Reversal of tolerance induced by transplantation of skin expressing the immunodominant T cell epitope of rat type II collagen entitles development of collagen-induced arthritis but not graft rejection

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Collagen-induced arthritis (CIA) is induced in H-2^q mice after immunization with rat type II collagen (CII). The immunodominant T cell epitope on heterologous CII has been located to CII256–270. We have previously shown that TSC transgenic mice, which express the heterologous epitope in type I collagen (CI), *e.g.* in skin, are tolerized against rat CII and resistant to CIA. In this study we transplanted skin from TSC transgenic mice onto non-transgenic CIA-susceptible littermates to investigate whether introduction of this epitope to a naïve immune system would lead to T cell priming and graft rejection or instead to tolerance and arthritis protection. Interestingly, TSC grafts were accepted and not even immunization of recipient mice with CII in adjuvant induced graft rejection. Instead, TSC skin recipients displayed a reduced T and B cell response to CII and were also protected from arthritis. However, additional priming could break arthritis protection and was accompanied by an increased T cell response to the grafted epitope. Strikingly, despite the regained T cell response, development of arthritis was not accompanied by graft rejection, showing that these immune-mediated inflammatory responses involve different mechanisms.

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1 Introduction

Collagen-induced arthritis (CIA) is an animal model of human rheumatoid arthritis (RA), and is induced by immunization with type II collagen (CII) in adjuvant [1]. Development of CIA has been shown to be both B and T cell dependent [2, 3]. Similar to RA [4, 5], susceptibility to CIA is genetically linked to the MHC class II region and only mice of the H-2^q or H-2^r haplotype will develop severe arthritis after CII immunization [6]. In H-2^q mice this association has been mapped to the Aβ^q gene [7]. An H-2^q-restricted immunodominant T cell epitope in heterologous CII has been located to position 256–270 (CII256–270) [8, 9]. Furthermore, this epitope contains two lysine residues, at position 264 and 270, that can become posttranslationally modified by hydroxylation and if this occurs, hydroxylysine can further become glycosylated with a mono- or a disaccharide. We have shown earlier that each modification of K264 is specifically recognized by a distinct subset of A^q-restricted T cells [10].

CII is a conserved molecule [11] and the only difference within CII256–270 is a conservative substitution from aspartic acid (D) in the mouse to a glutamic acid (E) in heterologous CII. Although murine CIA can be induced by immunization with mouse CII [12, 13], incidence of arthritis is increased if heterologous CII is used instead, for example rat, human or bovine CII. To address the role of heterologous CII in CIA we have previously described two strains of transgenic mice that express mutated CII with E at position 266 [14]. When mutated CII was expressed in cartilage, T cell response to rat CII was partially but not completely reduced, as non-proliferative T cells retained their ability to produce cytokines and give B cell help. These mice, called MMC, were still suscepti-

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Abbreviations: CIA: Collagen-induced arthritis **CI:** Type I collagen **CII:** Type II collagen **GalHyK::** β-D-Galacto-pyranose-5-hydroxy-L-lysine **WT:** Wild type

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ble to arthritis but incidence was reduced compared to non-transgenic littermates. In contrast, when the heterologous CII epitope was expressed in type I collagen (CI), which is expressed in all fibrous connective tissue, *e.g.* in dermis, CII-specific T cells displayed a more pronounced tolerogenic phenotype and these mice, called TSC, were completely resistant to CIA [14]. These observations not only show that normal interaction between T cells and joint-restricted antigen will lead to incomplete tolerance, but also stresses the importance of availability of the autoantigen in tolerance induction and protection from autoimmune disease.

