T cell receptor-transgenic mouse models for studying cellular immune responses to *Salmonella* in vivo

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Abstract

Cellular immune responses are crucial both for protective immunity against salmonellosis, and for the immunogenicity of oral vaccines based on avirulent live *Salmonella* as antigen carriers. The crucial early steps of T cell induction are difficult to investigate in conventional animals, but recently developed T cell receptor (TCR)-transgenic models allow visualization of antigen-specific T cells in vivo while they become induced. In this review, the results obtained with four different TCR-transgenic *Salmonella* infection models are described, and advantages and potential limits of each of the different models are compared.

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1. Introduction

Salmonella causes important human and veterinary diseases and remains a worldwide health concern [1]. Because of an increasing incidence of multi-resistant strains, vaccination appears to be the most attractive long-term solution for control of this pathogen [2]. Various vaccines with moderate protective efficacy have been developed and licensed for human use, and other, potentially more effective vaccines are currently being tested in clinical trials [3]. In addition, avirulent *Salmonella* have been shown to be promising live vaccine carriers of heterologous antigens for oral immunization against a wide range of infectious diseases [4].

Cellular immune responses seem to play a crucial role for protective immunity against salmonellosis and other important infectious diseases [5,6]. However, important aspects of the early inductive steps of CD4$^+$ T cells to *Salmonella*-encoded antigens remain incompletely understood because the rare antigen-specific precursor T cells are difficult to detect. In particular, it is unknown where and when T cells become initially activated and how *Salmonella* properties might modulate these early inductive steps. T cell receptor (TCR)-transgenic mouse models offer unique advantages for this purpose [7], as such mice can be used as a source for naive, almost pure mono-specific T cells. The artificially high frequencies of specifically responding T cells in TCR-transgenic mice (typically 50–90%) might result in non-physiological conditions, hence adoptive transfer of a few TCR-transgenic T cells to syngenic non-transgenic recipient mice is generally being used to obtain a small (0.2–1%) but detectable population of naive CD4$^+$ T cells that can be tracked in vivo during induction using clonotypic monoclonal antibodies [7], MHC-II tetramers [8], or CFSE-labeling prior to transfer.

TCR-transgenic models have been successfully used to monitor T cell induction in a wide variety of experimental models. However, potential limits of this approach should be kept in mind for data interpretation [9]. First, transgenic T cell precursor frequencies in the recipients are usually some 100-fold higher than normal. Among other effects, this could cause T cell competition for antigen-presenting cells resulting in weak and abnormal responses [10]. Secondly, most transgenic T cell receptors recognize model antigens such as ovalbumin or invertebrate cytochromes that have not been subject to pathogen/host co-evolution, and thus might not be representative for relevant cellular responses to infection [9]. As a consequence of these potential limitations, a comparative evaluation of several models is very useful. Indeed, four different MHC

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class II-restricted TCR-transgenic models for murine *Salmonella* infections have been established so far. Except for the most recent SM1/*fliC* system [11], all systems use recombinant *Salmonella* expressing a model antigen recognized by the corresponding transgenic TCR. The results obtained as well as advantages and potential limits of each model system for investigating immune responses during salmonellosis or optimizing *Salmonella*-based live vaccines are summarized through this review (Table 1).

2. Cytochrome C-*fliC* model antigen

The MHC class II-restricted moth cytochrome C (MCC) epitope containing residues 88–103 was inserted in the hypervariable region of the flagellin-encoding gene *fliC* [12]. *Salmonella enterica* serovar Dublin expressing this modified flagellin from the P_{fliC} promoter had a motility defect but flagella with normal appearance were detected by electron microscopy of broth-grown *Salmonella*. In vivo cellular responses were analyzed for attenuated *Salmonella* strains after intraperitoneal infection of mice transgenic for a MCC-specific class II-restricted TCR [13]. *Salmonella* with flagella carrying the recognized MCC epitope were cleared more rapidly compared to control *Salmonella* with normal flagella, while clearance in normal mice was similar for both *Salmonella* strains [12]. This suggests that MCC fused to flagellin was able to induce the transgenic T cells to become protective effector cells. Inductive sites and activation kinetics have not been characterized.

