44, BEAR-1	+	+	+	+
CD14	M5E2	+	-	+	+
CD16	3G8	+	+	+	+
CD20	2H7	+	+	+	+
CD25	M-A251	ND	+	+	+
CD34	581	+	-	-	-
	563, 566	+	+	+	+
CD35	To5, E11	+	+	+	+
CD38	OKT10	ND	+	+	ND
CD41a, CD41b	HIP8, HIP2	+	+	+	+
CD45	Tü116,	+	-	+	+
CD45	D058-1283[a]	ND	+	+	+
CD45RA	5H9, 2H4, B-C15	ND	+	+	+
CD49d	9F10	+	+	+	+
CD49e	VC5	+	+	+	+
CD56	B159	+	+	+	+
	MY31	ND	+	+	+
CD61	VIPL2, Y2/51	+	+	+	+
CD90	5E10	ND	+	+	+
CD133	AC133	ND	-	-	-
CXCR4	12G5	ND	+	+	+
HLA-A, B, C	G46-2.6	+	+	+	+
HLA-DR	G46.6, B8.12.2, CR3/43	+	+	+	+

N.B. This is a non-exhaustive presentation. For more data regarding immuno-cross-reactivity in rhesus monkey, see [12]. ND: not determined. a: specific anti-nonhuman primate mouse Ab.

knowledge of possible pathological consequences of hyperleucocytosis over $100x10^9$ white blood cells (WBC)/L or thrombocytosis over $1x10^{12}$ platelets (PLT)/L appears crucial to determine the therapeutic index of HGFs [31]. Moreover, long-term treatment with G-CSF or G-CSF + SCF has been evaluated in rhesus macaques and does not seem to result in stem cell exhaustion or clonal dominance [32]. Rhesus monkeys and baboons have also proved to be good models for exploring vascular biology and hemostasis [33, 34].

Numerous radiobiological studies have been performed with primates and show that radiation exposure results in early, systemic inflammatory reaction and hemostasis disorders preceding BM aplasia. Baboons are very pertinent models in radiopathology since they mimic the intrinsic heterogeneity of accidental irradiations, characterized by an absorbed radiation dose gradient, mainly related to body thickness [35]. Residual HSPC can be collected in monkeys and then enumerated, using blood sampling and multiple-site BM aspirations. Thus, the FLT-3 ligand serum level has recently been proposed as a prognostic bioindicator of BM damage [36]. Chemotherapeutic drugs have also been tested in primates. Unfortunately, most of the toxicopharmacological studies performed by pharmaceutical companies have remained unpublished. Monkeys appear to be very sensitive to specific drugs such as cyclophosphamide which must be administered at reduced doses in comparison to humans (FH & MD, unpublished data).

Therapeutic models

Large NHP models are very useful in evaluating pharmacological agents or cell therapy in radiation or chemotherapy-induced myelosuppression used in oncological settings. The safety and/or efficacy of anti-B lymphoma cell Abs [37, 38] have been studied in monkeys. Unfortunately, to date, there is no suitable simian model in oncology in spite of spontaneous or virus-induced lymphomas in macaques and baboons [39].

The safety and efficacy of cytokines in healthy and myelosuppressed NHPs

Cytokines are used clinically to stimulate hematopoiesis in myelosuppressed patients and to mobilize HSPC for stem cell transplantation. Since GM-CSF has been cloned, numerous preclinical studies have been performed with rhu cytokines and HGFs. The proteic homology of numerous NHP and human cytokines has been studied. For IL-3 and Epo, there is 98.5% and 91% homology respectively between humans and primates (chimpanzee and macaque [40, 41]). Toxico-pharmacological studies in NHPs are guidelines for clinical pharmacology in spite of some