As most A^q-restricted T cells generated after priming with heterologous CII recognize the CII256–270 epitope, and as TSC transgenic mice express this epitope in CI, we were interested to see if grafting of tissue from TSC transgenic mice, containing the epitope, on naive mice would lead to priming of epitope-specific T cells and rejection of the transplanted tissue. Alternatively, it was possible that transplantation would lead to tolerance and arthritis protection, as TSC transgenic mice are strongly tolerized against rat CII and resistant to CIA induced with rat CII. To investigate this, we transplanted skin, which contains large amount of CI, from TSC transgenic mice to naive, arthritis-susceptible littermates. TSC skin was not rejected when grafted on adult CIA-susceptible mice, not even after immunization with rat CII in adjuvant. Instead, we observed that transplantation of TSC skin led to a reduced susceptibility to CIA, which was accompanied by a specific reduction in the anti-CII IgG2a response. Furthermore, TSC skin recipients failed to mount an antigen-specific T cell response after immunization with CII in adjuvant. Together, these data indicate that T cells specific for the grafted epitope had been tolerized, resulting in a reduced capability to supply B cell help. However, protection against CIA was only transient and development of arthritis correlated with an increased T cell response to the grafted epitope. Strikingly, recovered susceptibility to CIA was not accompanied by graft rejection showing that arthritis development and graft rejection was mediated through different mechanism. Finally, we found that protection against CIA occurred only if recipients were immunized within 4 weeks of transplantation. This suggested that the grafted epitope must be continuously available for immune recognition until the time of immune priming if tolerance is to be maintained.

2 Results

2.1 Mutated CI is expressed in the skin of TSC transgenic mice but glycosylation of the T cell epitope is age dependent

TSC transgenic mice express mutated CI, including the heterologous CII256–270 epitope, and are tolerized to the CII epitope as well as resistant to CIA when immunized with rat CII [14]. To confirm that the mutated CI was expressed in TSC transgenic mice, we first prepared collagen from the skin of adult transgenic mice. When this preparation was presented to T cell hybridoma clones HCQ.4 and HCQ.10, specific for the non-glycosylated and glycosylated CII256–270-epitope, respectively, only the HCQ.4 hybridoma responded (Fig. 1), showing that the glycosylated epitope was not present in skin. This was surprising, as we have earlier found that mutated CI in newborn TSC mice includes the glycopeptide [14]. Since it is plausible that the level of glycosylation varies with age, we also prepared collagen from younger TSC transgenic mice and tested the expression in the T cell hybridoma assay (Fig. 1). The HCQ.10 hybridoma only responded significantly to mutated CI prepared from newborn mice. In contrast, the HCQ.4 hybridoma, specific for the non-glycosylated epitope, responded well to all batches independently of age of the mice from which

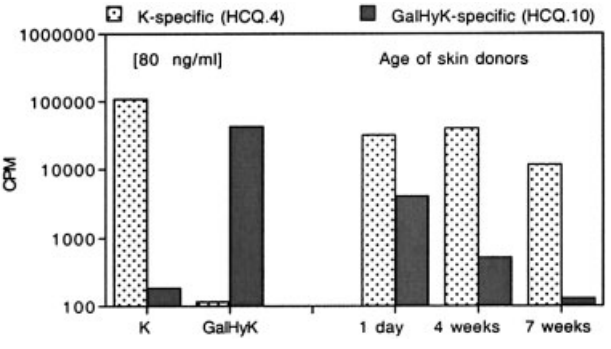


Fig. 1. The glycosylation level of mutated type I collagen is age dependent in TSC transgenic mice. Type I collagen (CI) was prepared from skin of mice of different ages. Transgenic expression of mutated CI was detected using T cell hybridomas specific for the glycosylated CII epitope (HCQ.10) or the non-glycosylated CII epitope (HCQ.4). As controls, hybridoma cells were stimulated with the non-glycosylated (K) and the glycosylated (GalHyK) CII-peptide (80 ng/ml). These hybridomas are highly specific for their epitopes and do not crossreact to the other epitope at peptide concentrations of 50 μg/ml. Comparable results were obtained in repeated, but separate experiments. Preparation of CI and the T cell hybridoma assay were performed as described in Sect. 4.

the collagen was prepared. Thus, the level of glycosylation in the skin of TSC mice is age dependent and with increasing age the expression of non-glycosylated epitope becomes dominant.

