ZKBS
Zentrale Kommission für die Biologische Sicherheit

Ref. 08020204.0002.0018[1] updated February 2026

Position statement of the ZKBS on the classification of genetic engineering operations, in which nucleic acid fragments with immunomodulatory potential are inserted into the genome of replication-competent microorganisms

Introduction

The hazard potential of replication-competent microorganisms (e.g. viruses or bacteria) can be significantly altered by the introduction of subgenomic nucleic acid fragments capable of modulating the immune system. If the inserted transgene encodes a stimulatory immunomodulator, this may reduce the residence time of the recombinant microorganism in the host organism and, where applicable, its pathogenicity. However, this could also intensify pathogenic inflammatory reactions. Conversely, an immunosuppressive transgene may increase the survival rate and pathogenicity of the microorganism. However, the impact on the hazard potential cannot be predicted across the board for every introduced immunomodulator, but must be determined in the context of the respective pathogenicity mechanisms of the recipient organisms and the specific effect of the transgene.

The transferred transgenes may encode various types of immunomodulatory proteins or non-coding nucleic acids, which are presented below:

Cytokines

Cytokines primarily regulate the proliferation and differentiation of their target cells. They include interferons, interleukins, colony-stimulating factors, tumour necrosis factors and chemokines. A large number of these immunomodulatory proteins are currently known [1].

Cytokines often have a stimulating effect on the immune system, e.g. interleukin-2 (IL-2), which activates lymphocytes [2]. However, there are also cytokines with immunosuppressive effects, e.g. the anti-inflammatory IL-10, which inhibits T cell proliferation and the production of other cytokines [3]. Furthermore, some cytokines also have pleiotropic effects (IL-6); thus depending on the target cell, such a cytokine can have different and in some cases even opposing effects on various processes of the immune system [4–6]. For example, IL-6 stimulates the activation, proliferation and differentiation of T cells (pro-inflammatory), but on the other hand it also suppresses the formation of IL-1 and tumor necrosis factor (TNF) (anti-inflammatory) [6].

[1] former ref.: 6790-03-05

Furthermore, the context in which a cytokine is expressed is crucial to its effect. For example, the administration of tumour cells expressing the *IL-4* gene can elicit a systemic T cell-dependent immune response against the tumour cells [7]. However, a study by Jackson *et al.* found that IL-4 can significantly suppress the cellular immune response against *Orthopoxvirus ectromelia* (formerly Ectromelia virus, ECTV) [8]. Following infection of mice with a recombinant, replication-competent ECTV into whose genome the murine *il-4* gene had been inserted, existing immunity was circumvented [8]. The mice infected with this recombinant ECTV died, whereas mice infected with a corresponding control virus survived the infection. The insertion of the murine *il-4* gene thus significantly increased the hazard potential of ECTV.

Another example of an increase in hazard potential is the infection of mice with an otherwise attenuated *Lyssavirus rabies* strain, into whose genome the gene for the chemokine IP-10 (CXCL10) had been integrated. This led to severe weight loss, neurological deficits and the death of three out of ten test animals, whereas infection with the parental strain resulted in only minor weight loss and transient symptoms such as ruffled fur in one out of ten test animals [9].

Antibodies and antibody fragments

Antibodies are integral components of the adaptive immune response and play a role in various processes. For example, they specifically bind to the epitopes of antigens and block them (neutralisation), mark pathogens for elimination by phagocytes (opsonisation), and activate the complement system as well as immune cells such as natural killer cells (cell-mediated cytotoxicity). Depending on the requirements for their function or structure, recombinantly expressed antibodies may include not only complete antibodies but also antibody fragments such as Fab fragments, single-chain variable fragments (scFv), single-chain antibodies (nanobodies) or fragments derived from them, such as monobodies or bispecific T cell engagers (BiTEs). The extent to which the encoded gene products can modulate the immune system depends on their target-recognition structure. If the antibodies or their fragments are intended to bind specific epitopes (e.g. in the case of expressed tumour antigens), this does not generally constitute immune modulation, as this represents a natural function of the immune system. However, if the antibodies (or their fragments) are directed against regulatory or functional components of the immune system and aim to block or inhibit them, an immunomodulatory effect can be assumed. For example, BiTEs consist of two scFv fragments linked by a peptide bridge. One fragment is specific for an antigen, whilst the second fragment binds specific surface molecules of T cells, such as the T cell receptor. Consequently, the T cell and the antigen-presenting cell are brought into close proximity to one another, triggering a T cell-mediated immune response [10]. For example, the BiTE blinatumomab, approved in the European Union (EU) as Blincyto® for the treatment of acute lymphoblastic leukaemia (ALL), binds the surface molecule cluster of differentiation 19 (CD19) on B cells and simultaneously CD3 on T cells [11]. Other targets may also include immune checkpoints or their inhibitors, which themselves regulate the immune system, i.e. have pro- or anti-inflammatory effects. A well-known example is the programmed cell death protein 1 (PD-1), a surface protein expressed on B and T cells, and its ligand PD-L1. Binding of PD-L1 to PD-1 results in the inhibition of the activation and proliferation of immune cells. The monoclonal antibody atezolizumab, approved in the EU as Tecentriq® for the treatment of various tumour types, binds to PD-L1 and thus prevents it from binding to PD-1. This triggers an enhanced immune response against the tumour cells [12]. Known side effects of both BiTEs and monoclonal antibodies include cytokine release syndrome (CRS) and immune