This model uses attenuated *Salmonella* and is thus appropriate for studying immune responses to avirulent *Salmonella*-based vaccine constructs but not for salmonellosis. The recombinant *Salmonella* express high levels of the flagellin fusion protein in vitro, hence large amounts of antigen were administered by i.p. injection of 10^6 broth-grown *Salmonella* cells. On the other hand, *Salmonella* probably lack flagella during the predominantly intracellular growth in systemically infected hosts [14,15], suggesting that the flagellin-linked model antigen might be rapidly down-regulated after infection [15]. T cell induction would thus be mainly due to the large initial antigen dose contained in the *Salmonella* inoculum. This model uses the artificial i.p. route, inducing immune responses that are different from those induced by the regular oral route of infection. Moreover, TCR-trangenic mice with very high frequencies of mono-specific T cells (ca. 90%) were used [13], possibly resulting in non-physiological T cell induction [10].

3. Ovalbumin model antigen

One of the most widely used TCR-transgenic models is

the Do11.10 mouse that is transgenic for a TCR recognizing a class II-restricted ovalbumin epitope containing residues 327–337 at least [16,7]. This model system has been used with *Salmonella* expressing either full-length ovalbumin [17] or green fluorescent protein (GFP) fused to the cognate ovalbumin epitope [18,19].

3.1. Full-length ovalbumin

Full-length ovalbumin has been expressed in virulent *S. enterica* serovar Typhimurium from a plasmid stabilized by balanced–lethal complementation [17]. In vivo cellular responses to ovalbumin were analyzed in subcutaneously infected BALB/c mice that had previously received 2.5×10^6 TCR-transgenic T cells from Do11.10 mice. Transgenic T cells accumulated in the draining lymph nodes and spleens of mice infected with ovalbumin-expressing *Salmonella* but not in mice infected with control *Salmonella*, indicating a specific induction and proliferation of cognate T cells. Surprisingly, T cell accumulation was only transient, with peak numbers around day 5 in spite of an ongoing *Salmonella* infection. The initial T cell induction was similar in susceptible (Ity^s) and resistant (Ity^r) BALB/c mice but only transgenic T cells in resistant mice retained the capacity to produce IFN-γ later in the infection. Ovalbumin expression and the presence of TCR-transgenic T cells did not affect the *Salmonella* colonization in both susceptible and resistant mice, suggesting that clearance was not limited by the number of responding CD4$^+$ T cells.

This model uses virulent *Salmonella* lethal to susceptible mice and is thus appropriate for studying salmonellosis. The expression of the model antigen ovalbumin has some deleterious effect on Gram-negative enterobacteriaceae including *Escherichia coli* and *S. enterica* [20], and ovalbumin expression plasmids are rapidly lost during infection unless they are stabilized by balanced–lethal complementation [17,21]. The stabilized *Salmonella* strain expressing ovalbumin under the control of the constitutive P_{trc} promoter was reported to produce about 70 µg per 10^8 bacteria, corresponding to about 7 000 000 ovalbumin copies per cell [17], but this exceedingly high amount might reflect an overestimation. The in vivo ovalbumin expression level is unknown. While 10^8 *Salmonella* cells have been applicated systemically, less than 10^6 *Salmonella* cells persisted in lymph nodes and spleen throughout infection, suggesting that the initial ovalbumin dose present in the inoculum was by far larger than the amount that might have been produced in situ by the surviving *Salmonella*. T cell induction might thus reflect mainly the initial stage of infection in this model, explaining the transient T cell accumulation in spite of the ongoing infection. In addition, in this model the artificial s.c. route is used that induces immune responses different from those induced by the normal oral route of infection.