2.2 TSC skin grafts are accepted when transplanted onto wild-type mice and induce partial protection to arthritis

Previous studies have shown that most T cells generated after immunization of H-2^q-mice with heterologous CII recognize the CII256–270 epitope [8, 10, 14]. Since adult TSC transgenic mice express this epitope in dermis and are resistant to CIA, we were interested to see if transplantation of skin from TSC mice to wild-type (WT) mice would activate CII-specific T cells and induce graft rejection or alternatively alter susceptibility to CIA in graft recipients. Consequently, we transplanted 2–3 cm^2 of full thickness skin from adult (>7 weeks old) TSC mice onto the back of adult CIA-susceptible littermates. Age-matched WT skin was grafted as control. TSC skin grafts appeared intact and healthy 2 weeks after transplantation with only minor areas (<10%) of unhealed tissue around the edges of the graft and by 4 weeks after transplantation graft appeared to have healed completely, with the appearance of fur-growth in the graft (not shown). Some mice have been followed for more than 6 months without any signs of graft rejection (not shown). As grafts appeared fully healed by 4 weeks of transplantation we choose to immunize recipient mice with CII in adjuvants at this time to investigate if this would prime CII-specific cells sufficiently to induce graft rejection or if the transplanted graft instead would influence susceptibility to CIA. Strikingly, no signs of graft rejection were observed as a consequence of immunization but instead TSC skin recipients were protected from arthritis (Fig. 2). However, protection was only partial as 35% of TSC skin recipients still developed arthritis.

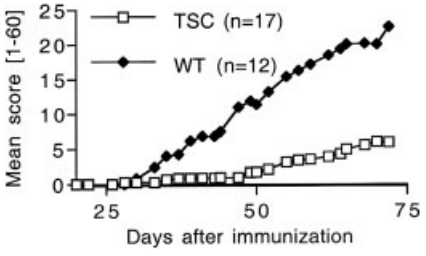


Fig. 2. TSC skin grafts are not rejected and recipients are less susceptible to collagen-induced arthritis. Mean score of mice grafted with skin from WT or TSC-transgenic (TSC) mice 4 weeks before immunization with CII in CFA. Mice were also given a boost injection of CII in IFA 5 weeks after immunization. At 10 weeks after immunization, arthritis incidence was reduced in TSC skin recipients (35%), compared to control-grafted mice (92%, p=0.002). Cumulative mean score of arthritis (severity), at the end of the experiment, was lower in TSC skin recipients (p=0.0003, Mann-Whitney U test, healthy animals are included in the statistic calculation).

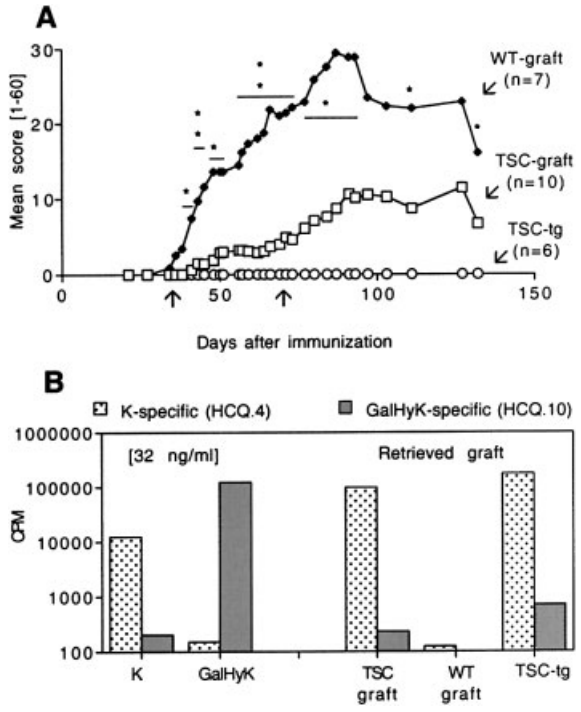
2.3 Development of arthritis does not promote graft rejection

The observation on partial protection from CIA in TSC skin recipients was also confirmed in a second arthritis experiment (see legend to Fig. 3). Similar to the first experiment, transplantation of TSC skin did not protect against CIA development in all recipients as 20% of TSC skin recipient had developed arthritis by 10 weeks after immunization. Because of this, we next wanted to investigate how reliable protection was in mice that appeared healthy at this time, and mice were therefore given a second boost immunization and arthritis development was monitored for an additional 9 weeks. At the end of this long-term experiment, the majority (90%) of TSC skin recipients had developed arthritis (Fig. 3a). Thus, although the initial protection mediated by TSC skin grafts was quite efficient, protection could be overcome by a second boost immunization. As a positive control, naive TSC transgenic mice were also immunized and monitored for arthritis development. As expected, TSC transgenic mice were fully resistant to CIA (Fig. 3a).

