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Wang, N.

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SUMMARY

Interplay of the T cell receptor/CD3 complex and the co-stimulatory molecule SLAM in immune responses

SUMMARY

The immune system is our defense against the outside world. It is formed by a highly complex interplay of different cell types and molecules that as a whole enable the body to fight constant external threats. Two main arms with extensive interaction define the system: the innate and the adaptive immune response. T cells are responsible for the cell-mediated immune responses in adaptive immunity. T cells recognize antigen in the context of MHC molecules presented by professional APCs. Antigen specificity is defined by the T cell receptor. The T cell receptor is a multimeric complex composed of the antigen specific subunits, TCR-α and β (or the related TCR-γ and -δ) and six noncovalently associated signaling subunits, CD3-γε, CD3-δε and CD3-ζζ. All of the TCR subunits are the products of distinct genes that are assembled in a highly orchestrated fashion.

T cell development can be categorized through the phenotype of their surface markers CD25, CD44, TCR, CD4 and CD8, which are sequentially expressed. These markers indicate the progress from the double negative (DN, $CD4^-CD8^-$) stages DN1 to DN4, then become double positive (DP, $CD4^+CD8^+$) and select finally a single positive fate (SP, $CD4^+$ or $CD8^+$) before emigrating from the thymus. A feature common to all of the TCR invariant signaling subunits is the presence of one or more copies of conserved amino acid sequence motif termed immune receptor tyrosine-based activation motif (ITAM). In the ITAM two tyrosine residues are surrounded by a series of semi conserved amino acids and are separated by 6-8 amino acids (YxxLx6-8L). The phosphorylation of both tyrosine residues located within the ITAMs upon stimulation of the TCR initiates the well characterized signaling cascade in T cells. CD3-γ, -δ and –ε each contain one

ITAM, and CD3-ζ homodimer contains 6 of the 10 ITAMs present in the TCR/CD3 complex. For this reason, it is widely believed that the CD3-ζ chain is the most important signaling component of the TCR/CD3 complex.

Based on these findings, we went to dissect the individual roles of CD3 components in vivo by generating mutant mice that either lack specific CD3 subunits entirely or express human CD3 transgenes in the CD3-deficient mice models. Since CD3-γ and CD3-δ genes are located only 1.4 Kb in mouse from one another in the mouse, which are organized in a head-to-head configuration, we take the advantage of deleting the first exons of both the CD3-γ and CD3-δ genes by the same targeting construct (**Chapter 2**). The absence of CD3-γ and CD3-δ resulted in severe defect in the T cell development. Total cellularity of the thymi of CD3-γδ-deficient mice was 2-5% of that in wild-type mice. TCR-αβ T cell development is blocked at the same DN 3 ($CD4^{-}CD8^{-}CD44^{-}CD25^{+}$) check point as in Rag-deficient mice. Despite normal development and germline rearrangement of TCR-β, TCR-γ and -δ loci, the lack of CD3-γδ in thymocytes prohibited surface expression of the pre-TCR and early γδ TCR complex. In contrast to the single deficienct CD3-δ or CD3-γ mice, CD3-γδ-deficient mice exhibit a complete block in γδ T cell development. Thus, these studies indicated that CD3-γ and CD3-δ pay a critical, yet partially overlapping, role in the development both αβ and γδ T cell lineages.

It appears that CD3-γ chain plays a differential biological role in T cell development between human and mouse as CD3-γ-deficient murine models fail to recapitulate human CD3-γ immunodeficiency. Early blockage of T cell development was observed in CD3-γ-deficient mice, whereas humans lacking CD3-γ exhibits a mild αβ T lymphopenia, which suggests that highly homologous CD3-γ and CD3-δ genes might replace each other in the human TCR/CD3 complex. To prove the hypothesis, the CD3-γδ-deficient mice were crossbred with human CD3-δ transgenic mice (**Chapter 3**). Unexpectedly, the resulting mutant mouse strain, γδ- x hδTg, shows a phenotype similar to that of CD3-γ-deficient patients. The expression of human CD3-δ chain efficiently supports pre-TCR-mediated progression from the DN to the $CD4^{+}CD8^{+}$ double-positive stages. However, αβTCR-mediated positive and negative selection was less efficient in γδ- x hδTg mice than in

wild-type mice. The further studies exhibit the impairments in TCR-initiated signal strength in regulating positive or negative selection in γδ- x hδTg thymocytes. In addition, direct support for a unique role for human CD3-δ gene in γδ- x hδTg mice has been provided by the observation that the development and function of CD3-γδ-deficient T cells were not restored when the CD3-γδ-deficient mice were reconstituted with a mouse CD3-δ transgene. These findings, therefore, demonstrate that CD3-γ and CD3-δ play a different role in human and mice in pre-TCR and TCR function during T-cell development.

