7 Discussion

In this study, the mechanisms underlying the cross-recognition of peptides derived from mycobacterial and murine hsp60 were investigated on the basis of the hsp60 crossreactive CD8+ T cell clone UZ3/4. This T cell clone was originally activated by mycobacterial hsp60, and shows cross-reactivity with murine hsp60 peptides (Schoel et al., 1994; Zugel et al., 1995). Adoptive transfer of this T cell clone into TCR $\beta^{-/-}$ mice induces an autoimmune intestinal pathology by specific recognition of the self-hsp60 peptides by UZ3/4 in an adoptive transfer model (Steinhoff et al., 1999). However, these results were obtained with an *in vitro* cultured CD8+ T cell clone that represents a valid model for activated effector CD8+ T cells. Yet, this model was not suitable to analyze the maturation and selection as well as the requirements for the activation of such potentially hazardous hsp-crossreactive T cells *in vivo* and hence the physiological meaning of this pathology remained incompletely understood.

7.1 Promiscuous peptide recognition of a singe TCR combination is responsible for the induction of autoimmune pathology

The TCR chains of the CD8+ CTL clone UZ3/4 were analyzed with the intent to generate TCR transgenic mice as a model for the activation of crossreactive T cells by mycobacterial hsp60. Thereby, one TCR β chain and two TCR α chains were found and cloned. The result, that two TCR α chains are expressed in this T cell clone was not surprising, because in contrast to the TCR β chain, TCR α chain allelic exclusion is incomplete. It is not known why the immune system tolerates the potential expression of two TCR α chains that together with one TCR β chain can participate in the formation of two TCR with different ligand specificities in one single T cell. Increased probability for positive selection in the thymus and increased diversity of the T cell repertoire have been discussed to favor such T cells to mature and populate the periphery. In fact, a recent report by He et al. has shown that dual TCR cells can be beneficial for the immune system by extending the TCR repertoire for foreign antigens (He et al., 2002).

The finding that one αβ TCR T cell could have two distinct ligand specificities (Padovan et al., 1993) also implied that these dual TCR cells may have an autoimmune potential. The notion was that positive maturation signals delivered to a developing T cell through one harmless TCR could traffic a second, selfreactive TCR through thymic selection. However, when surface expressed during negative selection, the same criteria must apply for both TCR α chains leading to thymic deletion of T cells bearing any TCR that recognizes self-peptide carrying MHC molecules with high avidity. It was argued that different expression levels of the two TCR α chains in the thymus and in the periphery could nevertheless undermine effective negative selection of dual TCR T cells (Elliott, 1999). So far, studies aiming at proving an involvement of dual TCR T cells in autoimmune diseases have failed. Mice hemizygous at the *TCRa* locus and therefore unable to express two rearranged *TCRa* genes have not been found to be less susceptible to autoimmune disease in non-obese diabetic mice (Elliott and Altmann, 1996) or in the collagen-induced arthritis mouse model (Corthay et al., 2001).

It has been shown that productive rearrangements of two α chain loci can either result in surface expression of both α/β TCR combinations, i.e. a dual TCR, or lead to surface expression of only a single TCR through posttranslational allelic exclusion mechanisms (Kuida et al., 1991; Couez et al., 1991). With regard to the generation of hsp60 specific TCR transgenic mice, the observation that the T cell clone UZ3/4 contained two TCR α chains posed additional questions. Which TCR α chain was involved in the recognition of the two defined peptides from mycobacterial and murine hsp60? Or was each of the two conceivable TCR αβ combinations specific for one of the two peptides?