To ensure that the loss of protection against CIA was not a consequence of late graft rejection, grafts were recovered from recipients at the end of the 19-week CIA experiment for both histological analyzes and for crude preparation of collagen. In histological analyzes of TSC skin grafts, we found no evidence of cellular infiltration and these grafts were comparable to control grafts (data not shown). In agreement with earlier results (Fig. 1), collagen from TSC skin grafts failed to stimulate the glycosylated CII-specific T cell hybridoma clone (HCQ.10). Most importantly however, collagen prepared from TSC grafts was able to stimulate the HCQ.4 hybridoma (specific for the non-glycosylated CII256–270 sequence) and thus confirmed that the grafts had not been rejected (Fig. 3b). Therefore, even if the majority of the recipients were affected by arthritis, the transplanted epitope was still expressed in the grafts and thus appeared ignored by the immune system.

2.4 Reduced B and T cell response in arthritis protected TSC skin-grafted recipients

To search for mechanisms that could explain the initial protection in TSC skin-grafted mice, the CII-specific antibody response in skin-grafted mice was investigated



◀ *Fig. 3.* Discontinued arthritis protection does not lead to graft rejection. (A) Mean score of mice grafted with skin from WT or TSC mice (TSC) 4 weeks before CII immunization and non-grafted TSC-transgenic (TSC-tg) mice. Arrows along the X-axis indicate boost injections of CII in IFA, 5 and 10 weeks after the first immunization. Asterisks indicate significant differeces in arthritis index between WT and TSC skin-grafted mice (*$p<0.05$; **$p<0.01$, Mann Whitney U test). At 10 weeks after immunization arthritis incidence was reduced in TSC skin recipients (20%), compared to control-grafted mice (100%, $p=0.001$). However, at 19 weeks after immunization, nine out of ten (90%) TSC skin grafted mice had developed arthritis. Cumulative mean score (including all animals) of arthritis as well as mean maximum score (including arthritic animals only), at termination of experiment, was lower in TSC skin recipients ($p=0.0047$ and $p=0.019$, respectively, Mann-Whitney U test). (B) CI was prepared from skin grafts recovered from TSC- or WT-grafted mice as well as from skin from TSC-transgenic mice (TSC-tg) at termination of the arthritis experiment (4 months after transplantation). Transgenic expression was detected as described in Fig. 1. As control, T cells were stimulated with the non-glycosylated (K) and the glycosylated (GalHyK) CII-peptide at a concentration of 32 ng/ml.

5, 10 and 19 weeks after immunization and the results are summarized in Table 1. TSC skin recipients had reduced anti-CII IgG levels compared to controls at both 5 and 10 weeks after immunization. Reduction in total anti-CII IgG levels correlated to a significant reduction of the antigen-specific IgG2a isotype, whereas the IgG1 isotype did not differ compared to control-grafted mice. In contrast, anti-CII IgG levels in sera collected from mice 19 weeks after immunization (Experiment 2) did not differ significantly between mice grafted with skin from WT or TSC transgenic mice. Interestingly, CII-primed TSC transgenic mice had significant anti-CII IgG antibody titers 5 weeks after immunization. These were comparable to those of TSC skin grafted recipients. However, when measured 10 weeks after immunization, antibody titers were reduced in TSC transgenic mice, while remaining at a similar or elevated level in TSC-grafted mice.

The most obvious explanation for a reduced anti-CII IgG response in TSC skin recipients would be that CII-specific T cells had become tolerized and therefore had

Table 1. Anti-CII antibody response in grafted mice 5, 10 and 19 weeks after immunization[a)]

		5 weeks post immunization			10 weeks post immunization			19 weeks post immunization[b)]		
Exp	Graft (*n*)	IgG	IgG1	IgG2a	IgG	IgG1	IgG2a	IgG	IgG1	IgG2a
1 + 2	WT (19)	424	28	175	478	15	151	501	10	68
	TSC (27)	133	8	18	168	4	19	314	10	58
		$p < 0.001$		$p < 0.001$	$p = 0.002$		$p < 0.001$			
2	None (6)[c)] (TSC-tg)	118	1	10	82	1	9	42	1	6

a) Mean total anti CII-IgG levels are calculated as μg/ml, using anti-CII polyclonal sera with a known antibody concentration as positive controls. Mean IgG subclass levels are calculated as arbitrary units/ml (U/ml), using anti-CII-specific monoclonal antibodies with a known concentration as positive controls.
b) Only including Exp 2; WT, *n*=7; TSC, *n*=10; TSC-tg, *n*=6.
c) TSC transgenic mice (TSC-tg, no graft) were immunized and monitored for arthritis development in parallel to grafted mice.

