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Rational Genome Engineering – on the Generation of Cell and Mouse Models for Infection

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The need for cell and animal models for infection

In many areas of biomedical research, including infection research, experiments on cells and animals are conducted to get a deeper understanding of the function and regulation of genes, in the origin and molecular pathogenesis of diseases and also to develop novel therapeutic strategies.Such model systems are often limited, *e.g.* when the experimental settings do not reflect the situation in patients, in case of host restrictions (such as found for hepatotropic viruses) or if certain experimental conditions need to be modulated over time. The genetic modification of cells and animals represents a unique tool to rationally design model systems so that they can provide experimental conditions that are naturally not available. Thereby, tailor-made experimental systems with a plethora of different properties become conceivable and can pave the way for new avenues to address open questions.

The research group Model Systems for Infection and Immunity (MSYS) at HZI is focusing on the establishment of novel cellular and mouse based model systems. For this purpose, novel experimental approaches are developed for applications in infection research.

In vitro cell models with physiological properties

Cell cultures are of particular interest since they allow the investigation of biological questions in the smallest autonomous entity and thereby avoid the complexity of an animal. However, the application of primary cells isolated directly from the organism is limited due to the low number and limited availability of many cell types. This accounts in particular for human cells. Moreover, most primary cells cannot be expanded *in vitro* to the numbers required for the various applications. Immortalized cell lines of different tissues and species have been isolated from tumor material. They can also be generated by transfer of genes that promote cell proliferation, *i.e.* by immortalization. Cell lines can be cultivated unlimitedly and thus represent a valuable source for many *in vitro* studies. In most cases, however, these cell lines have lost their original properties and thus no longer mirror the respective cells in the body. Accordingly, the relevance of data obtained with these lines is limited and restricted to specific questions. Moreover, the fact that these cell lines proliferate in an uncontrolled manneris

an important difference to primary cells which cycle only rarely in their natural niche in the organism. This limits the benefit of immortalized cell systems for many applications *in vitro*, but also for potential regenerative approaches.

To attack this dilemma, a novel immortalization strategy has been developed. The strategy implies the immortalization of cells in a way that the proliferation activity is controlled so that one can put a time limit on cell growth. To this end, synthetic control elements that provide tight transgene regulation (May et al., 2008) are employed for expression of proliferation control genes. Once introduced into primary cells these synthetic tools allow to control onset and cessation of the expression of proliferation inducing genes and thereby the cell cycle. By using externally added small molecules that regulate the introduced control elements, the cells can be maintained in the expansion phase. The strategy used is depicted in Fig. 1. A Doxycycline controlled promoter was used to drive expression of different immortalizing genes. In the presence of Doxycycline, cell expansion is achieved while in the absence of the inducer the cells undergo proliferation arrest (May et al., 2004). Using this procedure, a number of different cell lines of mouse and man could be established (Table 1). Importantly, it could be demonstrated that following this procedure, the cells can be expanded on demand but still retain their original properties (May et al., 2010). This was proven by functional implementation of the human cells in mice. It could be demonstrated that conditionally immortalized human endothelial cells can form functional vessels and overtake their intrinsic function once exposed