Experiments blocking the cytolytic activity of the T cell clone UZ3/4 with TCR α8 and β8 chain specific mAb gave a first hint that promiscuous peptide recognition and activation of the T cell clone is mediated by a single TCR rather than by a dual TCR. Each of the TCR α8 and β8 chain specific mAb was sufficient to block recognition of both mycobacterial and murine hsp60 peptides. These results were confirmed and extended using an efficient retroviral system for the transduction of TCR deficient thymoma cell lines with the individual TCR chains of UZ3/4. Transduction of these

cells with the two potential α/β TCR combinations and subsequent analysis of T cell receptor surface expression clearly showed that TCR α8, but not TCR α7, is able to form a functional surface receptor with the TCR β8 chain. Moreover, TCR β8/ α8 double transfectants were stimulated by both mycobacterial and murine peptides. Therefore, promiscuous peptide recognition of hsp60 peptides from different species and induction of autoimmune pathology by the T cell clone UZ3/4 was not mediated by a dual, but by a single TCR although the two TCR α chains were successfully rearranged and expressed. Since the TCR α7 chain was not expressed on the cell surface, it was clearly not involved in peptide recognition of the T cell clone UZ3/4. Influences of the intracellular TCR α7 chain on the thymic selection of the T cells are thus unlikely but cannot be formally excluded by these *in vitro* data .

The mycobacterial and murine peptides recognized by the T cell clone UZ3/4, SALQNAASIA and KDIGNIISDA, respectively, are not sequence homologues and share only 3 amino acids including the anchor residue Asn in position 5 that is considered essential for the binding to the H2-D^b binding groove (Rammensee et al., 1993). Apart from the peptide KDIGNIISDA, five additional murine hsp 60 peptides were reported to be recognized by the T cell clone UZ3/4 (Schoel et al., 1994; Zugel et al., 1995). It is tempting to speculate that the ability of hsp to associate with various peptides regardless of their amino acid sequence may depend on “sticky” patterns in the hsp sequence. Thus, the finding that several hsp peptide fragments readily bind to the MHC molecules and confer TCR recognition may reflect the physiological role of hsp, which is binding of peptides. Moreover, analogous to the specific recognition of hsp60 by innate immunoreceptors (Janeway, Jr. and Medzhitov, 2002), the generation of self-hsp peptides that are recognized by specific T cells may be part of a “danger signal” which is emitted at sites where hsp are upregulated due to cellular stress.

Here it was shown that a single TCR of the T cell clone UZ3/4 crossrecognizes peptides of the murine and mycobacterial hsp60 which share only intermediate sequence homology. This is in agreement with reports that show degenerate TCR recognition of peptide-MHC complexes, which does not require primary structure

homology between two peptides presented by a given MHC molecule (Wucherpfennig, 2001; Maverakis et al., 2001). TCR cross-recognition of peptides that share only minimal or no sequence homology has been shown *in vitro* by the substitution of distinct amino acids in the sequence of recognized peptides (Evavold et al., 1995) or by the use of synthetic combinatorial peptide libraries (Hemmer et al., 1998a). *In vivo*, evidence for the relevance of T cell epitope cross-recognition for the onset of MS has been presented (Martin et al., 2001). Furthermore, the development of autoimmune pathology resembling human RA has been shown to be due to recognition of unrelated peptides on distinct MHC molecules by KRN TCR transgenic T cells (Basu et al., 2000; Kouskoff et al., 1996). In this context our model of intestinal autoimmune pathology represents a further example for the induction of autoimmune pathology by promiscuous T cell recognition.

A prerequisite for the crossreactive recognition of (hsp derived) self-antigens by CD8+ T cells is the effective processing and presentation of the peptides. Recent experiments from our laboratory show that proteasomal processing of antigens varies in different tissues (Kuckelkorn et al., 2002). Interestingly, processing of the pathology related hsp60 T cell epitope of murine origin was most efficient in the small intestine, the target organ of crossreactive hsp60 specific CD8+ T cells. Differential processing and presentation of defined self-peptides in certain tissues above a critical threshold could thus be a further mechanism to control or activate autoreactive CD8+ T cells and protect from or induce organ-specific pathology.

Taken together, the TCR crossreactivity of the T cell clone UZ3/4 can thus either be seen as an epitope mimicry of the mycobacterium that tries to evade immune recognition or in the light of a tolerated recognition of self-hsp peptides by the host. The latter may be just inevitable due to the high degree of evolutionary conservation of hsp or may reflect a mechanism of stressed cells to present the stress signal to T cells in the form of stimulating self-hsp peptides.