a reduced capability to supply B cell help for antibody production. To test this, grafted mice were immunized 4 weeks after transplantation and the *in vitro* response of lymph node cells to the non-glycosylated [with a non-modified lysine (K) at position 264] and the glycosylated [with a β-D-galactopyranose-5-L-lysine (GalHyK)] CII-peptide, was examined 10 days later. Proliferation as well as IFN-γ production was reduced against the non-glycosylated peptide in TSC skin recipients, whereas the response towards the glycopeptide did not differ significantly from WT grafted mice (Fig. 4). To further evaluate and compare T cell responses in TSC- and control-grafted mice, we used A^q-multimers loaded with the non-modified CII peptide (Aq+CIIp) to detect antigen-specific T cells. This multimer stains in an antigen-specific manner as it stained the non-glycosylated CII-specific HCQ.4 hybridoma but failed to stain T cell hybridomas HDBR.1 and HCQ.10, specific for the hydroxylated and glycosylated CII epitope, respectively, or the OVA-specific HOB.6 T cell hybridoma (Fig. 5a). Thus, using the multimer allowed us to determine the frequency of $CD4^+$ non-glycosylated CII-specific T cell that participated in the recall response in TSC skin grafted mice, irrespective of their cytokine profile. WT- and TSC skin grafted recipients were immunized with CII 4 weeks after transplantation and the recall response was investigated 10 days after immunization. Lymph node cells from both groups were stained with an anti-CD4 antibody, multimer (or a PE-conjugated control antibody) and an anti-N418 antibody (to exclude unspecific multimer binding by dendritic cells) and analyzed by flow cytometry (Fig. 5b). The number of $CD4^+$ antigen-specific T cells in draining lymph nodes was significantly reduced in TSC skin recipients compared to control grafted littermates (Fig. 5b, c). However, as differences appeared rather small between the groups, 0.7% compared to 1% for mice grafted with TSC- and WT-skin, respectively (Fig. 5c), an aliquot of the cells used in multimer analysis was also used for determination of the number of IFN-γ-producing cells upon CII restimulation *in vitro*. In agreement with earlier *in vitro* experiments (Fig. 4), the number of IFN-γ-producing cells was dramatically reduced for TSC skin-grafted mice, upon stimulation with the non-glycosylated CII peptide (Fig. 5d). As expected, since the

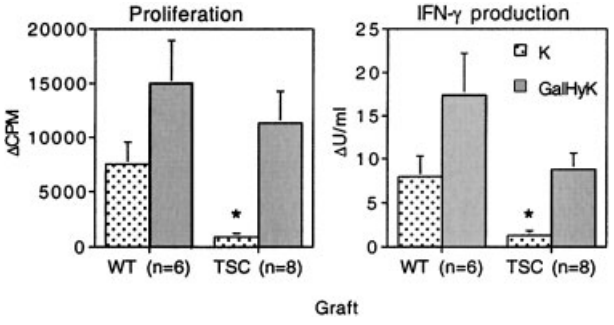


Fig. 4. Reduced recall response to the non-glycosylated CII peptide in TSC skin graft recipients. Mice were grafted with skin from either WT or TSC transgenic (TSC) mice. Four weeks later mice were immunized with CII in CFA. Ten days after immunization, lymph node cells were restimulated *in vitro* with the non-glycosylated (K) and the glycosylated (GalHyK) CII-peptide (50 μg/ml) for 4 days and the proliferation was measured (left). After restimulation, supernatants were collected for determination of IFN-γ content (right). Data are presented as mean ± SEM. (ΔCPM and ΔU/ml, response with antigen – response without antigen); *$p<0.05$ compared to WT-skin grafted mice, Mann Whitney U test.