effector cell-associated neurotoxicity syndrome (ICANS) [13, 14]. Due to the occurrence of these toxicities, some of which are severe [15, 16], an increased hazard potential cannot be ruled out in the context of a replication-competent microorganism.

Antigens, superantigens and antimicrobial peptides

Antigens (e.g. small protein fragments or cryptic peptides) that have been inserted into the genome of replication-competent microorganisms for expression can interact with the immune system in several ways. In intracellularly replicating microorganisms, the processing of the expressed peptides leads to the presentation of antigens by the major histocompatibility complex I (MHC-I) [1]. MHC-I is ubiquitously present on all cells and forms part of the immune system's strategy against intracellularly replicating pathogens. The immune system is activated via the binding of cytotoxic $CD8^+$ T cells to the antigen-loaded MHC-I molecules, resulting in the elimination of the antigen-presenting cell infected by the microorganism. Extracellularly replicating organisms or parts thereof are usually taken up by antigen-presenting cells (APCs, e.g. dendritic cells, macrophages, B cells) via endocytosis, phagocytosis or pinocytosis and neutralised in endosomes/lysosomes [1]. The resulting small protein fragments of the microorganism, including the expressed foreign proteins, are presented as antigens to MHC-II molecules, which are subsequently displayed on the surface of the APCs. If a $CD4^+$ T cell recognises the antigen, it subsequently differentiates and expands clonally, e.g. into a T-helper cell (T_H1 or T_H2), and is able, among other things, to trigger a stimulating cell-mediated or humoral immune response through the production of cytokines.

The presentation of the expressed small protein fragments or cryptic peptides as antigens via MHC-I and MHC-II leads to activation of the immune system, which is specifically directed against the peptide-presenting cells. Where applicable, this is associated with immunomodulation, which is, however, specific to the respective epitope and has no pleiotropic effect. It can be assumed that the expressed antigens have no further effects on the immune response. Consequently, there is no increase in the hazard potential of the replication-competent microorganism.

In contrast, so-called superantigens can lead to the overactivation of T cells regardless of processing by antigen-presenting cells; these T cells then proliferate polyclonally and release cytokines uncontrollably, which can result in toxic shock [17]. In this process, the superantigen forms a bridge between immune cells through non-specific binding to MHC-II molecules and T cell receptors. Examples of these non-glycosylated proteins include the staphylococcal enterotoxins (SE) and toxic shock syndrome toxin-1 (TSST-1) produced by *Staphylococcus aureus,* as well as the streptococcal pyrogenic exotoxins A and C (SPEA and SPEC) and the streptococcal mitogenic exotoxin Z (SMEZ). Although most superantigens are produced by bacteria, the existence of viral superantigens is under discussion, e.g. in *Lentivirus humimdef* (formerly Human immunodeficiency virus, HIV) and the *Betaretrovirus murmamtum* (formerly Mouse mammary tumour virus, MMTV) [18, 19]. In replication-competent microorganisms into whose genome nucleic acid fragments encoding superantigens have been inserted, an increase in the hazard potential cannot be ruled out.

Other peptides for which an immunomodulatory effect has been demonstrated include antimicrobial peptides, also known as host defence peptides (HDPs) [20]. These peptides, ranging in

length from 10 to 50 amino acid residues, are found in both prokaryotic and eukaryotic organisms and form part of the innate immune response in animals. In humans, defensins, cathelicidins and histatins, amongst others, are known to influence the differentiation of dendritic cells, the recruitment of phagocytic macrophages and mast cells, the secretion of anti-inflammatory cytokines, wound healing, apoptosis and the suppression of pro-inflammatory cytokines [21]. HDPs are effective against viral, bacterial and fungal pathogens as well as parasites [22]. The role of HDPs in tumour biology has not yet been fully elucidated [23], and there is also occasional discussion of a stimulating effect of defensins on tumour growth and invasion [24]. In the case of replication-competent microorganisms into whose genome nucleic acid fragments encoding HDPs have been inserted, an increase in the hazard potential cannot be entirely ruled out.