Table 6
Examples of cytokines evaluated in myeloablated primates

Cytokine	Animal model	Frequency and duration of administration	Effects observed	References
G-CSF[1], GM-CSF[1], IL-3[1,2], IL-6[2], IL-11[2], TPO[2]	Monkey, baboon TBI 5 to 8 Gy	QD for 15 to 23 days	Reduction of neutropenia[1] and/or thrombocytopenia[2]	[35, 43-46]
TPO[3] + G-CSF	Monkey TBI 7 Gy	QD for 23 days	Reduction of neutropenia and thrombocytopenia	[47]
Synthokine ±G-CSF	Monkey TBI 7 Gy	QD for 14 to 23 days		[48]
Fusion molecules[4]				
GM-CSF/IL-3 (PIXY321)	Monkey TBI 7 Gy	QD for 15 days	Highly efficient multi-lineage recovery	[49]
IL-3/G-CSF, IL-3/TPO, FLT-3 ligand/G-CSF				[50]

(3): TPO or MGDF; Synthokine: IL-3 variant; (4): dual cytokine-receptor agonists of cited cytokines.

limitations. In fact, because of species specificity, efficacy of rhu cytokines is reached in monkeys with higher doses than those needed in humans. This is the case for G-CSF, which has to be administered at 40 to 100 µg/kg/day in the baboon when used alone or in combination with SCF, to obtain high levels of blood $CD34^+$ cell mobilization. Moreover, adverse effects of cytokines such as immunogenicity with rhuTPO/Peg-MGDF have not initially been disclosed in primates. In fact, administration to healthy volunteers revealed, in rare cases, the production of anti-TPO Abs responsible for severe thrombocytopenia. Since the granulopoietic activity of G-CSF and GM-CSF [42] was demonstrated, numerous native or modified cytokines (*i.e.* truncated, pegylated or chimeric molecules; *table 6*) have been evaluated in NHPs [46-48]. Some of these cytokines have been engineered as recombinant rhesus macaque cytokines, which refines these animal models. In classical schedules, treatment is initiated 24 hours after myelosuppression and prolonged until neutrophil and/or platelet recovery. Moreover, the efficacy of early administration has been demonstrated. This is the basis of emergency treatments which could be offered in a nuclear accident scenario [51, 52]. In a graft context, the benefit of HGF administration is still debated [53].

Finally, NHP models which are closer to humans than mice and dogs, have clearly shown that the extent of BM damage is the limiting factor for HGF efficacy. In fact, high grade myelosuppression is a major indication for stem cell therapy.

Transplantation models of unmanipulated or ex vivo, *expanded hematopoietic stem cells*

NHPs are pertinent models for BM or PB hematopoietic stem cell transplantation (HSCT), allowing global evaluation from the mobilization phase to post-graft, long-term reconstitution.

Several agents mobilizing $CD34^+$ cells have been evaluated in monkeys and have shown a variable efficacy: HGF, combined or not with, myelosuppressive drugs, chemokines, glycans, Abs directed against adhesion molecules *(table 7)*. However, these normal animal models imperfectly mimic the situation of a reduced HSPC pool of "poor mobilizers" in oncology.

Numerous autologous but very few allogeneic [18] HSCT models have been developed so far in monkeys. In fact, the dog is the best animal model for allogeneic graft studies, as abundant litters provide related animals in both DLA-matched and haploidentical situations [60]. The canine model is also the gold standard for non-myeloablative conditioning regimen evaluation. However, the relevance to humans of dog and NHP models, respectively, remains to be determined in terms of MHC allelic polymorphism.

Transplantation models of *ex vivo,* expanded autologous $CD34^+$ cells have been developed in NHPs *(table 8)*. Interestingly, graft efficacy can be ethically assessed in monkeys without co-administration of an unmanipulated rescue graft [9]. To date, the benefit of *ex vivo* expansion remains under debate as it depends on different parameters