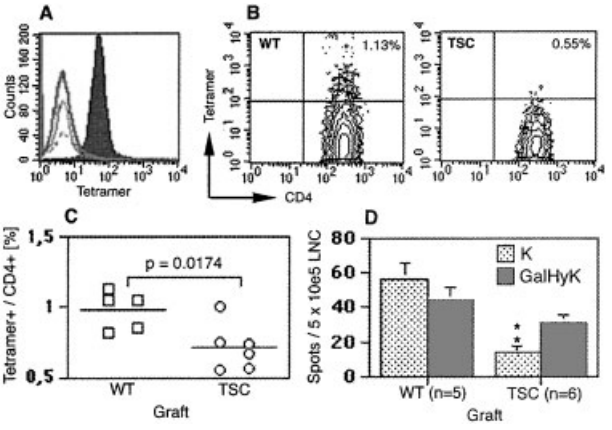


Fig. 5. Deficiency in mounting a T cell response to the non-glycosylated CII-peptide in TSC skin-grafted mice. Mice grafted 4 weeks earlier with either WT or TSC skin (TSC) were immunized with CII in CFA. Ten days later antigen-specific T cells from lymph nodes were enumerated using an A^q-multimer that presents the covalently bound non-glycosylated CII256–270 peptide and an IFN-γ ELISPOT assay as described in Sect. 4. (A) Control multimer stainings using T cell hybridomas with known specificities; HCQ.4 (filled peak), specific for the non-glycosylated CII peptide; HCQ.10 (thick line), specific for the glycosylated CII peptide; HDBR.1 (thin line), specific for the hydroxylated CII peptide; HOB.6 (hatched line), specific for an OVA peptide. Dead cells were excluded by gating out PI^+ cells. (B) Example of multimer staining of lymph node cells from immunized WT and TSC skin-grafted mice. Cells were gated on forward and side scatter and CD4. Numbers indicate the percentage of multimer-positive cells within the $CD4^+$ population. (C) Pooled results of multimer staining of $CD4^+$ T cells from WT or TSC skin-grafted mice (*p* value is calculated with Mann-Whitney U test). (D) An aliquot of lymph node cells used for multimer staining was also tested for IFN-γ production against CII peptides in an IFN-γ ELISPOT assay (data are presented as mean ± SEM. * *$p<0.01$, Mann-Whitney U test).

glycosylated CII epitope appeared to be absent in skin from adult TSC transgenic skin donors, the response towards the glycopeptide did not differ significantly in TSC skin recipients. Hence, the most likely explanation for the initial protection observed in the majority of TSC skin grafted mice is that T cells become tolerized to the grafted epitope. Subsequently, the reduced T cell response will influence the priming of CII-specific B cells, leading to an impaired B cell response as recipient are immunized with CII.

2.5 Loss of T cell tolerance to the transplanted epitope abolish arthritis protection but do not induce graft rejection

In addition to our finding that arthritis susceptibility of TSC skin recipients can be regained by a second boost immunization (Fig. 3a), we also observed that there was failure of initial protection in approximately 30% of the mice (Fig. 2). We wanted to explore if complete heeling of the graft before the time of immunization could have reduced the amount of available antigen for maintaining the induced tolerance. Accordingly, mice were grafted with TSC skin, but to ensure complete healing in all mice, recipients were immunized 8 weeks after transplantation and monitored for arthritis development. Indeed, TSC skin recipient mice developed arthritis to the same extent as control-grafted mice when immunized 8, instead of 4 weeks after transplantation (Fig. 6a). Furthermore, anti-CII IgG levels in sera collected 5 weeks after immunization were fully comparable to those of control-grafted mice (Fig. 6b). Again, control TSC-transgenic mice were resistant to CIA (data not shown) and had reduced, but still measurable, humoral responses to CII compared to skin-grafted mice (Fig. 6b).

To see if tolerance to the non-glycosylated epitope was still present in arthritic mice immunized 8 weeks after transplantation, the recall response was investigated at the end of the experiment. This was an interesting question, as TSC grafts were still accepted, although all mice were suffering from arthritis at this time. In contrast to the reduced *in vitro* response seen in TSC skin recipients immunized 4 weeks after transplantation (Fig. 4 and 5), arthritic TSC skin-grafted mice had regained their ability to respond to the non-glycosylated epitope (Fig. 6c), indicating that the transplanted epitope must be available until the time of immunization to achieve the initial arthritis protection. In addition, these results suggest that different mechanisms are involved in mediating graft rejection and the development of arthritis, as loss of T cell tolerance to the grafted epitope is strongly correlated to arthritis susceptibility but not to graft rejection.