7.2 Hsp60 specific TCR transgenic mice

Having determined the TCR chain composition of the hsp60 specific CD8+ T cell clone UZ3/4, the TCR α8 and the TCR β8 chain were used to generate hsp60 specific TCR transgenic mice. The cDNA based expression vector pHSE3' was chosen because it had proven suitable in other systems (Pircher et al., 1989) and it was convenient to handle due to its moderate size of 6.2 kb.

To generate transgenic mice that express more than one transgene it is possible to co-inject several constructs at a time. This procedure usually results in co-integration of the constructs at the same site of a chromosome leading to co-transmission to the next generation (Rülicke, 2001). Thus, co-injection of a TCR α and a TCR β chain can be a useful strategy for the expression of a transgenic TCR. In the case of the hsp60 specific TCR of UZ3/4 we avoided co-injection of the two TCR expression constructs because this TCR had a potential reactivity to self-antigens. Although the one T cell that later became the T cell clone UZ3/4 had originally passed positive and negative selection, it was not clear whether this would be the case for the T lymphocytes in TCR α8 and TCR β8 double transgenic mice. This single T cell could have had accidentally escaped from thymic deletion, possibly somehow mediated by the expression of the second TCR α7 chain. Upon screening potential founder mice for TCR expression, it would therefore be impossible to discriminate between clonal thymic deletion of autoreactive T cells and insufficient expression of the transgene. Furthermore it was conceivable that TCR α8 and TCR β8 double transgenic mice developed lethal autoimmune pathology that would complicate breeding of the transgenic mouse lines. These objections were supported by the finding that one TCR α8 and TCR β8 double transgenic founder obtained from co-injection of the two constructs spontaneously died at an age of 8 weeks, possibly due to the adverse effects of the transgenic expression of autoreactive T cells. Therefore, we preferred to inject the TCR α8 and the TCR β8 constructs separately.

A great advantage for the analysis of these single transgenic mice was the availability of specific mAb directed against the TCR V α8 and V β8 segments, which allowed us to screen for the expression of the transgenic TCR chains. In single transgenic mice the TCR α8 and TCR β8 chains paired with endogenous partners and thus these

T cells most likely differed from the self-reactive T cell clone UZ3/4 in peptide specificity. In the case of the TCR α8 chain, expression levels of the transgene amongst the 13 TCR α8 chain founders were intermediate, varying from 10% – 50% of the αβ T cells. In contrast, an "all or nothing" expression pattern of the transgenic TCR β8 chain was observed. The founder mice expressed the transgenic TCR β8 chain either at no significant levels, or on almost every αβ T cell. This finding may reflect the ability of a rearranged TCR β chain to induce a complete allelic exclusion of other TCR β chain loci. The latter notion is further supported by the different FACS profiles of TCR α8 and TCR β8 transgenic mice (results section 6.4, figures 19 + 20). While the TCR β8 transgenic T cells emerged as one discrete population, TCR V α8 levels were not proportional to the TCR β chain levels and the cells appeared as more than one distinct population. This indicates that additional endogenous TCR alpha chains were expressed in a part of the TCR α8 transgenic T cell population.

The approach of random integration of the transgenic construct does not lead to a direct correlation between the copy number and the expression level of the transgene (Hogan et al., 1986; Rülicke, 2001). Thereby, the accessibility of the integration site seems to be the critical parameter for transgene expression. However, transgene integration at multiple sites on different chromosomes is possible. Thus founder mice were backcrossed to C57BL/6 mice for several generations to ensure stable expression of the transgene, and in addition, to yield a pure C57BL/6 genetic background.

Nevertheless, informative conclusions can be drawn from analysis of the offspring from preliminary crosses of TCR α8 and TCR β8 mouse lines (β8-C and α8-P). Double transgenic offspring so far showed no pathological findings, although TCR α8 and TCR β8 double transgenic T cells were found in their periphery. These T cells were expressing the CD8 coreceptor and specifically recognized the same mycobacterial peptide in the context of H2-Db MHC class I as the parental T cell clone UZ3/4. Taken together, these results clearly demonstrate that T cells exhibiting the TCR of UZ3/4 are not negatively selected as immature autoreactive T cells

through central or peripheral deletion. These findings also imply that the additional intracellular TCR α7 chain had no influence on the thymic maturation of UZ3/4. It remains to be determined which factors control or activate the autoimmune potential of the transgenic hsp60 crossreactive T cells.

