Assessing T cell differentiation at the single-cell level

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SUMMARY AND GENERAL DISCUSSION

SUMMARY

This thesis describes the development and use of a novel technology for single-cell fate mapping, called cellular barcoding (**chapter 4**). The use of this technology allowed us to address multiple questions aimed at the acquisition of detailed knowledge on several aspects of T cell differentiation:

I) *Do individual T cell clones produce both effector and memory subsets?* By comparing the presence of unique genetic tags (barcodes) in antigen-specific effector and memory T cell populations in systemic and local infection models, at different anatomical sites and for TCR – pMHC interactions of different avidities, we revealed that under all conditions tested, individual naïve T cells yield both effector and memory $CD8^+$ T cell progeny (**chapter 5**). This indicates that naïve T cells are not yet committed to an effector or memory fate and thus that the decision to develop into either subset is not taken before the first cell division.

II) *Do T cells commit early in the response to a short-lived effector or a long-lived memory fate?* By providing the first three daughter generations with unique barcodes and subsequently analyzing their fate in infection-matched recipients, we found that under these experimental conditions the vast majority of the cells is not yet committed towards either a short- or long-lived fate (**chapter 6**). These data show that asymmetry of the first cell division, which was recently suggested to be the fate determining event[1], is unlikely to be responsible for imprinting effector and memory fates. Instead, the decision between longevity or death after antigen clearance appears to be a relatively late event after T cell activation and might require signals acquired by more downstream progeny.

III) *How is the magnitude of the T cell response regulated?* Using the cellular barcoding technology, we could measure whether the fraction of naïve T cells recruited into the response, and thereby the clonal composition of the response, changed depending on the severity of infection. We demonstrated that independent of the pathogen and its dose, recruitment of naive T cells into the response was near constant. Furthermore, recruitment was shown to be close to complete upon high-dose *Listeria monocytogenes* infection (**chapter 7**). This shows that in spite of their scarcity, antigen-specific T cells are recruited very efficiently, and that the magnitude of antigen-specific $CD8^+$ T cell responses is therefore primarily controlled by clonal expansion.

IV) *To what extent do individual naïve T cells contribute to the overall response?* Second-generation sequencing of barcodes demonstrated that individual naïve T cells produce highly variable numbers of daughter cells after *Listeria monocytogenes* infection, in spite of the fact that these T cells harbor the same TCR (**chapter 8**). The disparity in progeny sizes increased when T cells were activated by low affinity antigen.

Our data are consistent with a model in which the expansion of each individual antigen-specific T cell clone is influenced by signals received early during T cell activation.

GENERAL DISCUSSION

Fate mapping by cellular barcoding – strengths and limitations

Many aspects of T cell responses have been elucidated after following the behavior of transferred antigen-specific T cell populations that could be distinguished from the host by the expression of fluorescent or congenic markers. However, as with a group of humans, not necessarily all members of a group of cells behave in a uniform manner. Understanding T cell differentiation into divergent subsets therefore requires technologies to track the behavior of single cells rather than cell populations.

Since the advent of intra-vital microscopy, single cells have been tracked *in vivo*. The potential of this technology is however limited to a spatially confined area and a relatively short time frame, and motile cells are likely to migrate out of the imaging field. In 2007, the group of D. Busch described a single-cell transfer technology that allowed a single congenically marked antigen-specific T cell and its progeny to be followed over time *in vivo*[2]. Recently, this system has been refined to enable simultaneous tracking of up to 8 single cells that can be identified by a unique combination of congenic markers (D. Busch, personal communication). Development of the cellular barcoding technology has provided the means to perform *in vivo* single-cell fate mapping on a much larger scale. As this technology utilizes the introduction of unique DNA sequences (barcodes) to mark individual cells, the multitude of single-cell behaviors that can be assessed simultaneously is only limited by the diversity of barcode sequences available for cell labeling. Currently, hundreds of cells and their progeny can be distinguished within one mouse by cellular barcoding.

As a downside to the identification of T cells by DNA tags, each cell can only be analyzed once, as it has to be lysed to allow access to its DNA for barcode amplification. After single-cell transfer, individual T cells can theoretically be analyzed and sorted by flow cytometry and subsequently be re-injected for further tracking, although this is practically very challenging due to the small cell numbers. Clearly, intra-vital microscopy is the most suited technology to follow the exact same cell over time, although the time span during which this can be realized is relatively short. Also events occurring early after T cell activation can best be monitored by microscopy, as the two other technologies require some extent of clonal expansion before the progeny of the initially labeled cell can be detected either by flow cytometry or barcode analysis. The sensitivity of barcode detection has recently been improved by implementation of a new PCR protocol and second-generation sequencing as readout system. Nevertheless, efficient isolation of the transferred cells from the tissues remains a crucial step in both the single-cell transfer and the barcoding system.