One explanation for the failure of TSC skin recipients to reject their grafts could be that the transplanted epitope

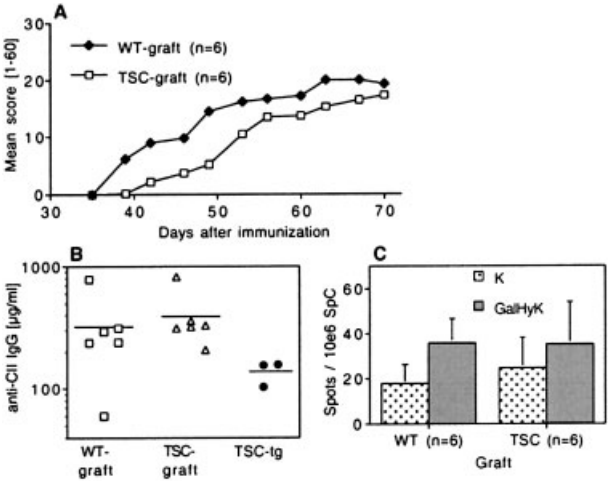


Fig. 6. Transient tolerance and CIA protection in TSC skin-grafted recipients. Mice were grafted with skin from WT (WT-graft) or TSC-transgenic mice (TSC-graft). Eight weeks later grafted mice and non-grafted TSC-transgenic mice (TSC-tg, *n*=3) were immunized with CII in CFA and again boosted 5 weeks later with CII in IFA and monitored for arthritis development. (A) Mean score of grafted mice. (B) Serum levels of anti-CII IgG-levels 5 weeks after immunization. Open symbols represent mice that developed arthritis during the experiment. Closed symbols represent healthy animals. Horizontal lines indicate mean values. For comparison with other experiments, mean titers were: 322 μg/ml for WT skin recipients, 393 μg/ml for TSC skin recipients and 138 μg/ml for non-grafted TSC-transgenic mice. (C) The number of antigen-specific T cells in the spleen of animals 10 weeks after immunization was determined in an IFN-γ ELISPOT assay (data represent mean ± SEM).

was not sufficiently presented in the healed graft. Alternatively, the available T cell repertoire that could respond to the epitope, with only one amino acid non-self change at position 266 within the CII256–270 epitope, may be too limited to mediate graft rejection. To explore these possibilities, we grafted arthritic mice with skin from WT or TSC transgenic mice (7 weeks after immunization with CII in adjuvant and 2 weeks after boost injection with CII in IFA). Even though mice were affected by severe arthritis at the time of transplantation, TSC skin grafts were accepted (not shown). We also grafted mice with TSC skin 10 days after priming with CII in adjuvant. Also here, TSC skin grafts healed as efficiently as control grafts (not shown) despite the fact that T cells specific for the non-modified CII-epitope are clearly present and activated in CII-primed mice at this time (see control-grafted mice in Fig. 4 and 5). Therefore, limited antigen disparity rather than restricted T cell accessibility of CII-specific T cells to the healed graft is likely to explain why arthritic TSC skin recipients fail to reject their grafts.

3 Discussion

The immune system is normally only partially tolerant to CII in cartilage — a finding that may be of fundamental importance for understanding development of autoimmune arthritis. To further investigate the nature of tolerance to CII and its importance for the development of arthritis, we have transplanted skin expressing the immunodominant T cell epitope integrated in CI. We found that the transplanted skin was not rejected, not even after CII immunization, and T cells specific for the grafted epitope were tolerized and mice were protected from arthritis. However, protection was transient and after complete healing of the graft, tolerance was lost and the mice were again susceptible to arthritis. The recovered arthritis susceptibility correlated with an increased T and B cell response to CII but development of arthritis did not cause graft rejection, although the CII epitope was still expressed. Hence, this study shows that T cell tolerance against an explicit CII epitope can be sufficient to protect the individual from arthritis development. However, to maintain tolerance and disease protection, the epitope needs to be constitutively available for the immune system.