7.3 TCR Vα 7 chain transgenic mice

Given that the TCR α8 and the TCR β8 chain constitute the functional TCR of the T cell clone UZ3/4, and having shown that the TCR α7 chain is unable to pair with the TCR β8 chain, the question remained whether the TCR α7 chain could pair with any TCR β chain other than TCR β8 or if it was generally unable to form a functional TCR.

The VJ-rearrangement of this individual TCR α 7 chain was in-frame and contained no irregular stop codons. However, the variable V gene segment of the TCR α7 chain, named ADV7.2 has also been found as a component of TCR δ chains (Okazaki and Sakano, 1988). The TCR δ chain locus resides within the TCR α chain locus and some V segments are shared between both types of TCR chain (Arden et al., 1995). To date, it is not clear whether these V gene segments can also be functionally expressed both in αβ and γδ TCR. Furthermore, this TCR α 7 chain lacked N-glycosylation sites (Koning et al., 1988) which are normally found in TCR α chains (Rudd et al., 1999). Therefore, the correct folding, posttranslational processing or TCR assembly of the TCR α 7 chain with TCR β chains and the CD3 components may be impaired.

To test whether the TCR α7 chain of the T cell clone UZ3/4 was able to pair with any TCR β chain, TCR α7 chain transgenic mice were generated and backcrossed with TCR $\alpha^{-/-}$ mice (α -/- mice). In TCR α 7 transgenic α -/- mice (α 7.2 mice) every peripheral TCR αβ T cell should thus contain a TCR β chain that had successfully paired with the transgenic TCR α7 chain.

In this study it was shown that transgenic expression of the TCR α 7.2 chain in α -/- mice did not result in the development of normal TCR αβ T cells as described for

other TCR α chains (Brandle et al., 1995), but surprisingly increased frequencies of highly activated CD4+ TCR $\alpha^{-}\beta^{+}$ T cells were found. These CD4+ TCR $\alpha^{-}\beta^{+}$ T cells probably express TCR β chain homodimers at their surface (Takahashi et al., 1999) and have been shown to be responsible for the induction of IBD in α -/- mice (Takahashi et al., 1997; Mizoguchi et al., 1997b).
Instead of assembling normal αβ T cell receptors, the TCR α 7 chain probably acted as a "private molecular chaperone", protecting newly synthesized TCR β molecules from degradation and thus facilitating the assembly of TCR β dimer/ CD3 complexes. This mechanism is reminiscent of a report by Barber and colleagues (Barber et al., 1998), who suggested that expression of $pT\alpha^{b}$, a splice variant of pTα, stabilizes surface expression of TCR β in CD4+ TCR $\alpha^{-}\beta^{+}$ T cells. Presumably due to the chaperone function of the TCR α7 chain, the number of peripheral CD4+ TCR $\alpha^{-}\beta^{+}$ T cells in α 7.2 mice was markedly increased compared to α -/- mice. As a consequence of the expanded CD4+ TCR $\alpha^{-}\beta^{+}$ T cells population, the course of IBD in α 7.2 mice was exacerbated and in some mice lethal at an age of two month when α -/- mice showed no signs of disease. The pathological picture found in α 7.2 animals resembled the IBD previously described for aging α -/- mice (Mombaerts et al., 1993; Mizoguchi et al., 1999; Iijima et al., 1999).

Our α 7.2 mouse model demonstrates that productively rearranged, but non-pairing TCR α chains promote the development of CD4+ TCR $\alpha^{-}\beta^{+}$ T cells. Since unproductive TCR α chain rearrangement is a normal event occurring in thymocyte development, we assume that these unconventional T cells are also present in normal individuals that have an intact TCR α locus. However, the question which physiological role the CD4+ TCR $\alpha^{-}\beta^{+}$ T cells may play has not been answered to date. These chronically activated CD4+ TCR $\alpha^{-}\beta^{+}$ T cells may represent some form of "innate lymphocytes" (Bendelac et al., 2001) that either provide baseline activation to the immune system or serve to recognize conserved microbial structures analogous to natural killer T cells (NK-T cells). Interestingly, the development of the NK-T cells depends on the rearrangement of invariant TCR α chains (Lantz and Bendelac, 1994). It is therefore fair to hypothesize that the Vα 7.2 segment of the

TCR α7 chain which is not able to form a functional αβ TCR is not just "evolutionary trash", but represents a mechanism to expand the functional diversity of αβ T cells beyond the recognition of peptides in the context of MHC molecules.