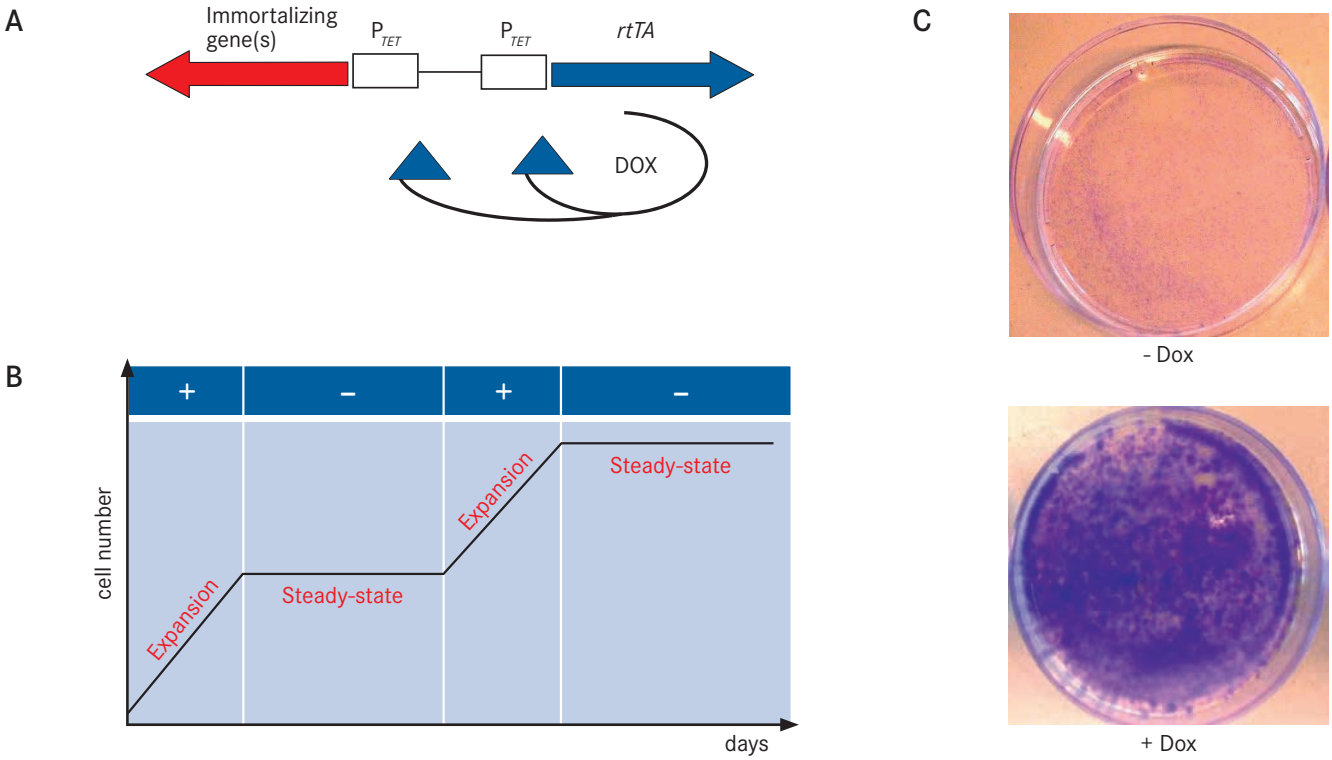


Fig. 1. Strategy for controlled expansion of cells by conditional immortalization
A: An autoregulated, Doxycycline (Dox) dependent expression vector for Dox controlled expression of immortalizing genes. In presence of Dox, the transactivator rtTA binds to the Ptet promoter and activates the immortalizing genes in a reversible manner.
B: In presence of Dox (+) cell expansion is achieved, while with drawal of Dox (-) results in maintenance of the cell number.
C: Conditionally immortalized fibroblasts were cultivated for 5 days in presence or absence of Dox and subsequently stained.
Figure: HZI

Cell type			Immortalization constitutive	Immortalization conditional
Fibroblasts	mouse	embryonal	+	+
	mouse	ear	+	+
	man	foreskin	+	+
Endothelial cells	mouse	lung	+	+
	mouse	liver	+	+
	human	lung	+	+
	human	cord	+	+
Osteoblasts	human	bone	+	not done
Hematopoietic stem cells	murin	bone marrow	+	-
Hepatocytes	mouse	liver	+	+

Table 1. Characterized cell lines

to a suitable niche *in vivo* (Fig 2). Moreover, the novel endothelial cell systems of man and mice proved to be susceptible to infection of various pathogens including Group A Streptococci (S. Talay, unpublished observations), KSHV (Alkharsarh et al., 2011) and MCMV (Dag et al., submitted) which demonstrates the potential of these cells for infection research. Based on this proof of concept, current activities focus on the development of growth controlled cells from hepatic tissues and epithelial cells. The results confirm the benefit of the conditional immortalization approach for expansion of these cell types.