Table 1
TCR-transgenic mouse models for *Salmonella* infection

Salmonella strain	Antigen	In situ expression	Route of infection	Conc. of tg T cells[a]	Inductive site	Activation kinetics	Reference
Avirulent *S. dublin*	flagellin-cytochrome	unknown	intraperitoneal	90%	?	?	[12]
Virulent *S. typhimurium*	ovalbumin	unknown	subcutaneous	0.6% (dLN)	dLN, spleen	transient	[17]
Avirulent *S. typhimurium*	GFP-ovalbumin	measurable	oral	0.2% (PP)	PP	continuous	[18]
Virulent *S. typhimurium*	flagellin	unknown	oral	0.14% (PP)	PP, mLN	transient	[11]

[a]Concentration of TCR-transgenic CD4^{+} T cells among all lymphocytes in relevant organs of adoptive transfer-recipients. dLN, draining lymph nodes; PP, Peyer's patches, mLN, mesenteric lymph nodes.

3.2. GFP-ovalbumin

To avoid the toxic effects of full-length ovalbumin on *Salmonella*, only a 25mer peptide was expressed that contains the class II-restricted epitope recognized by the Do11.10 TCR [18]. To obtain an antigen that can be quantified in situ, this epitope was fused to the GFP detectable by confocal microscopy [18] or flow cytometry [22]. In wild-type or attenuated *S. enterica* serovar Typhimurium up to 200 000 copies of this GFP_OVA fusion protein do not alter virulence [23]. In vivo cellular responses to attenuated *Salmonella* expressing GFP_OVA were analyzed in orally infected BALB/c mice that had previously received 4×10^6 TCR-transgenic T cells from Do11.10 mice [18]. Ovalbumin-specific T cells in the Peyer's patches up-regulated the very early activation marker CD69, became larger, and divided after infection with *Salmonella* expressing GFP_OVA (but not with control *Salmonella* expressing GFP alone), indicating an antigen-specific T cell induction. In contrast to the subcutaneous *Salmonella* infection model [17], there was a continuous activation of transgenic T cells which closely correlated with the in situ amount of the fluorescent antigen as determined by flow cytometry. Throughout induction, most ovalbumin-specific T cells resided in the interfollicular T cell regions of the Peyer's patches, suggesting that these are the inductive sites. Interestingly, most viable *Salmonella* resided several 100 μm apart in the sub-epithelial dome area. This system also allowed a quantitative comparison of in vivo antigen expression from a series of different promoters with the immunogenicity of the respective construct [19]. As a result, an improved strain was identified for which 1000-fold smaller inocula are sufficient for potent T cell induction compared to the first prototype construct. Other parameters for optimization of *Salmonella*-based vaccines, such as antigen localization, are currently being investigated. To estimate the impact of the artificially high precursor frequency of ovalbumin-specific T cells (see Section 1) that might result in abnormal responses, the concentration of transgenic cells among the Peyer's patches lymphocytes was titrated over a range of 0.1–0.6% (unpublished data). Within this range, there was no effect on the proportion of activated TCR-transgenic cells after *Salmonella* infection, suggesting that intra-clonal T cell competition may be of minor importance.

This model uses attenuated *Salmonella* and is thus appropriate for optimization of live *Salmonella*-based vaccines but possibly less suited for investigating salmonellosis. The model antigen GFP_OVA is very well tolerated and has the unique advantage that its in situ level can be easily quantified. However, it is an artificial antigen and may not reproduce properties of autologous *Salmonella* antigens that have been subject to pathogen–host co-evolution. In contrast to the models described previously, the natural oral route of infection is used. The results clarify the important role of interfollicular regions of Peyer's patches as inductive sites for cellular responses to enteric pathogens [24].