The peripheral CD4+ TCR $\alpha^{-}\beta^{+}$ T cells in both α -/- and α 7.2 mice show a chronically activated phenotype with regard to the expression of the cell surface activation markers CD62L, CD44 and CD69. These novel observations support the notion that an unusual TCR structure and TCR composition of the CD4+ TCR $\alpha^{-}\beta^{+}$ T cells allows T cell activation with less stringent stimulation requirements. It remains to be determined whether this TCR consists exclusively of β chain homodimers. Despite the phenotypical similarity of the CD4+ TCR β^{low} T cell population in α -/- and α 7.2 mice we cannot rule out that the TCR α 7 chain participates in the formation of unconventional TCR complexes on the cell surface. Supposed the TCR α 7 chain does not only facilitate the increased frequencies of CD4+ TCR $\alpha^{-}\beta^{+}$ T cells in α 7.2 mice by the stabilization of TCR β chains but alternatively can be part of the TCR complex itself, the missing N-glycosylation sites of the TCR α 7 chain may provide an explanation for the low activation threshold of these cells. Structural studies have shown that the sugar moieties on the TCR αβ protein surface prevent nonspecific protein-protein interactions between adjacent complexes and, therefore, nonspecific TCR clustering (Rudd et al., 1999; Garcia et al., 1996). Moreover, targeted mutations of the glycosylation machinery have been found to exaggerate cellular immune responses, comprising inappropriately robust and sustained T cell activation in the absence of CD28 cosignaling (Lowe, 2001; Demetriou et al., 2001; Chui et al., 2001). We propose that the special composition and structure of the TCR complexes on CD4+ TCR $\alpha^{-}\beta^{+}$ T cells lowers the threshold for the TCR response, thereby increasing their autoreactive potential.

In the healthy organism, regulatory mechanisms have to suppress the harmful activities of such autoreactive T cells. It has been shown that IBD is more severe and develops faster in the absence of regulatory cells (Mizoguchi et al., 1997a; Mizoguchi et al., 2000). Under immunocompromizing conditions, activated Th2

cytokine producing CD4+ TCR $\alpha^{-}\beta^{+}$ T cells can thus be detrimental. This is consistent with the fact that UC, a Th2 cytokine associated disease, is a frequent complication following bone marrow transplantation (Proujansky, 1999), while remission of CD, an inflammatory disease associated with Th1 cytokines, was observed in patients who received bone marrow transplants (Lopez-Cubero et al., 1998). It is possible that CD4+ TCR $\alpha^{-}\beta^{+}$ T cells are involved in the induction of UC as transplant complication because they are readily activated and thus have a proliferative advantage to repopulate the gut in a host deprived of lymphocytes. Most recent data from our laboratory show that IBD could be induced in TCR $\beta^{-/-}$ mice by adoptive transfer of CD4+ TCR $\alpha^{-}\beta^{+}$ T cells from α 7.2 mice, proving the "colitogenic" potential of these cells. To clarify the role of chronically activated CD4+ TCR $\alpha^{-}\beta^{+}$ T cells in the pathogenesis of UC, further characterization of this unique T cell population is necessary. Currently this is not possible because TCR $\alpha^{-}\beta^{+}$ T cell specific markers are not available. Normal CD4+ T cells downregulate TCR expression upon activation and are therefore indistinguishable from CD4+ TCR $\alpha^{-}\beta^{+}$ T cells that constitutively express low levels of surface TCR β chain. A mAb recognizing the constant region of any surface expressed TCR α-chain would represent a tool to visualize CD4+ TCR $\alpha^{-}\beta^{+}$ T cells that could greatly enhance the understanding of the frequency and role of these unconventional T cells.

