SYNOPSIS

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Characterization of thymocyte subpopulations and mechanistic insights during various modes of thymic atrophy

T lymphocytes along with B lymphocytes constitute the adaptive immune system, which is critical for host defence. The progenitors of T cells arise in the bone marrow and the sequential development of T cells occurs in the thymus, a primary lymphoid organ. Developing thymocytes undergo selection and maturation processes in the thymus, post which only fully mature, non-self-reactive and immunocompetent T cells egress to the periphery. In the peripheral lymph nodes, T cells recognise antigenic peptides presented on major histocompatibility molecules by antigen presenting cells. Subsequently, T cell activation occurs to mount an effective immune response.

Thymic atrophy i. e., reduction in the cellularity of the thymus, is well known to occur with ageing. It is also observed during other conditions such as malnutrition, trauma, infections, cancer chemotherapy, etc. As a consequence, the thymic output may be affected, leading to lower number of naïve T cells with limited T cell receptor diversity in the periphery. Although the process of thymic atrophy has been widely reported, a detailed characterization of the thymocyte subpopulations that are affected and the underlying mechanisms governing them has not been performed.

In this study, we utilized an acute and lethal infection model wherein C57BL/6 mice orally infected with *Salmonella* Typhimurium (*S*. Typhimurium) undergo extensive thymic atrophy. *S*. Typhimurium is a Gram-negative bacterium that causes gastroenteritis in humans but in mice leads to a Typhoid-like disease, similar to that

caused by *Salmonella* Typhi (*S.* Typhi) in humans. Upon oral gavage of *S.* Typhimurium, severe thymic atrophy develops in mice. On the other hand, the cellularity of the mesenteric lymph node is much less affected during the course of infection. Using multicolour flow cytometry, we systematically studied the thymocyte subpopulations during *S.* Typhimurium-induced thymic atrophy. The major thymocyte subpopulations can be developmentally categorized on the basis of the cell surface expression of the T cell co-receptors, CD4 and CD8. The most immature cells lack CD4 and CD8 expression and are thus termed $CD4^-CD8^-$ double negative (DN). These cells give rise to cells expressing both CD4 as well as CD8 i. e., double positive (DP) cells. Post selection and maturation, the DP cells either give rise to $CD4^+$ single positive (SP) or $CD8^+$ SP. During *S.* Typhimurium-induced thymic atrophy, in C57BL/6 mice where the thymic cellularity drops to about ~8-10 fold, we observed a great loss of DN and DP cells and to a lesser extent, loss of $CD4^+$ SP cells. Using additional developmental and maturation markers, we subsequently studied the DN, DP and SP subsets. CD44 and CD25 expression aid in distinguishing the four stages of DN thymocyte development. The most immature $CD44^+CD25^-$ DN (DN1) cells give rise to the $CD44^+CD25^+$ DN (DN2) cells, which in turn produces the $CD44^-CD25^+$ DN (DN3) cells, which further gives rise to the $CD44^-CD25^-$ DN (DN4) cells. Infection resulted in a profound loss of DN2-DN4 cells, while the DN1 cells appeared to be unaffected during the atrophy process. Next, during T cell development, the DN4 cells generate cells known as immature single positive (ISP) cells which rapidly proliferate and are phenotypically characterized as $CD8^+CD3^-CD24^{hi}$. These cells were found to be completely depleted during infection. Next, we studied the DP subset using the maturation markers, CD5 and CD3. The $CD5^{lo}CD3^{lo}$ (DP1) cells are pre-selected thymocytes, which give rise to the $CD5^{hi}CD3^{int}$ (DP2) cells. The DP2 cells can either produce the $CD4^+$ SP cells or the $CD5^{int}CD3^{hi}$ (DP3) cells. On the other hand, the DP3 can only produce the $CD8^+$ SP cells. Infection-induced thymic atrophy led to an extensive loss of DP1 and DP2 cells. Surprisingly, the DP3 cells were comparatively more resistant, which is probably associated with higher levels of the anti-apoptotic molecule, Bcl2. Additionally, the residual DP cells were found to express higher amounts of activation and maturation markers e. g., CD44 and CD69. Subsequently, we studied the SP cells using the markers, CD24 and CD3. Upon acute atrophy, we observed accumulation of $CD24^{hi}$ cells in both $CD4^+$ as well as $CD8^+$ SP cell subsets. Further characterization using maturation markers including CD44, CD69, CD62L and MHC class I, the $CD24^{hi}$ cells

were identified as the least mature cells, while the CD24^{lo} being the most mature, in the SP cell subsets. Apart from these observations, we also observed differential susceptibilities of the thymocyte subpopulations to depletion. The ISP cells were the most susceptible cell population, followed by DN2-4 and DP1-2 and then the DP3 and CD4$^+$ SP cells. The CD8$^+$ SP cells were the most resistant to depletion during thymic atrophy.