4. Flagellin

To investigate T cell responses to an autologous *Salmonella* antigen, a new transgenic mouse model (SM1) was recently generated using a TCR specific for a class II-restricted flagellin (*fliC*) epitope containing residues 427–441 [11]. In vivo T cell responses to virulent *S. enterica* serovar Typhimurium were studied in orally infected B6 mice that had previously received 2×10^6 TCR-transgenic SM1 T cells. Flagellin-specific T cells in the Peyer's patches and the mesenteric lymph nodes up-regulated the very early activation marker CD69, produced IL-2, became larger, and divided. Similar to previous observations with the GFP_OVA system (see above), the interfollicular regions of the Peyer's patches appeared to be the major inductive site for these cellular responses. Surprisingly, T cell activation was transient, with peak CD69 expression already at 12 h post-infection despite an increasing *Salmonella* colonization. Accumulation of flagellin-specific T cells was also transient, with peak numbers at day 3. No responses were observed in spleen although this organ also became heavily infected. Another unusual finding was the general collapse of the bacterial population in Peyer's patches, mesenteric lymph nodes, and spleen at day 5, while *Salmonella* infections of susceptible mice normally result in persistent high bacterial loads in the Peyer's patches and exponential proliferation in mesenteric lymph nodes and spleen.

This model is the only one that uses an autologous *Salmonella* antigen (FliC) previously shown to dominate

T cell responses during salmonellosis [25,26]. Together with the use of virulent *Salmonella* and the natural oral route of infection, this system thus appears to be optimally suited for studying relevant cellular immune responses during salmonellosis. However, flagellin expression is likely to be down-regulated during intracellular growth of *Salmonella*, which might contribute to the rapid, but transient activation of flagellin-specific T cells [15]. Moreover, flagellin itself induces vigorous innate immune responses mediated by Toll-like receptor 5 [27] and can stimulate gut epithelial cells to produce the chemokine CCL20 which triggers the migration of immature dendritic cells [28]. Broth-grown *Salmonella* express and secrete large amounts of flagellin [29], and the administration of 5×10^9 *Salmonella* thus introduces antigen amounts that are by far larger than what the maximally 10^6 intracellular *Salmonella* present in infected tissues until day 5 [11] can be expected to produce. The most likely explanation for the strictly local and transient T cell activation in Peyer's patches and mesenteric lymph nodes is, therefore, a massive initial dose of antigen. The much smaller amounts of antigen that may be produced in situ are obviously too small to induce cellular responses that are detectable in this system. As such a massive initial antigen dose corresponding to 50 000 LD_{50} is unlikely to occur during natural infections, it remains unclear whether the data might be representative for salmonellosis.

5. Conclusion

Cellular immune responses are crucial for protective immunity to *Salmonella* and other important pathogens. TCR-transgenic mouse models have provided new information about inductive sites and induction kinetics for $CD4^+$ T cells specific for *Salmonella*-encoded antigens, that could not be obtained with conventional animal models. Because of detection limits for flow cytometry, most TCR-transgenic systems use artificially high T cell precursor frequencies, which could result in non-physiological responses due to T cell competition. However, data for the GFP_OVA system suggest that this might not be a limitation at least of this particular model. Another potential disadvantage of TCR-transgenic models is the predominant use of model antigens that have not been subject to host–pathogen co-evolution and may thus not be representative for *Salmonella* antigens relevant during salmonellosis. This aspect has been addressed by application of the recent SM1/*fliC* system. However, flagellin is probably rapidly down-regulated after the initial invasion stage, resulting in an only transient T cell activation. Hence, TCR's specific for alternative *Salmonella* antigens known to be expressed during the course of infection [30,22] might be more suitable for studying cellular immune responses towards salmonellosis. On the other hand, systems using attenuated *Salmonella* expressing heterologous model antigens are probably most useful for rational optimization of oral vaccines based on avirulent *Salmonella* as live antigen carriers. In particular, the use of a fluorescent antigen such as GFP_OVA allows one to directly relate immunogenicity to in situ antigen expression and other *Salmonella* properties.

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