In summary, TCR α 7 transgenic α -/- mice show that productively rearranged but non-pairing TCR α chains directly associate with and stabilize TCR β chains leading to increased formation of unusual CD4+ TCR $\alpha^{-}\beta^{+}$ T cells. These T cells are chronically activated and induce IBD. Detailed knowledge of the frequency and role of these unconventional T cells in immunocompetent individuals may allow the design of new intervention strategies for IBD.

7.4 Concluding remarks and outlook:

Based on the TCR chains of a hsp60 specific CD8+ T cell clone we established two different TCR transgenic mouse models. On the one hand, the TCR α8/β8 transgenic mice may contribute to understand the general mechanisms of how potentially autoreactive T cells can be activated by infectious agents and how autoimmune T cell responses are controlled. On the other hand, the TCR α7 transgenic mice provide important new information about the development of an unconventional T cell population that is highly suspect to be involved in the etiology of ulcerative colitis.

To underline that the transgenic mice expressing TCR α8/β8 and the TCR α7 are completely unrelated models of T cell mediated pathologies, **Table 4** summarizes the differences of the two systems.

Table 4.

Transgenic TCR chains	**TCR α8/β8**	**TCR α7**
Specific antigen	Hsp60 peptides	unclear
MHC restriction	H2-D^b	unclear
Coreceptor	CD8	CD4
TCR structure	TCR $\alpha^+\beta^+$	TCR $\alpha^-\beta^+$
Pathology	Autoimmune inflammation	IBD
Organ specificity	Small intestine	Colon

TCR α8/β8 transgenic mice are valuable tool to analyze the basic mechanisms of CD8+ T cell mediated crossreactivity to mycobacterial and murine hsp60. In the first section of the introduction we considered the immune responses to microbial hsp a "double edged sword". This colloquialism was used to illustrate that hsp can either serve as targets for protective immunity or provoke undesirable immune responses to self-antigens. The two edges of the sword are now reflected by the diverse potential applications of the mouse model.

We have shown that the transgenic hsp60 specific CD8+ T cells are not negatively selected during thymic maturation, and thus stable TCR α8 and TCR β8 double transgenic lines will provide a source for large amounts of potentially selfreactive CD8+ T cells. The analysis of the latter can either be performed directly in the TCR transgenic mice, or these animals can serve as a donor for monoclonal hsp60 specific T cells that can be further analyzed in other systems *in vitro* and *in vivo*.

The following specific questions will be addressed:

1. What are the requirements for the activation of the naive transgenic T cells? Can naive T cells respond to stressed APC that present self-hsp60 peptides or is the cognate mycobacterial antigen and proper costimulation necessary?
2. Do transgenic crossreactive T cells that were activated by mycobacterial infection induce an autoimmune pathology and thus provide a direct link between infection and autoimmune disease?
3. What regulatory mechanisms control these autoreactive T cells?
4. Do the naive or the activated transgenic T cells accumulate in the small intestine where the self-hsp60 epitope is best processed?

In addition, it was shown that the mycobacterial hsp60 peptide SALQNAASIA is relevant for the activation of CD8+ T cells during BCG infection. Apart from their autoimmune potential, transgenic T cells specific for SALQNAASIA may thus represent a tool to study the principles of CD8+ T cell responses to mycobacterial infections. It will be interesting to see whether the TCR α8/β8 transgenic mice are protected against mycobacterial infection.

TCR α7 transgenic mice may serve as model to further investigate the mechanisms underlying the spontaneous development of IBD resembling ulcerative colitis. The focus will be on the characterization of the TCR in the unconventional CD4+ T cells found in these mice. Particularly interesting are the questions how the unusual composition and structure of this TCR influence the TCR signaling and whether the TCR α7 chain is part of a surface expressed TCR. On the long run, it is crucial to

verify the existence and role of such unconventional CD4+ T cells in healthy individuals. Therefore, it may be worthwhile to look for specific patterns of CD4+ TCR $\alpha^{-}\beta^{+}$ T cell markers, e.g. using micro-array technology.

Detailed knowledge of the frequency and role of these unique CD4+ T cell population in immunocompetent individuals may allow the design of new intervention strategies for IBD.