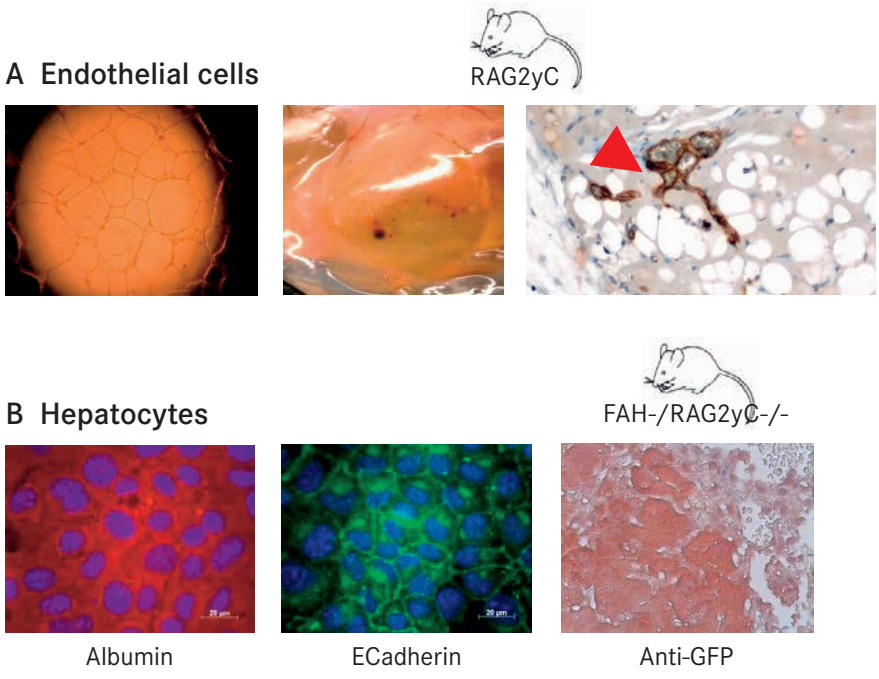


Fig. 2. Examples for novel immortalized cell lines
A. Conditionally immortalized human endothelial cells maintain the capacity to form tube like structures in matrigel in vitro. *Grafting of the cells in immune compromised mice results in formation of perfused vessels.*
B. Immortalized mouse hepatocytes show hepatic markers such as albumin and Ecadherin. Upon transplantation into FAH-/- mice, they show successful engraftment. Figure: HZI

While this activity started as a pure scientific activity more than a decade ago, it is now complemented by a start-up company (InScreenEx) funded by Dr. Tobias May and Dr. Roland Schucht, former members of the research group MSYS. InScreenEx focuses on the generation and commercialization of the novel cell systems in particular for screening purposes, thereby teaming up with MSYS to bundle up resources. To extend this concept, and to get hands even on cell types in which the above mentioned approach is not satisfactory, the current focus is on the screening and identification of particular (sets of) proliferation inducing genes that support immortalization and maintenance of the properties of specific cell types.

A novel development is the application of conditionally immortalized cells for expansion of iPS (induced pluripotent stem) cell derived cells. iPS cells have been recently shown to provide a source for generation of various patient-specific cell types upon *in vitro* differentiation. However, both the efficiency of generation of iPS cells (reprogramming) and also their differentiation to the tissues of choice are inefficient, highly stochastic processes. Moreover, the generated differentiated cells do not proliferate. Accordingly, only limited numbers of the differentiated cell types can be achieved. We could demonstrate that the conditionally immortalized cells are susceptible for reprogramming to induced pluripotent stem cells. Upon expression of reprogramming factors, conditionally immortalized fibroblast (CI-MEFs) and endothelial cells (CI-LSECs) have been reprogrammed to iPSCs. These cells can then be subjected to *in vitro* differentiation. It could be demonstrated that in the differentiated cells the proliferation cassettes can be activated again. Thereby, the differentiated progeny can be expanded (Maeda, Wirth et al., manuscript in preparation). This paves the way for the application of conditionally immortalized patient cells for research, for screening of novel compounds and also opens new perspectives for regenerative approaches.

Road to precision: predictable transgene expression by targeted chromosomal integration

The generation of genetically modified cells involves the transfer of recombinant DNA (*e.g.* plasmids) encompassing the regulatory elements (promoters) as well coding sequences for proteins of choice. Upon transfer to the cells and concomitant activation of cellular repair mechanisms that sense and eliminate broken DNA, the recombinant DNA is integrated into the cellular chromosomes. As a result, the recombinant DNA will be transduced to daughter cells upon cell division as part of the cellular chromosome. However, for stable expression, the nature of the integration site turns out to be one crucial factor that affects the level, stability and regulation of transgene expression. Although the genome of various species has been sequenced, our knowledge on the sequence-based gene regulation mechanisms and constituting regulatory cascades is limited. Thus, the impact of a specific integration site on transgene expression remains an unpredictable parameter. To overcome this limitation, screening is used to identify those integration events that meet the requirements for transgene

expression (*e.g.* a stable or a regulated expression). This imposes a problem, in particular when screening is limited, such as the identification of appropriate integration sites for the generation of transgenic mice (see below).