Immunomodulatory gene products of pathogens

Over the course of evolution, genes that modulate the host's immune system in order to evade the immune response emerged in certain pathogens. Strategies for immune evasion include, amongst others, the inhibition of the humoral immune response, the suppression of the interferon response, the inhibition and modulation of cytokines and chemokines, the prevention of apoptosis, as well as evasion of immune cells and the alteration of MHC function [25]. For example, mastadenoviruses utilise several of these strategies: various gene products from the E1A, E1B and E3 regions exert immunomodulatory effects by inhibiting the interferon response or TNF-mediated apoptosis and downregulating the expression of MHC-I proteins [26]. Other viral gene products (e.g. from *Mammarenavirus choriomeningitidis* (LCMV), *Orthohepadnavirus hominoidei* (HBV), *Orthohepacivirus hominis* (HCV), HIV), on the other hand, influence the expression regulation of IL-10 or PD-L1 [27]. Furthermore, pathogens can also express immunomodulatory proteins that resemble the host's cytokines or have been directly acquired from them in the course of evolution. For example, members of the *Herpesviridae* family express orthologues of IL-10 to suppress the host's immune system [28]. For replication-competent microorganisms into whose genomes nucleic acid fragments encoding immunomodulatory gene products from pathogens have been inserted, a higher hazard potential cannot be ruled out, as their elimination by the immune system might be impaired.

Non-coding nucleic acids

Short non-coding nucleic acids, such as small hairpin or small interfering RNAs (shRNAs, siRNAs), can also influence the immune system when they target genes encoding regulatory or functional components of the immune system. Both shRNAs and siRNAs interact with the transcribed mRNA of a target gene via the process of RNA interference (RNAi). Consequently, the expression of the corresponding gene products is downregulated or completely silenced. The effect of non-coding nucleic acids cannot be generalised and depends, amongst other things, on the target gene, the function of the gene product within the context of the immune system, and the extent of inhibition achieved. Non-coding nucleic acids are used, e.g. (i) in tumour therapy, whereby oncolytic viruses can additionally express an shRNA against an immune checkpoint [29, 30], or (ii) in anti-inflammatory immunotherapy, whereby, e.g. shRNAs against CD40

expressed by *Saccharomyces cerevisiae* are being investigated for the treatment of autoimmune diseases [31].

Recommendation

For genetic engineering operations in which nucleic acid fragments with immunomodulatory potential are expressed by replication-competent microorganisms, a general statement regarding the mode of action of these expression products cannot be made on the basis of general criteria, but only on a case-by-case basis. Such operations are therefore generally to be regarded as non-comparable within the meaning of the Genetic Engineering Act (see below for exceptions).

Consequently, a case-by-case assessment of genetic engineering operations in which nucleic acid fragments with immunomodulatory potential are inserted into the genome of replication-competent microorganisms is, in principle, required by the ZKBS.

In summary, these are nucleic acid fragments coding for:

- cytokines
- antibodies and antibody fragments (including mono- or nanobodies) directed against regulatory or functional components of the immune system (e.g. immune checkpoints, immune receptors)
- peptides with immunomodulatory effects (e.g. superantigens)
- immunomodulatory gene products of pathogens
- non-coding nucleic acids targeting regulatory or functional components of the immune system, with the aim of downregulating or silencing them

This list is not exhaustive.

In the following cases, a case-by-case assessment by the ZKBS is not required:

1. Recipient organisms are used that do not pose a risk to humans and animals and cannot permanently colonize or infect humans and animals.
2. Combinations of recipient organisms and nucleic acid fragments with immunomodulatory potential are used that have already been assessed by the ZKBS (e.g. transfer of the gene for the human protein TRAIL to *Mastadenovirus caesari*).

   A list of combinations that have already been assessed is available on the ZKBS website.
3. Subgenomic nucleic acid fragments are transferred which encode antibodies, antibody fragments, mono- or nanobodies that are not directed against regulatory components of the immune system in order to block or inhibit them.
4. Subgenomic nucleic acid fragments are transferred which encode protein fragments or cryptic peptides that, as antigens, trigger a specific immune response directed against these protein fragments or cryptic peptides and which are not superantigens.

The current data do not allow to provide a definitive list of nucleic acid fragments with immunomodulatory potential that do not require a case-by-case assessment.

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