Table 7
CSPH mobilization models in primates

Primate	Mobilizing agent	Peak mobilized progenitor blood levels	References
Baboon	G-CSF [b]	> 100 ^{d5} $CD34^+$ /µL	[54]
	Cytoxan + G-CSF [a]	100 – 500 ^{d10} $CD34^+$ /µL	[9]
	G-CSF [b] + SCF	180 ^{d5} and 420 ^{d10} $CD34^+$ /µL	[54]
Rhesus monkey, baboon	RhIL-8 (30-100 µg/kg)	1.4 x 10^3 CFU/mL (30 min post-inj.)	[55, 56]
Nemestrina monkey, baboon	FLT-3 L (200 µg/kg)	4.6-7.3 x 10^3 CFU/mL (day 12)	[57]
	FLT-3 L (200 µg/kg) ± G-CSF	41-95 x 10^3 CFU/mL	
	anti-VLA4 antibody	2.2 x 10^3 CFU-GM/mL (3d post-inj.)	[58]
Rhesus monkey	GRO-β	< 0.5 x 10^3 CFU-GM/mL (1h post-inj.)	[59]

G-CSF: 40[a] or 100[b] µg/kg/d; SCF: 25 µg/kg/d. Base line CFU-GM blood level: 10 to 100 CFU/mL. Peak of mobilization expressed in days.

Table 8
Primate models of expanded or unmanipulated HSCP transplantation

Primate	Cell source	*Ex vivo* expansion	*In vivo* associated HGF	References
Monkey	Auto-BMT	No	GM-CSF ± TPO	[61, 62]
Monkey	IL-3/G hybrid mob AutoPBPC	No	No	[63]
Baboon	Auto-BMT	No	No	[14]
Baboon	Auto-BMT	Yes, on porcine stroma	No	[64]
Baboon	G-CSF + SCF mob AutoPBPC	No	No	[54]
Baboon	G-CSF mob AutoPBPC	Yes	± G-CSF and MGDF	[53]
Baboon	Cytoxan/G-CSF mob AutoPBPC	Yes	No	[9, 65]
Baboon	Allo-BMT	No	No	[18]

such as cytokine combination (early-acting versus late-acting cytokines), culture media (liquid culture without or with stromal cell layers), and culture duration. Thus, several preclinical models [9] *(table 8)* have shown that *ex vivo* expansion using cytokines permits wide amplification of progenitors and precursors, but achieves only low amplification of LTC-IC and NOD-SCID repopulating cells [66]. NHP models could also be used to evaluate the safety and efficacy of grafts resulting from co-cultures performed on allogeneic or xenogeneic stromal cell layers [64, 67]. Moreover, the ability of mesenchymal stem cells (MSC) to modulate alloreactivity *in vivo* in an allogeneic skin graft model has been studied [68]. Finally, optimisation of *ex vivo* stem cell expansion may be achieved through other strategies based on activation of stem cell regulatory pathways [69]. In this context, the overexpression of transcription factors (TF) such as HoxB4 or the use of recombinant proteins such as TAT-HoxB4 has been proposed. *Ex vivo* expansion of human hematopoietic stem cells can also be achieved in a co-culture system using stromal cells genetically engineered to secrete HoxB4 [70]. The activation of Wnt signalling in HSCs has been shown to increase expression of HoxB4 and Notch1. The efficacy and safety of such approaches could usefully be assessed in NHPs.

Monkey models of in utero *HSPC transplantation*

In utero HSCT is a promising treatment for immune and hematologic diseases of fetuses and newborns. NHP models have been developed to establish long-term chimerism in such a context. For example, *in utero* transplantation of allogeneic fetal liver HSPC in monkeys resulted in long-term multilineage chimerism without GVH in the absence of myelosuppressive therapy [71], and a study using cytokine-primed, haploidentical BM $CD34^+$ cells as a stem cell source was aimed at investigating the effect of the number of allogeneic T cells infused on engraftment after *in utero* transplantation in NHPs. [72].

This strategy was used to induce tolerance in a context of solid organ grafts. *In utero* HSPC transplantation has been shown to extend the survival of postnatal kidney transplants in monkeys [73]. However, immunosuppressive therapy remained necessary in this model to counteract long-term host *versus* graft reaction.

The induction of tolerance to donor could make organ transplantation safer and more uniformly successful. One of the most promising approaches currently being investigated involves the induction of deletional tolerance through the establishment of mixed chimerism [74].