For the development of novel cell or mouse based systems, strategies are required to implement genetic modifications in a predictable, reliable, and, most importantly, effective way. We have developed highly effective protocols to guarantee that genetic modifications will have the desired effect in cells and/or transgenic mice. As such, genetic elements to control gene functions – so-called expression cassettes – are not randomly integrated into the cell's chromosomes but targeted to pre-selected chromosomal loci. This allows ruling out the negative impact of regulatory elements that are frequently associated to randomly chosen chromosomal sites. Moreover, this allows to ensure tight regulation of synthetic expression cassettes that can be externally controlled and switched *on* or *off* on demand. Different methods are available to specifically target expression cassettes to chromosomal loci of choice (Fig 3). Among these, homologous recombination is a complex and *per se* rather inefficient method which is masked by illegitimate recombination in many cell lines. Its efficiency is increased upon introducing double strand breaks that can be introduced by employing upcoming methods based on the design of site specific nucleases such as Zn finger nuclease (ZFN), TAL nucleases or the CRISPR/Cas9 nuclease. However, the particular chromosomal site that provides the expected expression phenotype after integration of vector cassettes cannot be predicted and has to be identified experimentally.

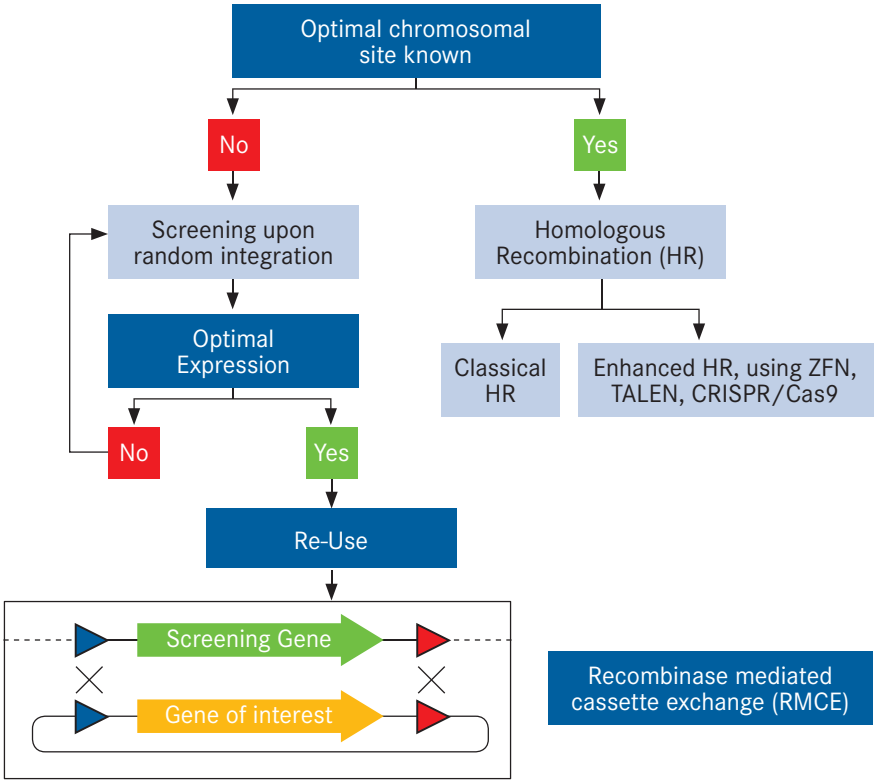


Fig. 3. Strategies for controlled genetic manipulation of cells and mice
If the chromosomal site for genetic modification is known, it can be targeted by homologous recombination. Recently emerging methods for generation of specific DNA double strand breaks such as Zn finger nuclease, TALE nuclease or the CRISP/CAS9 based nuclease systems are employed to increase the efficiency of homologous recombination. In case the optimal site is not defined, screening is performed to identify the chromosomal sites that give rise to the desired expression pattern. To re-use the identified chromosomal site, the screening cassette will be flanked with recombinase target sites (blue and red triangle) that allow for efficient targeting of the gene of interest flanked by the same set of sites.