The CD3-ε subunit is required for early T cell development, as its targeted deletion arrests development at the DN 3 stage. T cell development in the CD3-ε-deficient mice was blocked prior to the completion of TCR-β selection, leaving thymocytes unable to express the pre-TCR on the cell surface. Since the CD3ε subunit associated with the CD3-γ and –δ during TCR assembly, it is not surprising that assembly and surface expression of the entire TCR complex would be disrupted by elimination of CD3-ε. However, our CD3-ε-deficient mice display a complex disruption of CD3-γ and CD3-δ expression, which makes it difficult to evaluate a role of CD3-ε gene alone in T cell development (**Chapter 4 and Chapter 5**). On the other hand, the unexpected disruption of CD3-γ and CD3-δ raises question as to whether CD3-ε gene regulates CD3-γ and CD3-δ expression or insertion of PGK-*Neo* cassette in targeted locus affects the expression of CD3-γ gene and CD3-δ gene. For this reason, we introduced a full length human CD3-ε gene into the CD3-ε-deficient mice. The reconstitution of CD3-ε^{null} mice (CD3$\varepsilon^{-/-}$x hTgε) with the human CD3-ε transgene did not restore CD3-γ and CD3-δ expression (**Chapter 5**). The thymic phenotype of CD3$\varepsilon^{-/-}$x hTgε mice still resembles very much that of CD3-ε-deficient mice. However, an impressive finding is that a very small population of prothymocytes expressing CD3-γ and CD3-δ from CD3ε-/-x hTgε mice is able to overcome first checkpoint (transition from $CD44^{-}CD25^{+}$ to $CD44^{-}CD25^{-}$) and progress to $CD4^{+}CD8^{+}$ or SP thymocytes, which eventually lead to a significant T cell population in the periphery. Taken together, our studies demonstrate that CD3-ε does

not regulate CD3-γ and CD3-δ expression and the severe suppression of CD3-γ and CD3-δ expression in CD3-ε-deficient mice is most likely due to PGK-*Neo* affection.

It is clear that CD3-γ, -δ, -ε, and –ζ chains, as invariant subunits of pre-TCR-αβ and TCRs, are crucial for assembly of TCR/CD3 complex, but how the integration of the signals from four individual CD3 proteins affect pre-T cell development remains to be elucidated. Because the insertion of PGK-*Neo* cassette in CD3-ε locus abolished CD3-δ expression and almost completely suppressed CD3-γ expression in CD3-ε-deficient mice (**Chapter 5**), the breeding of this mouse strain with CD3-ζ-deficient mice generated CD3-γδεζ-deficient mouse strain (**Chapter 4**). In *de facto* CD3-γδεζ-deficient mice, thymopoiesis were arrested at the $CD44^{-}CD25^{+}$DN stage as observed in RAG-deficeint mice. The fact that thymocytes found in the CD3-γδεζ-deficient mice are capable of reaching a stage during which TCR-β gene rearrangements normally happen led us to determine the impact of the CD3-γδεζ mutation on the occurrence of TCR-β gene rearrangement. Using a DNA-PCR assay, we showed that the absence of *de facto* CD3 polypeptides had no measureable effect on the extent of both Dβ to Jβ and Vβ to DβJβ rearrangement. Thus, these studies demonstrate that CD3 complex collectively is required for the transition from DN2 ($CD44^{+}CD25^{+}$) to DN3 ($CD44^{-}CD25^{+}$) in T cell development, but they appear to be dispensable for earlier thymocyte development prior to DN2 thymocytes.

T cell immune responses are initiated at peripheral tissues where immunogenic peptides bound to MHC molecules are presented on the surface of professional APC as antigen. The MHC molecule hereby gets into close contact with the T cell receptor (TCR). This interaction (signal 1) leads to T cell activation by the initiation of a multistep intracellular signaling pathway. In addition to the signal delivered by the TCR-MHC:peptide, a second signal (signal 2) provided by costimulatory molecules is required for full T cell activation. The first signal provides antigen specificity and is necessary but not sufficient to induce complete T cell activation. For complete and sustained activation the costimulatory signal is required. The second signal is antigen independent and can be generated by a number of distinct molecular interactions that

occur at the APC-T cell interface. There is now a large of number of T cell costimulatory molecules, with distinct and overlapping function. One of the current challenges in this area is to understand the functions of each of these T cell costimulatory pathways. Costimulation is provide by three major families: the B7:CD28 superfamily, a TNF:TNFR subfamily that lack death domains, and the CD2 superfamily, as well as some integrins.

Costimulatory receptors of the SLAM family belong to the CD2 subset of the Ig superfamily, which function in the immune synapse between T cells and antigen-presenting cells. The nine members of the SLAM family (SLAM, CD48, CD229, CD244, CD84. Ly108/NTB-A, CD319, SLAM8, and SLAM9) have been identified in hematopoietic cells. The cytoplasmic tails of six of these receptors carry one or more copies of a unique intracellular tyrosine-based switch motif (ITSM), which has a high affinity for the single SH2-domain signaling molecules SLAM-associated protein (SAP) and EAT-2 (EWS/FLI activated transcript-2). The notion that SLAM-family receptors operate as co-stimulatory molecules was initially based upon the function in T cell activation ascribed to the cell-surface receptor SLAM and its association with the adaptor SAP. SAP/EAT2-dependent and –independent recruitment of intracellular molecules leads to the inhibitory and stimulatory signals, which ensure upon engagement of these receptors. Integration of signaling networks determine the outcome of several effector functions depending on the cell type and state of activation. In this thesis, our studies have focused on SLAM (CD150) that is a prototypic molecule in the SLAM family.