Gene marking and gene therapy

HSPCs are pertinent targets for gene therapy due to their potential for self-renewal and multilineage differentiation. Gene transfer into NHP HSPC has been designed either to evaluate *in vivo* cell trafficking after reinfusion (analytical gene marking) or to develop therapeutic tools (gene therapy). When long-term gene expression is required, viral vectors are the most appropriate tools to allow gene integration into the host's genome. So far, retroviruses and lentiviruses have been assessed [75-77]. NHPs are valuable models for improving gene transfer and gene expression efficiency. Gene marking is particularly useful in identifying the hematopoietic clones responsible for short-term recovery and those supporting long-term reconstitution. Thus, prolonged, high-levels of retrovirally marked hematopoietic cells have been detected in NHPs after transduction of $CD34^+$ progenitors [78]. Moreover, long-term repopulating stem cell clones have proved to be polyclonal [28]. Andrews *et al.* showed the differential engraftment of genetically modified $CD34^+$ and $CD34^-$ hematopoietic cell subsets in a lethally irradiated baboon model [79]. In fact, gene marking revealed impairment of long-term engraftment potential in a monkey model of an *ex vivo* expanded graft in which macaques were reinfused with cells cultured for 14 days in the presence of IL-3, IL-6, SCF and FLT-3 [80].

NHP models are used to study host tolerance of transduced HSPCs. Xenogenes that are efficient in gene marking could induce high immunological conflicts leading to transduced cell elimination mainly due to cell lysis [81]. Autoimmune anemia has recently been reported in macaques following homologous Epo gene therapy using a skeletal muscle route, in correlation with the appearance of neutralizing Abs against the endogenous Epo [82]. However, prolonged murine or human adenosine deaminase expression was observed after transduction of macaque HSPCs using retroviruses. Moreover, macaque $CD34^+$ cells have been successfully transduced by different teams using an EGFP xenogene [83]. In fact, reduced HSPC transduction efficacy could be counterbalanced by the *in vivo* expansion of immature cells. Such a goal could be achieved in NHP models using a cell growth gene co-transfection strategy. Efficient and durable gene marking of HSPCs in monkeys can be achieved after non-myeloablative conditioning [84]. Moreover, gene transfer into fetal hematopoietic progenitor cells can be performed in baboons, providing an efficient model for human stud-

ies, since both species exhibit similar, terminal, hematopoietic differentiation during the last three months of pregnancy [85]. An *in vivo* transduction strategy targeting BM stromal cells using adenoviruses has also been tested in NHPs and could be useful in stimulating hematopoietic recovery, post-myelosuppression through transient cytokine gene expression [86].

Furthermore, it has been shown in baboons that autologous transplantation of genetically modified HSPCs is more pertinent for the evaluation of long-term repopulating cells than xenotransplantation to NOD/SCID mice [87]. Linear amplification-mediated PCR has been developed in NHPs to identify and track vector-genomic DNA junctions. Each individual junction is a unique tag for a stem or progenitor cell and all its progeny. This strategy can be used to study proviral insertion sites, and to look for potential carcinogenic side effects, the knowledge of which is mandatory from a therapeutic perspective. In conclusion, NHP models are very useful to check the safety of viral constructs. To date, gene marking studies are far more common in research than in preclinical settings.

NHP models and pluripotent stem cells

Embryonic stem cells. NHP embryonic stem cell (ESC) lines have been either isolated from embryos [88, 89] or produced following *in vitro* parthenogenic development [90]. Indeed, ESCs have proved to be very useful models for studying the mechanisms of hematopoietic differentiation (*e.g.* genes involved in the early stages of hematopoiesis, regulative factors of hematopoietic potential) [91, 92]. ESC potential for tissue regeneration is currently being evaluated (*e.g.* in hematopoiesis, vasculogenesis, cardiomyogenesis, neurogenesis) [93]. By contrast, NHP cloning failed and the development of a human hematological disease model using transgenic technology (*i.e.* gene deletion) remains a problematic, long-term end-point.