Previous studies from our laboratory have identified roles of glucocorticoids (GCs) and Interferonγ (IFNγ) in depletion of DP thymocytes during *S.* Typhimurium-induced thymic atrophy. To study their involvement in modulation of the thymocyte subpopulations, we used the GC receptor antagonist, RU486, and *Ifnγ$^{-/-}$* mice respectively. There was a modest but significant rescue in the number of thymocytes obtained from mice administered with RU486 post infection, as well as in infected *Ifnγ$^{-/-}$* mice. In the DN cell subset, RU486 treatment was able to rescue the DN2-4 cells, while in *Ifnγ$^{-/-}$* mice, further rescue could be observed. Therefore, the loss of DN2-4 cells was dependent on GCs as well as on IFNγ. A similar observation was found in the DP subset, where the DP1 and DP2 cells were rescued post RU486 treatment and also in *Ifnγ$^{-/-}$* mice. Surprisingly in *Ifnγ$^{-/-}$* mice, the comparative resistance of DP3 cells was not observed, which contrasted to that observed in C57BL/6 wild type mice. Nonetheless, administration of RU486 in *Ifnγ$^{-/-}$* mice could reduce the loss of DP3 to some extent. Therefore, the depletion of DP1, DP2 and DP3 cells was regulated by both GCs and IFNγ, but IFNγ exerted a dominant role in resistance of the DP3 cells. In the CD8$^+$ SP subset, the ISP cells were neither recovered post RU486 treatment nor in the *Ifnγ$^{-/-}$* mice. However, administration of RU486 in *Ifnγ$^{-/-}$* mice was successful in recovering the cellularity of ISP cells to a modest yet significant extent. Thus, a synergistic action of both GCs and IFNγ was required for depletion of ISP cells. In both the SP compartments, the accumulation of CD24^{hi} cells was IFNγ-dependent but GC-independent. A model demonstrating the roles of GCs and IFNγ during infection-induced thymic atrophy has been proposed.

Apart from infection, the phenomenon of thymic atrophy is also observed during various stress conditions like malnutrition, cancer chemotherapy and trauma. Therefore, we compared the thymocyte subpopulations during atrophy induced by infection to other conditions, such as administration of lipopolysaccharide (LPS), the cancer

chemotherapeutic drug, etoposide (Eto) and the synthetic glucocorticoid analog, dexamethasone (Dex) in BALB/c mice. Although these treatments did not severely affect the cellularity of the mesenteric lymph nodes, significant thymic atrophy was observed during the above mentioned treatments. This was primarily due to the depletion of the DP thymocytes, which make up most of the cellularity of the thymus. Similar to what was observed during infection, the DN1 cells remained largely unaffected, while the DN2-4 cells were greatly depleted during thymic atrophy induced by LPS and Eto treatment. In the DP subset, the cellularity of DP1-3 subsets was depleted to equal extents during thymic atrophy induced by LPS and Eto. This is in contrast to what was observed during infection, where DP3 cells were more resistant to depletion. In the $CD8^+$ SP compartment, the ISP cells were completely depleted during LPS and ETO-induced thymic atrophy. In both the SP compartments, $CD4^+$ SP as well as $CD8^+$ SP, the build-up of $CD24^{hi}$ SP cells was less pronounced in mice treated with LPS and Eto. In addition, the $CD24^{int}$ $CD4^+/CD8^+$ SP cells were reduced during Eto-induced thymic atrophy. Although LPS and Eto induced similar changes in thymocyte subpopulations, Dex selectively depleted the DP subsets, the $CD24^{int}$ $CD8^+$ SP and the $CD24^{int/lo}$ $CD4^+$ SP cells without affecting the DN subsets. In addition to these observations, the roles of reactive oxygen species (ROS) in modulating the changes in thymocyte subpopulations during atrophy were also investigated. Upregulation of ROS was observed during *S*. Typhimurium and LPS-induced thymic atrophy but not upon Eto or Dex treatment. Oral administration of the ROS quencher, N-acetyl cysteine (NAC) reduced the extent of *S*. Typhimurium and LPS-induced thymic atrophy and the cellularity of DN and DP subsets along with ISP cells were increased post NAC treatment.

Overall, this study is on the detailed characterization of thymocyte subpopulations during various modes of thymic atrophy and the underlying mechanisms involved. The output of the thymus is strongly correlated to the outcome of various clinical conditions, such as progression of HIV patients to AIDS and immune reconstitution post bone marrow transplantation. Thus, augmentation of thymic output may reduce the morbidity and mortality associated with numerous clinical conditions. Therefore, studies on the thymocyte subsets during development and identification of thymopoietic drugs may aid in boosting the cellular immune response in various clinical conditions.