The SLAM-family genes are located on chromosome 1 in human and mice; seven of the genes are located in a contiguous ~400kb fragment in both mouse and human chromosome 1. In Chapter 6, we characterized the mouse and human SLAM genes, each of which comprises seven exons spanning approximately 32 Kb. Mouse SLAM are expressed in thymoytes, memory $CD4^+$ and $CD8^+$ T cells. High levels of SLAM were found in Th1 cells and only small amounts of SLAM are detected in Th2 cells. Whereas expression of mouse SLAM is the highest on double positive ($CD4^+CD8^+$) thymocytes, single positive T cells express low levels of the receptor. Like in the human, mouse SLAM is rapidly up-regulated upon activation of peripheral T cells (**Chapter 6**).

SLAM demonstrates homotypic binding and functions as costimulatory molecule. Initial studies showed that treatment of anti-SLAM mAb enhanced IFN-γ production by human T cells and redirected Th2 cells to a Th0/Th1 phenotype, but did not affect Th2 cytokine secretion, suggesting an important role of SLAM in the Th1 cell differentiation. The striking finding that SAP with high affinity binds to the first phosphorylated or nonphosphorylated ITSM of the cytoplasmic tail of SLAM implies that SAP is involved in the SLAM-mediated signaling. However, the observation that SAP-deficient mice exhibit the severe defect in the Th2 cytokine production raises the possibility that SLAM positively regulates Th2 differentiation instead of Th1 differentiation. Consistent with this idea, the analysis of SLAM-deficient mice reveals that the levels of Th2 cytokine expression (IL-4 and IL-13) are low or barely detectable in response to triggering of the T-cell receptor (**Chapter 7**). This defect in IL-4 production is also evident from stimulation studies of antigen-specific TCR-transgenic T cells with APCs and peptides. In contrast to the effect of anti-mSLAM mAb on the induction of IFN-γ, the deficiency in SLAM only marginally affects the capacity of polarized Th1 cells to enhance IFN-γ production. Therefore, the evidence that SAP recruits FynT to SLAM and the individual mutation of SLAM, SAP or FynT leads to the defect in the Th2 cytokines supports that SLAM/SAP/FynT signaling pathway plays a critical role in controlling Th2 differentiation.

Clearly, as SLAM is widely expressed on hematopoietic cells in human and mouse, it is most likely that its immune regulation is not just limited to T cells. As expected, severe impairment in the production of IL-12, TNF-α and nitric oxide was observed in SLAM-deficient macrophages in response to LPS (**Chapter 7**). To confirm if SLAM deficiency results in the alteration in T cell differentiation and macrophage function in vivo, SLAM-deficient mice were infected with *Leishmania major* (*L. major*). This is one of well-established models to study the differential development of $CD4^+$ T helper cell subsets (Th1 and Th2) and macrophage functions. We compared *L. major* infection in mice lacking SLAM on the C57BL/6 and BALB/C backgrounds. $SLAM^{-/-}$C57BL/6 mice displayed susceptibility to infection with *L. major* in marked contrast to resistant wild-type mice. This impaired ability to clear the parasites was confirmed by increased

parasite titers in infected SLAM-deficient mice compared with wild-type mice (**Chapter 7**). The inability of SLAM-deficient C57BL/6 to heal the *L. major* lesions is consistent with defective IL-12, TNF-α, and nitric oxide (NO) production by SLAM-deficient macrophages.

T-helper type 2 (Th2)-derived cytokines such as IL-4, IL-5, IL-9 and IL-13 play an important role in the synthesis of IgE and in the promotion of allergic eosinophilic inflammation and airway wall remodeling. Based on the deviation of $CD4^+$ SLAM-deficient T cell response, I determined the importance of SLAM in allergic inflammation in a murine model (**Chapter 8**). The SLAM-deficient mice and their BALB/c wild-type counterparts were sensitized and repeatedly exposed to ovalbumin (OVA) aerosol. After three challenges with the OVA antigen, the mice were assessed for airway hyperresponsiveness (AHR) and inflammation. It was found that SLAM-deficient mice had significantly decreased AHR in OVA-immunized mice. Allergen-induced Th2 and Th1 cytokines were decreased in SLAM-deficient mice. These results suggest that the deficiency in SLAM strongly attenuates antigen-induced airway inflammation and hyper-responsiveness in mice.

The results contained within this thesis have contributed to an increasing understanding of the role of the CD3 components in the TCR/CD3 complex in early T cell development as well as further defining the critical role of SLAM in T cell activation and macrophage function. We hope that our work will contribute to development of specific therapies for diseases such as XLP, measles virus infection and SLE, in which SAP/EAT-dependent or –independent SLAM signaling may involve.