Since barcode-labeling occurs by retroviral transduction, the barcoding technique brings with it the concern that the site of retroviral integration might influence

the behavior of the cell. This is something we cannot rule out, although it seems unlikely on the basis of theoretical arguments. Retroviral integrations occur at roughly random locations and as we are dealing with only ~400 integrations in each mouse (in each mouse ~400 uniquely labeled T cells are tracked), the chance is low that part of these barcodes would integrate in regions whose disruption would result in changes in T cell differentiation or proliferation. Furthermore, the observation that single naïve T cells give rise to both effector and memory cells and that the progeny sizes of different T cells are distinct was made both with our barcoding technology (chapter 5 and 8) and with the single-cell transfer system. If retroviral barcode integration would have substantially influenced T cell differentiation or proliferation, we would have obtained results distinct to those acquired after the transfer of single, untransduced T cells. To alleviate any concerns about the effect of retroviral integrations while maintaining the capacity for large-scale cell fate analysis, one could track the fate of cells that are marked by an endogenously generated barcode. Such barcoding without the need of retroviral transduction occurs in the BCM mouse that was recently developed in our lab by Jeroen van Heijst and Jos Urbanus. This mouse uses the Rag1, Rag2 and TdT enzymes to generate random barcodes at the junction of V, D and J gene segments that had been introduced into the Rosa 26 locus (unpublished).

The time of fate decisions

We (chapter 5) and others[2] have demonstrated that naïve T cells are not yet committed to a short-lived effector or long-lived memory fate, as individual antigen-specific naïve T cells produce both types of progeny *in vivo*. In my opinion, this one naïve cell – multiple fates mechanism makes a lot of sense in terms of host defense. It provides the host with both effector and memory cells of each activated clone and thereby ensures that those clones that are effective in the acute clearance of the pathogen will be preserved to provide protection upon renewed infection.

Our more recent experiments (chapter 6) furthermore provide evidence that short-lived and long-lived fates are not adopted until the 3rd cell division, as cells that had undergone 3 cell divisions produced both short-lived and long-lived progeny. This finding is in direct contrast with the claim that effector or memory commitment occurs during the 1st cell division through asymmetry of the division[1]. How can these seemingly opposed findings be explained? I consider the experimental data showing asymmetric partitioning of several molecules (including CD8) during the initial cell division to be strong[1,3,4] and they have been confirmed by an independent research group (E. Palmer, presentation at the NVVI Lunteren meeting 2011). Asymmetric inheritance of molecules does however not necessarily result in the acquisition of distinct fates.

The claim that asymmetric cell division produces one committed effector daughter and one committed memory daughter is based on an experiment in which 1x divided T cells were sorted into CD8^{hi} and CD8^{lo} populations (the putative effector and memory daughters respectively) that were then transferred into distinct recipient mice. Subsequently, the potential of both populations to reduce bacterial burden

after re-infection was assessed. When re-infection occurred 30 days after initial T cell activation, the $CD8^{lo}$ (assumed memory) population was superior in reducing the amount of bacteria. Nevertheless, both populations provided significant protection relative to naïve mice. If all transferred $CD8^{hi}$ cells would have been committed short-lived effector cells, they would have died by day 30 and thus been unable to provide protection at that time.

Other studies proposing short-lived and long-lived fates to be acquired early during the response relied on the transfer of sorted cell populations that expressed different levels of KLRG-1 or CD25 at ~4 days after infection[5-7]. The potential of these populations to survive long-term was shown to be different, but again the outcome was not absolute. Cell numbers of the putative short-lived cells were reduced 5 to 12-fold by 2 months after transfer, but clearly not all cells had died.

In my opinion, none of these studies therefore provides evidence for early fate commitment. Accordingly, I do not think that our data oppose those of Chang *et al.* or the other research groups. Instead of demonstrating fate commitment early during the response, these studies show that one of the two transferred cell populations gives rise to relatively larger numbers of long-lived memory cells. This could indicate the existence of two committed cell populations that are not accurately separated by CD8, KLRG-1 and CD25 expression profiles, but as we did not observe any fate commitment early during the response in our barcoding experiments, I consider this explanation unlikely. Probably, the observed difference in longevity reflects a 'bias' in the sorted populations to preferentially produce long-lived or short-lived progeny. A relatively minor bias is something we could not detect in our experiments, as the microarray-based barcode readout system only allowed us to visualize relatively large differences in barcode abundance between two populations of interest. It will be interesting to re-analyze these data with our new readout system that applies second-generation sequencing to quantify barcode sequences. Such analysis will reveal whether after a few cell divisions, some antigen-specific T cells produce relatively more long-lived progeny than others.

It can be argued that we did not observe any early fate commitment in our experiments, because an early imprinted fate could have been reset in (part of) the lymphocytes when they were isolated from their *in vivo* niche to introduce the barcode sequences. The same argument applies for the data of Chang *et al*. though, who isolated antigen-specific cells few days after activation for cell sorting. Formally, it therefore remains possible that effector and memory fates are imprinted into the different daughter cells during an asymmetric 1^{st} cell division, but so far, this has not been shown unambiguously in experiments.

