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BIOCHEMISTRY, BIOPHYSICS, AND MOLECULAR BIOLOGY

Neonatal Lethality and Inflammatory Phenotype of the New Transgenic Mice with Overexpression of Human Interleukin-6 in Myeloid Cells[1]

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Abstract—To model human interleukin-6 (hIL-6) associated diseases, unique mice with transgenic overexpression of human IL-6 and reporter fluorescent protein EGFP in cells of macrophage-monocyte lineage were generated using loxP–Cre system. High level of hIL-6 production by macrophages and monocytes, as confirmed in vitro in primary culture of bone marrow-derived macrophages, in vivo resulted in early postnatal death in vivo, presumably, due to the effect of overexpression of hIL-6 on hematopoiesis.

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Interleukin-6 (IL-6) is one of the most important proinflammatory cytokines with a range of immunoregulatory properties. It was established that increased levels of IL-6 are associated with various pathological conditions. Previously, we designed a transgenic system for Cre-dependent production of hIL-6 in eukaryotic cells [1] using loxP–Cre technology [2]. In the present study, this transgenic hIL-6 construct was further modified by introducing two copies of the insulator HS4 [3], as well as the sequences of the two new transcription terminators (Fig. 1). These terminators are segments of the genome containing 3'-regions of two closely located genes transcribed simultaneously towards each other. This approach allows maximum isolation of the transgene from genomic transcription, while blocking the transcription "leakage" from the transgene into the genome, thereby effectively protecting the transgene expression from the genomic repression.

We added internal ribosome entry site (IRES) and the green fluorescent EGFP cDNA in order to detect the cells with active transgene (Fig. 1). The resulting construct was tested in vitro and used to generate mice with a "humanized" cytokine IL-6 receptor system [1], a characteristic feature of which is the presence of both classical and trans-signaling cascades [4].

The hIL-6 transgenic founder mice were generated with the use of the equipment of the CCU IBG RAS by microinjection of the genetic construct (1 ng/μL) into the male pronucleus of the fertilized mouse egg hybrid F1 (CBA × C57BL/6). Microinjections were performed according to the previously described protocol [5]. Following microinjection, 234 surviving zygotes were transplanted to 25 pseudo-pregnant female recipients (from 4 to 10 zygotes per female). We detected the integration of the transgene by PCR in 21 pups out of 45 pups delivered from female recipients. It should be noted that among the transgenic individuals, as well as in their progeny, we did not find abnormal phenotypic manifestations caused by transgene expression, which presumably indicated the safety of the stop cassette in the absence of Cre-recombinase. Transgenic founder mice were mated with C57BL/6 mice. F1 offspring was genotyped by genomic PCR and demonstrated integration of the transgene in 11 individuals (100%). Approximately 50% of the F2 offspring and subsequent generations were bearing the transgenic insertion, which was due to the genomic mosaicism of the transgenic founder mice. For further work, we chose a founder that showed the best fertility.

To generate unique mice with tissue-specific expression of hIL-6 in myeloid cells, we crossed mice of the transgenic strain with mice bearing the Cre-recombinase gene under the control of macrophage

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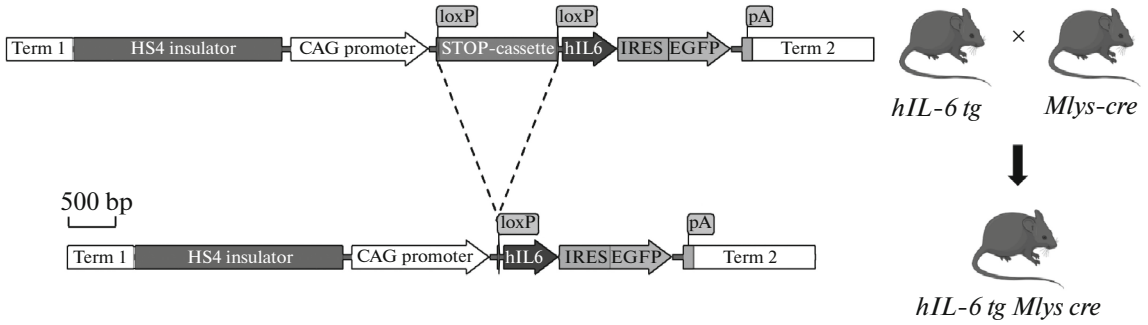