Stem cell plasticity. Recent studies have demonstrated the hematopoietic potential of non-hematopoietic tissues, mainly in mouse models. This may be related to the so-called pluripotency of somatic stem cells. Previous studies in baboons have focused on fetal/adult liver stem cells. The putative interest in extrahematopoietic stem cells is currently being explored by many teams. Interestingly, preliminary studies seem to indicate that stem cell plasticity would be more restricted in NHPs than has been seen in mice. Thus, our team recently evaluated the ability of mobilized stem cells to repair cardiac tissue injury in a baboon model of acute myocardial infarction [94]. In this model, stem cell mobilization was carried out, with SCF and G-CSF administered 4 hours after circumflex coronary artery ligation. This study suggests a mobilization of endothelial progenitors that promote angiogenesis in the infarcted myocardium without significant myocardial repair. As for the presence of hematopoietic stem cells in monkey and human muscle, it appears highly hypothetical [95]. MSCs can be considered to be a source of multipotent stem cells, since they are capable of producing osteoblasts, chondrocytes, adipocytes and neurons. Biodistribution of MSCs after systemic or intramedullar injection has been studied in NHPs in order to evaluate their level of engraftment and their possible contribution to injured tissue regeneration [96].

NHP MODELS IN IMMUNOLOGY AND VIROLOGY

Obtaining antibodies for prophylactic or therapeutic use

Abs intended for therapeutic use must display two essential properties: a biological activity (such as toxin or virus neutralization), which depends on its specificity and affinity, and good clinical tolerance after administration to humans, which in turn depend on the high degree of similarity between its sequences and those of human Abs. In order to meet this second requirement, several murine monoclonal Abs have been chimerized *i.e.* their variable (V) regions were expressed in fusion with constant regions of human Abs. The V regions however, represent thirty percent of an IgG, and such a large proportion of regions of murine origin often causes poor clinical tolerance of these chimerized Abs. Humanized Abs, which retain only the murine hypervariable regions (*i.e.*10% of the whole molecule), are nearly as well tolerated as human Abs, but alteration of the antigen-binding site frequently occurs during the process, so that the affinity of these Abs can be lowered by as much as several orders of magnitude (for a review of the topic, see [97] for instance).

Abs chimerization and humanisation have been performed because the production of Abs of entirely human origin has frequently given unsatisfactory results, such as the instability of human hybridomas or the poor affinity of Abs isolated from naïv libraries. Certain mouse strains (Xenomice®), whose immunoglobulin genes have been replaced by some of their human counterparts, have been developed to produce human IgGs using standard hybridoma technology, but they are available on a commercial basis only. Several Abs with the desired activities have, however, been isolated following human immunization, using phage technology to produce and screen immune libraries of human scFvs or Fabs (fragments of IgG that include the V regions), obtained from plasmocytes of immunized donors using molecular techniques [98]. The necessity of immunizing humans and sampling their plasmocytes (in peripheral blood, but more often in bone marrow or spleen) is indeed a major disadvantage of this technique, for practical and ethical reasons.

Immunization of NHPs has been proposed as a means of obtaining Abs sharing many similarities with their human counterparts, as it has even been assumed from somatic gene sequences that the differences between macaque and human IgGs are no greater than those found between the human IgGs from different individuals [99]. For various reasons, NHPs are more easily immunized, and their plasmocytes are more easily sampled, than humans. NHPs can also be very useful for the production of Abs against epitopes or antigens that do not elicit a humoral response in humans, for instance those of human origin. V domains of NHP origin can be fused to constant regions of human IgGs to produce primatized Ab [100], three of which (IDEC 112 directed against human CD4 to be used as immunosuppressive agents, IDEC 114 directed against human CD80/B7-1 to participate in treating autoimmune diseases such as rheumatoid arthritis and psoriasis and IDEC 152 directed against human CD53/RFcεII to counteract asthma) are currently undergoing clinical trials (Bio-