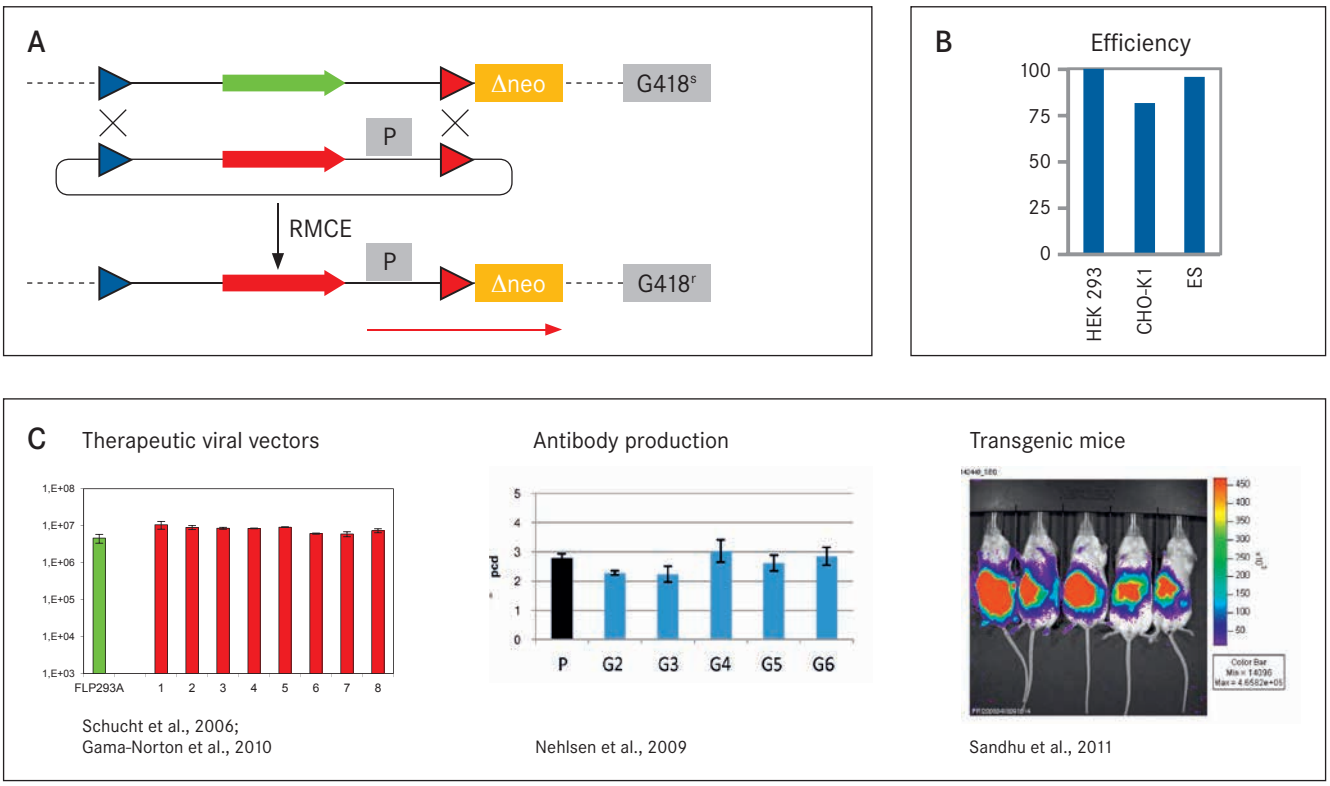
Figure: HZI

We followed a strategy that is based on the screening of integration sites with particular properties and the tagging of these sites with small (34 or 48bp) sequence tags that represent targets for the respective site specific recombinases such as Cre and Flp. By flanking the screening cassettes with sets of heterologous recombination target sites, recombinase based site specific cassette exchange is achieved (RMCE, recombinase mediated cassette exchange). Combined with an efficient selection protocol, this method confers targeting of virtually 100% (Fig. 4). Such strategies have been exploited for screening and exploitation of particular chromosomal sites for various applications (*e.g.* Nehlsen et al., 2009; Gama-Norton et al., 2010). It could be demonstrated that – as long as the main regulatory elements are preserved – the targeting of pre-screened sites indeed leads to predictable gene expression.