My interpretation of the currently available data is that antigen-reactive T cells accumulate a multitude of external signals throughout the course of the immune response – including those delivered during asymmetric cell division - and that the integration of these signals shapes the likelihood of the cells and their progeny to die or stay alive after pathogen clearance.

Recruitment of antigen-specific T cells into the response and the extent of their clonal expansion

In chapter 7 we conclude that 'Recruitment of antigen-specific $CD8^+$ T cells in response to infection is markedly efficient'[8]. One year later, La Gruta *et al.* state that: 'Primary CTL response magnitude in mice is determined by the extent of naïve T cell recruitment and subsequent clonal expansion'[9]. Unlike it might seem from these titles, both sets of data demonstrate that high-avidity $CD8^+$ T cells are efficiently recruited into the response to strong infections. This does not imply that recruitment into the response is equal under all conditions. Indeed, we found the efficiency of naïve T cell recruitment to be reduced by a factor of ~1.5, if T cells were stimulated by lower antigen doses, for shorter durations (chapter 7), by lower affinity antigens or in the absence of CD80 and CD86-mediated costimulation (chapter 8). La Gruta *et al.* found that for one of the two subdominant clones studied, only about one third of the MHC-tetramer-binding T cells had entered cell cycle by day 5. The capacity to bind MHC-tetramers and to proliferate upon antigen stimulation is however not the same. All antigen-responsive T cells usually bind tetramer, but the opposite is not necessarily true. T cells harboring TCRs of low antigen affinity might be able to bind tetramer, but not to proliferate after stimulation with a given antigen dose[10]. This means that in the study of La Gruta *et al.*, possibly not all of the tetramer-binding T cells could actually respond to the infection. In this case, the recruitment efficiency would have been underestimated.

If one nevertheless assumes that MHC-tetramer binding has accurately identified only antigen-reactive cells, these experiments would indicate a ~3-fold reduction in recruitment efficiency for one of the subdominant clones. The reduction in recruitment efficiency accounted in both studies however for only a minor part of the 10-30-fold reduction in overall response magnitude, demonstrating that the magnitude of the total antigen-specific response is primarily regulated by the average expansion that the antigen-specific clones undergo.

It has been estimated that $CD8^+$ T cells divide on average up to 14 times in response to LCMV infection[11,12], but the behavior of a group of cells does not imply that all members of the group display uniform behavior. While we demonstrated in chapter 5 that individual antigen-specific T cells behave similar in the sense that they produce both effector and memory progeny, we showed in chapter 8 that the amount of progeny per recruited cell is highly variable, even though the tracked cells possessed the same TCR. As a result of this, T cell responses were dominated by merely a fraction of the antigen-specific clones.

From this observation the question arises what the consequences of this dominance are for host defense. If all clones of similar TCR affinity for antigen were functionally comparable, one could argue that the exact clonal composition of the response (among cells with the same TCR) is irrelevant. Preliminary data of the group of D. Busch however indicate that clones harboring the same TCR differ in cytokine production and phenotype (D. Busch, personal communication). Therefore I consider it relevant to investigate whether there is a relation between clone size and cytotoxicity or cytokine

production. Experiments addressing this issue could be performed both by cellular barcoding and the single-cell transfer system. Using cellular barcoding, one could sort T cell populations producing different cytokines and subsequently assess whether the same order of clone sizes is found in both sorted populations. By performing this analysis on a large set of mice, one could establish whether particular functions are associated with clone size. Alternatively, donor T cell function and clone sizes could be compared in different recipients of single antigen-specific T cells.

So far, we have quantified T cell clone sizes only in secondary lymphoid organs. As it has been described that low-avidity T cells exit the priming lymph node prior to high-avidity clones[13], it would be appealing to assess whether in particular early after infection the distribution of clone sizes at a local effector site is different from the distribution in secondary lymphoid organs. If also some of the clones with TCRs of identical antigen affinity would leave the lymph node at an earlier time than others, these could dominate the early response at the effector site, while others could constitute the largest fraction in the lymph nodes. Knowing that functional and phenotypical differences exist between clones harboring the same TCR, it would furthermore be interesting to investigate whether potential differences in the preferential localization of the clones would be related to particular functional properties.

Our finding that the disparity in clone sizes is more pronounced in response to reduced T cell stimulation raises the question how vaccine-induced T responses are composed. Vaccines are usually less potent inducers of immune responses than infections with live bacteria, as used in our study in chapter 8. Does this mean that the dominance of a few clones is even more pronounced in vaccine-induced responses? Whether this is the case or not, the main remaining question is whether an increased clonal dominance correlates with a reduced potential to clear a pathogen. If so, I argue that in order to improve vaccine efficacy, one should aim to increase the expansion of the small clones. Obviously, this requires knowledge of the mechanisms underlying the unequal expansion of the antigen-specific clones, which should therefore be a focus of further research.

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