gen Idec Inc., Cambridge, MA, USA). Preliminary results show good tolerances of these primatized IgG [101, 102]. However, not much information about the generation of these primatized antibodies has been reported, and only one article [103] had, until recently, described the isolation of an NHP Fab (Fragment antigen-binding, including the V regions). This scarcity has been discussed [104]. We have described in detail how an immune library was constructed using peripheral blood as a template [105], after immunizing an NHP with a model antigen (tetanus toxoid, TT). Amplicons were obtained only on the 4^{th} day after the final boost, and none were obtained on days 7 and 12. This result led us to consider that these amplicons specifically coded for anti-TT Fabs, and we constructed and screened a small phage-displayed immune library ($5x10^5$ clones). A Fab, which was named 6-ATT, was successfully isolated from this library and it showed a high affinity for its antigen ($Kd = 4x10^{-10}$ M).

The 6-ATT sequence was recognized as originating from a gene similar to human germline genes, using on-line computational analysis with IMGT/V-QUEST and IMGT/JunctionAnalysis tools [106]. The match in identity between 6-ATT V regions and their most similar germline human counterparts was 89%, reaching 93% for the frameworks sub-regions, which are the most frequently involved in clinical tolerance of antibodies. Similar features have been obtained with another NHP Fab, which very efficiently neutralizes the anthrax toxin (data not shown), and they are the basis for good clinical tolerance of primatized IgGs. NHP may therefore be regarded as a rich source of an, as yet, almost untapped source of therapeutic molecules.

IL-7: the case of an immunostimulatory cytokine

A monkey IL-7 sequence was cloned (identical in *Rhesus macaca* and *Papio papio*) and a protein with an amino acid sequence very similar to human (96% homology compared to 59% homology with rodent IL-7 amino acid sequence) was found. With only a 6 amino acid difference, monkey IL-7 has a very similar sequence to that of human IL-7. Anti-human murine Ab directed against CD127 Ab, the specific receptor for IL-7, cross-reacts with monkey IL-7 receptors, allowing in-depth, *in vivo* immunological investigations.

In NHPs and humans, IL-7 has a specific immune response limited to T-cells, not involving major B-cell proliferation, in contrast to the situation in rodents. Recombinant simian IL-7 has a similar proliferative activity as rhIL-7 on human $CD4^+$ T cells. The efficacy of IL-7 immunostimulatory activity is currently under investigation in T-cell-depleted monkeys [107], in which naïve T-cell production can be assessed by the quantification of PB T-cell receptor excision circles (TREC). Interestingly, in simian immunodeficiency virus-(SIV) infected macaques, IL-7 treatment induced a significant increase in PB $CD4^+$ and $CD8^+$ T cells, even though declines in the frequency and absolute number of $TREC^+$ cells in the PB reflects effects on mature cells. Furthermore, in these SIV-infected animals that received continuing antiretroviral therapy during IL-7 treatment, no change in viral load was observed [108].

Models of vaccine

Microbiology (including HIV/AIDS) is one of the most common areas of research involving NHPs, and it represents 26% of the studies so far [109]. Thus, NHPs have primarily been used in vaccine research, where they appear to be the most appropriate models for humans. As a general principle, the pharmacological and toxicological characteristics of a candidate vaccine must be investigated in primates during the last stage of development, if available with a comparator which should be the original vaccine to be replaced. Primary pharmacodynamics are the primary endpoint used to demonstrate protection by the candidate vaccine against the challenge with the relevant pathogen. After a first step for evaluating induced protection against a pathogen challenge in rodents, good results prompted the evaluation of the longevity of protective levels of the neutralising Abs in macaques. This was the case for new adjuvanted measles vaccine candidates [110]. In fact, the main concern is to prove product safety, mainly when live viruses are used (*e.g.* measles, mumps, rubella, yellow fever vaccines...) for vaccine preparation. Yellow fever is a viral, hemorrhagic, mosquito-transmitted disease affecting 200 000 people in the world each year, and causes more than 30 000 deaths. The first vaccines were developed in the 1930s using the French neurotropic strain produced in mouse brain but were found to be associated with encephalitic reactions in children [111]. They were replaced by the "17D vaccine" prepared in embryonated chicken eggs, and millions of doses are administered each year. The attenuated live-virus vaccine has long been considered to be among the safest and most effective vaccines, even if rare but sometimes fatal adverse events associated with the vaccine have recently been recognized. Previously, post-vaccination multiple organ system failure was recognized as a rare, serious adverse event of yellow fever vaccination and 21 cases of post-vaccinal (YFV) encephalitis have been recorded [112]. This has prompted scrutiny of the traditional recommendations for using the vaccine and has raised questions about their pathogenesis in monkeys [113]. There was a direct correlation between the clinical scores in animals that exhibited signs of encephalitis and a higher degree of central nervous system (CNS) histological lesions, with more lesions in areas of the CNS such as the substantia nigra, nucleus caudatus, intumescentia cervicalis, and intumescentia ventralis. The European pharmacopoeia [114] and the WHO [115] continue to ask for viscerotropism, immunogenicity and neurotropism testing in *Macaca mulatta* or *Macaca fascicularis* before the qualification of new master and working seed lots.