Transgenic mouse models for infection research

RMCE (see above) has particular advantages for the generation of novel transgenic mouse models since it allows rapid and highly efficient integration of expression cassettes into defined loci of pluripotent embryonic stem cells. Embryonic stem cells represent the basic material for the generation of transgenic mice, which involves either their injection into blastocysts or the aggregation with tetraploid embryos and subsequent transplantation to foster mice. Thus, the genetic modification of embryonic stem cells will directly translate into the generation of genetically modified mice. Technologies that allow the specific modification of defined genomic sites in these cells are thus of pivotal interest. This in particular concerns homologous recombination. The increased efficiency

Fig. 4. Principle of RMCE and successful chromosomal integration
A. A pre-screened integration site is tagged with a set of two non-interacting recombinase target sites (blue and red triangle) and a non-functional resistance marker (ΔNeo). Targeted integration of cassettes of choice flanked with the same set of via RMCE can be achieved by specific activation of a selection marker that allows isolating correctly targeted cell clones.
B. The efficiency of this method was determined by PCR. The percentage of correctly targeted clones from G418R clones is indicated.
C. Applications of RMCE: Targeted integration of cassettes results in isogenic cells; the expression level of the generated clones is comparable to the parental clone in cell lines and also in transgenic mice.
Figure:HZI



upon combining this method with site specific induction of double strand breaks (see above) now paves the way for precise modification of virtually any loci. This particularly applies for the generation of knock-in and knock-out animals that represent specific modifications at particular sites. Beside this, synthetic expression cassettes are used in the context of the mouse genome for expression of a plethora of transgenes such as pathogen derived genes, dominant negative mutants of cellular genes but also the expression of regulatory RNAs such as shRNAs and microRNAs that is used to downregulate endogenous genes. For these applications, recombinase based RMCE is of benefit for exploiting the properties of a particular chromosomal site. Due to the unprecedented efficiency of recombinase based genetic manipulations and the short recombination sequences, this is the method of choice for these applications: indeed, screening is reduced to a minimum and the process of generation of transgenic mice speeds up. Importantly, the replacement of a reporter for a gene of interest at a particular chromosomal site will result in mice with the very same expression pattern. Thus, the expression characteristics are highly predictable. Importantly, the Flp recombinase based tag-and-target approach in embryonic stem cells can be combined with classical Cre/loxP based strategies for tissue specific transgene activation (Sandhu et al., 2011). Particular applications comprise the design of transgenic mouse models that allow mimicking features of complex diseases such as viral induced hepatitis in a controlled manner (Cebula et al., in revision). A number of different chromosomal sites in the mouse genome are available for recombinase based targeted integration. Due to the high advantage and robustness associated with this method, tagged embryonic stem cells have been implemented into the portfolio of the recently established service unit transgenic mice at the HZI (TGSM). This unit is dedicated to establish novel transgenic mouse models for infection research in collaboration with researchers from the centre.

Recent developments in molecular biology allow the design of cell and mice-based on controlled genetic manipulations. Together with the emerging field of synthetic biology these strategies will become interesting options for the design of tailor-made experimental models that allow to specifically addressing complex questions in biomedical research.

Dagmar Wirth studied chemistry in Braunschweig. Her interest in genetic engineering of mammalian cells started as part of her doctoral work at GBF – today's HZI – with a special focus on chromosomal elements that affect gene expression. As a Postdoc, she conducted research on recombination in mammalian cells and viruses and developed recombinant viruses for application in biotechnology and gene therapy. Since 2004 she is head of the R&D project 'Cellular Models', since 2007 she is head of the research group 'Model Systems for Infection and Immunity' and since 2012 she leads the service unit 'Transgenic Mice'. The current focus of her work lies within the development of cell and mouse systems that provide tight control of transgene expression.

Following her work as a scientist in the Department of Clinical Immunology at the Medical University in Hannover, she returned to GBF in 2004 to assume her current role as principal investigator for the project 'Cellular models' and as head of the research group 'Model Systems for Infection and Immunity'.

Dagmar Wirth and her work group.

Photos: HZI/Hallbauer & Fioretti

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