Fig. 1. Genetic construct for the regulated expression of hIL-6 in vivo and the scheme of engineering of transgenic mice. The construct contains a strong CAG promoter (consisting of an early human cytomegalovirus enhancer, a promoter and the first intron of the chicken beta-actin gene and an acceptor site from the intron of the rabbit beta-globin gene), a stop cassette flanked by loxP sites and cDNAs of the hIL-6 and fluorescent protein EGFP, separated by an internal ribosome entry site (IRES) of the encephalomyocarditis virus. pA is the SV40 polyadenylation signal. In front of the promoter the construct contains a pair of insulator HS4 sequences [3] and is flanked at the edges by transcription terminators consisting of fragments of the human genome located between the *SKIV2L* and *DXO* genes ("term 1," GRCh38, chr6: 31,969,373-31,970,143) and *PSMC5* and *SMARCD2* ("term 2," GRCh38, chr17: 63,831,740-63,833,140).

lysozyme M (Mlys-cre) promoter [6] on the C57BL/6 genetic background [7] (Fig. 1). Evaluation of the viability of the offspring from crossing hIL-6 tg × Mlys-cre mice was carried out by monitoring of developmental deviations in the progeny, as well as establishing the cause of neonatal deaths in each litter. Genotyping was carried out for all littermates, including those, which were found dead (F.D.) after birth. Of the 134 pups genotyped, 26 died neonatally (19.4%). 19 out of 26 F.D. mice were genotyped as hIL-6 tg Mlys-cre (73.1%) (Table 1). Half of all the descendants with the mutant genotype hIL-6 tg Mlys-cre died during the first four days of life. These double mutant mice were also characterized by signs of delayed development (Fig. 2a). In the liver of these mice, an increased level of hIL-6 mRNA expression was observed, while in mice without Cre we did not detect hIL-6 expression (Fig. 2b). Single mice with hIL-6 tg Mlys-cre genotype reached the age of more than 14 days and had signs of systemic inflammation and anemia, presumably as a result of the effect of overexpression of hIL-6 on hematopoietic cells [8, 9].

Since mice with the hIL-6 tg Mlys-cre genotype had a reduced viability and died during the first week of life, further in vivo analysis of these mice was very complicated. To study the effects of overexpression of hIL-6 at the cellular level, we established the primary cultures of bone marrow derived macrophages (BMDM) from hIL-6 tg Mlys-cre mice. In these primary BMDM cultures the proportion of cells that underwent excision of the stop-cassette and were expressing the reporter EGFP protein, as well as the effective production of hIL-6 in vitro was measured. For this purpose, BMDM cultures established from mutant and control mice were analyzed by flow cytometry and then, after 24 h, the level of hIL-6 production in the culture medium was determined using ELISA. The population of $CD11b^+CX3CR1^+$ macrophages isolated from the bone marrow of hIL6-tg Mlys-cre mice was characterized by about 20% of cells expressing EGFP. A high level of hIL-6 mRNA expression was also observed in these cells and high production of hIL-6 was detected in the culture medium. In the control samples expression of hIL-6 was not observed. (Fig. 2c).

Table 1. Neonatal lethality in hIL-6 tg × Mlys-cre litters

Genotype			
hIL-6 tg^+	hIL-6 tg^+	hIL-6 tg^-	hIL-6 tg^-
Mlys-cre^+	Mlys-cre^-	Mlys-cre^+	Mlys-cre^-
86.4% (19/22)	9.8% (5/51)	4% (1/25)	2.7% (1/36)

Percentage of neonatal lethality in mice of particular genotype. The number of mice that died neonatally is shown in the numerator, the number of all mice born is shown in the denominator.

Further investigation of the role of hIL-6 from myeloid cells will be carried out in mice bearing the hIL-6 transgene and the induced chimeric recombinase Cre-ERT2 [10] under the control of the *Cx3cr1* gene promoter [11]. These mice are expected to have normal viability in neonates, unlike the hIL-6 tg Mlys-cre mice described above, since in the absence of an inducer (tamoxifen) the Cre-ERT2 protein will remain in the cytoplasm of the myeloid cells. Deletion of the "floxed" STOP-cassette will occur only in adult mice following an injection of tamoxifen, which causes translocation of the chimeric protein into the nucleus of the cell [10].