Preclinical studies in NHPs play key roles in AIDS vaccine development efforts. In addition to their traditional application to gauge vaccine safety and immunogenicity, NHP models are currently employed to explore fundamental mechanisms of primate immune system regulation, to investigate pathogenic AIDS mechanisms, and to optimize immunization [116-118]. Macaques infected with pathogenic strains of SIV or related chimeras expressing the envelope of HIV-1 (SHIV), constitute a powerful model for studies of the fundamental mechanisms of HIV pathogenesis. Indeed, SIV/SHIV and HIV have similar biological properties, using similar cellular receptors (CD4) and

co-receptors (CCR5 for SIV, and both CCR and CXCR4 for several SHIV) and having identical target cells *in vivo*. In addition, infection of macaques with pathogenic SIV/SHIV isolates reproducibly induces an immunodeficiency strikingly similar to that observed in human AIDS [119]. In this context, it has recently been shown that severe BM damage commonly observed in macaques infected with SIV, as in HIV-seropositive patients, results in impaired T-cell production, which may contribute to the disruption of T-lymphocyte homeostasis [120]. These models have been of particular interest for understanding viral/host interactions including the installation of the immune response during early steps of infection and establishment of viral reservoirs. Major advances have been made in the description of viral transmission mechanisms, particularly at the mucosal level, emphasizing, for instance, the role of dendritic cells of the vagina [121]. However, to date there is no ideal animal model of human HIV-1 infection and, although considered as the most relevant, primate models of AIDS may have some limitations: 1) a model of pathogenic infection with HIV-1 is still lacking, chimpanzees could be chronically infected with HIV-1 but do not develop AIDS in most cases, and reliable infection of macaques with HIV-1 cannot be achieved although this species is susceptible to experimental infection with SIVmac or the almost identical HIV-2 and do develop AIDS; 2) host factor-driven resistance of macaques to HIV-1 infection are not fully understood. The contribution of TRIM5α factors has been recently suggested. Indeed, primate genomes encode a variety of innate immune strategies to defend themselves against retroviruses. Thus, if HIV-1 efficiently enters the cells of Old World monkeys, it encounters a block before reverse transcription. This species-specific restriction is mediated by a dominant repressive factor, TRIM5α a member of the tripartite motif (TRIM) family of proteins containing RING domains, B boxes, coiled coils and carboxyterminal B30.2 (SPRY) domains [122]. Small differences in the TRIM5α sequence between monkey species may account for the specificity for viral capsid proteins and subsequent breadth of the restriction. However, other cellular factors such as those of the APOBEC family [123] that restrict viral replication in different cell types, seem to have similar activities against HIV and SIV. In this way, comparison of lentiviral/host interactions in different primate species may help us find the clue to HIV-1 pathogenesis in humans. Indeed, natural infection of primates in Africa did not result in the development of AIDS, although chronic infection could be characterized by high viral load and replication rate [124]. African green monkey cells are able to restrict murine leukaemia virus N tropic (MLV-N), HIV-1, HIV-2, SIVmac and equine infectious anemia virus (EIAV), whereas macaque cells strongly restrict HIV-1 only [125]. The role of the SPRY domain sequence in TRIM5α specificity is currently under investigation. In fact, the SPRY domain, also found in the immunoglobulin superfamily, is a hot spot for insertions/deletions and positive selection, which have occurred throughout primate evolution [126]. As it has been demonstrated, using chimeras between the human and rhesus monkey TRIM5 genes [127], that a single amino acid residue controls HIV restriction, NHPs could be valuable models with which to examine the role of TRIM5α polymorphism in intraspecies variability of viral permissivity. Conversely, in addition to potentially providing resistance to animal retrovirus infection, human TRIM5α may limit the usefulness of certain retroviruses, particularly EIAV, as vectors for gene therapy. The use of experimental challenges of immunized NHPs with either SIV or chimeric SIV/HIV to generate preclinical vaccine efficacy data has emerged as an important criterion for facilitating the entry of a given vaccine candidate into early phase, clinical evaluation in humans. Primate models also represent important tools for the study of AIDS and viral pathogenesis, although some limitations should be pointed out. Additional efforts need to be devoted to generate challenge models that more closely recapitulate HIV-1 infection in humans.

For naturally occurring microbial threats, even those related to emerging diseases due to changes in human demographics and behavior, the last stage of vaccine evaluation is to perform human clinical trials from phases I to IV. However, another factor in microbial emergence has attracted an increasing degree of attention: the possible deliberate and criminal release of pathogenic microbes. Of the microbial agents that could be used for bioterrorism, most of them are very rarely (anthrax, plague...) or even no longer observed in humans (smallpox...). This threat requires the protective efficacies of former or new vaccines against highly infectious aerosol exposure, to be evaluated. This prompted the US Food and Drug Administration (FDA) to adopt a regulation that permits the demonstration of efficacy using animal models when efficacy trials in humans are impossible [128]. This is the case for smallpox, which disappeared in the 1980s thanks to the WHO eradication program. The cynomolgus monkey model infected by means of intravenous injection or aerosols of monkeypox virus was used to determine the efficacy of available vaccinia virus vaccines [129], and the screening of new, safer vaccine candidates such as the highly-attenuated, modified vaccinia Ankara (MVA) [130].

ETHICAL RULES REGARDING THE EXPERIMENTAL USE OF NHPS

Since NHPs are mammals very similar to humans as regards intelligence and social complexity, their use in experimental research is regulated by strict rules [131]:

– substitute animals of other orders for NHPs as often as possible,

– only use the species of NHP appropriate to the experimental aim,

– primate cohorts should be restricted in quantity consistently with statistical analysis.

Primates for experimental research must not be captured from the wild, but must come from accredited breeding facilities. Each primate enrolled in a protocol should be followed as an individual (identification with chips, personal file), and every effort made to enrich its own environment. Researchers must comply with official guidelines for animal care and use. Any protocol must receive the approval of both a scientific review board and an accredited ethics committee.

CONCLUSION

The great similarity of NHPs to humans justifies their use in the investigation of pathophysiological mechanisms in hematology, immunology and virology and in the evaluation of tolerance and efficacy of candidate therapeutics. Recent data have confirmed that experiments in rodents are not sufficiently relevant to be able to predict human responsiveness to biological modifiers, pathogens and potential therapeutics, notwithstanding the advantages conferred by the diversity of congenic strains, transgenic and knock-out murine models. Following a screening step in rodents, the use of sophisticated cell and gene therapy tools leading to transient or permanent modification of self (autologous) or non-self (allogeneic), makes it compulsory to validate them in preclinical trials with NHPs. AIDS studies have shown that breakthroughs in vaccine developments depend on the exploration of fundamental mechanisms of primate immune system regulation. Moreover, whenever efficacy trials are impossible in humans, the demonstration of efficacy using NHPs is an unavoidable means of validation. In the absence of *in vitro* models which take into account the complexity of the networks involved *in vivo* in humans, the use of NHPs remains justified "as a last step to human", provided that primate research is restricted in quantity and that the experimental environment is enriched.

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