Thus, in the present study, we uncovered the pathogenic role of hIL-6 overexpression, resulting in early postnatal death in mice and a pronounced inflammatory phenotype in vivo. These pathophysiological effects were associated with a specific cellular source of hIL-6—the myeloid cells. It should be noted

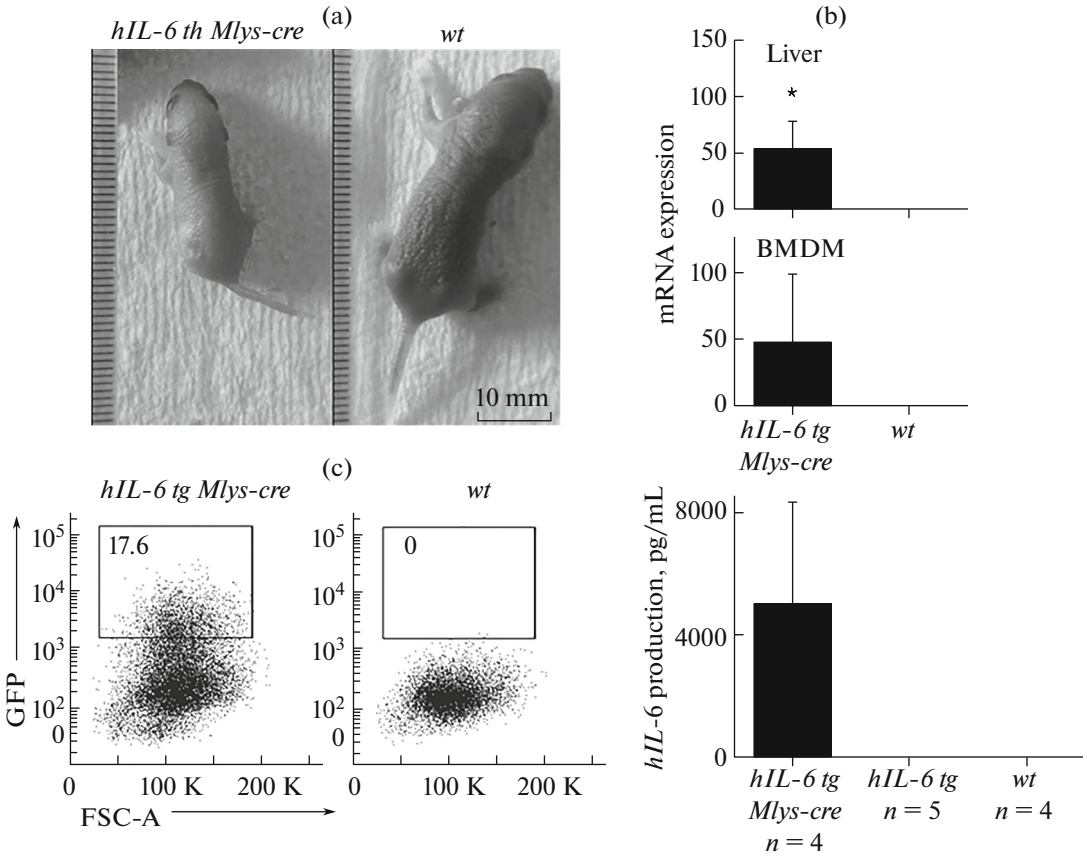

Fig. 2. The primary characterization of the hIL-6 tg Mlys-cre mouse phenotype. (a) Marked developmental delay in hIL-6 tg Mlys-cre mice at 3 day after birth. (b) The expression ($M \pm m$) of human mRNA *IL6* in hepatocytes and in bone marrow derived macrophages (BMDM); (c) representative dot plot and percentage of $CX3CR1^{+}CD11b^{+}EGFP^{+}$ live cells (left) and human IL-6 production in BMDM culture of hIL-6 tg Mlys-cre mice and wild-type control mice (right).

that the human IL-6 is able to bind mouse IL-6 receptor and transduce the downstream signal. Therapeutic inhibitors of hIL-6 or its receptor are widely used to treat several autoimmune diseases [4]. Thus, these newly generated mice, as well as primary cell cultures established from these mice, provide a useful experimental platform to evaluate the effectiveness of IL-6 blockers in lethal toxicity models. More subtle genomic editing using inducible Cre-ERT2 system will help extend the applicability of this transgenic model for further biomedical research.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflict of interest.

Statement on the welfare of animals. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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