The present investigation is based on a transgenic mouse strain, the TSC mouse, which has previously been investigated for arthritis susceptibility and immune responsiveness [11, 14]. TSC mice were completely resistant to CIA and had a dramatically reduced response to CII. Since this mouse has the CII260–270 epitope integrated into CI, which is expressed in all fibrous tissue from the birth, it was not possible to determine age-, time- and tissue-dependent effects of the tolerance. However, this was possible by transplanting TSC-derived skin. Coincidentally, we found that the epitope was only post-translationally modified during collagen synthesis in the newborn mice and not in adult life. This led to the situation that skin taken from adult TSC mice contained only the non-glycosylated epitope and not the immunodominant post-translational modified epitope. Consequently, only T cells specific for the non-glycosylated peptide and not the glycosylated peptide became tolerized. Furthermore, the biased tolerance to the non-glycosylated CII peptide induced by TSC skin transplantation was sufficient to protect the recipients from developing arthritis if primed within 4 weeks after transplantation. This was somewhat surprising, as results from earlier investigations suggested that the level of tolerance to the glycopeptide correlates positively to the degree of CIA protection [11, 14, 15]. In addition, glycosylated CII is more arthritogenic than non-glycosylated CII [9, 16], and adoptive transfer of glycopeptide-specific T cells into CII-immunized DBA/1 mice increases the incidence and severity of arthritis as well as the antibody response to CII [17]. As these findings implicate an important role for glycopeptide-specific T cells in the CIA model, it was unexpected that a reduced T cell response to the non-glycosylated epitope still had such prominent effects on the entire network of events that eventually lead to arthritis. In this report, we could make no conclusion on whether induction of tolerance was due to deletion or anergy of antigen-specific T cells, as both mechanisms will result in a failure to mount a significant recall response of such cells. Nevertheless, it is possible that the majority of T cells specific for the non-glycosylated epitope are related, leaving only a small population of anergic T cells behind, reducing the CII-reactive population sufficiently to prevent arthritis development [18]. Although glycopeptide-specific T cells were not significantly altered in their *in vitro* recall response, there was a tendency towards a reduced T cell response in TSC skin recipients. Theoretically, incomplete deletion of non-glycosylated peptide-specific T cells may leave a pool of antigen-experienced regulatory cells behind [19, 20], which are able to prevent arthritis despite the presence of a relatively large population of glycosylated CII-specific T cells.

Additional priming *in vivo* was able to break protection from CIA in TSC-graft recipients and the regained arthritis susceptibility was characterized by a recovered response to the non-glycosylated peptide, and yet transgenic skin grafts were not rejected. While no obvious cellular infiltration into the graft was observed in TSC skin recipients at this time, we speculated that failure to reject the graft could be due to the fact that activated T cells specific for the transplanted epitope had only limited access to the graft once it has healed. However, as priming of CII-specific T cells, prior to transplantation, did not elicit graft rejection, this explanation seems unlikely as activated T cells should in general have access to non-lymphoid tissue, and since transplantation of skin will induce local inflammation to attract such cells. We could not detect any significant antibody response in TSC-graft recipients before immunization and antibodies to CII did not bind transgenic CI (data not shown), suggesting that mutated CI does not contain a B cell epitope. This could possibly explain the lack of graft rejection in arthritic TSC skin recipients. However, B cell-deficient B6 mice can reject skin from BALB.B mice [21], and female mice expressing a transgenic TCR specific for the male H-Y antigen can reject male skin grafts independently of both B cells and CD8 T cells [22]. Moreover, a single and explicit CTL epitope may be sufficient for induction of late but spontaneous skin graft rejection [23]. Although CTL have been implicated in murine CIA [24–26], it has yet to be shown that the CII256–270 peptide is presented by MHC class I molecules or that it can

be recognized by T cells with cytotoxic functions. In fact, CD8-deficient mice mount an intact T cell response to both non-glycosylated and glycosylated CII256–270 peptide [27] and all examined CII256–270-specific T cells clones have been found to be $CD4^+$ (Bäcklund, unpublished observation). Furthermore, rapid rejection of skin grafts, displaying only minor antigen disparities, has been shown to depend completely upon the presence of $CD4^+$ cells [28]. However, antigenic disparity of TSC skin grafts is restricted to one single amino acid exchange and the target antigen in the graft is mainly extracellularly expressed. Hence, it is possible that antigenicity of the graft is too limited to elicit CD4-mediated graft rejection without the "help" of either graft-specific antibodies or cytotoxic T cells. However, antigenicity of mutated CI is nevertheless sufficient to mediate tolerance in antigen-specific T cells and initial protection from arthritis induction. That breaking of tolerance against a self-expressed antigen does not routinely lead to the breakout of autoimmunity has been shown in a double transgenic animal model for $CD8^+$ cells [29]. Instead, the microenvironment in the target tissue influences the exposure of the autoantigen and decides whether activation of autoreactive T cells will result in organ-specific autoimmunity [29]. Anti-CII antibody production is T cell dependent and humoral responses to CII are crucial in development of CIA. Therefore, even if antibodies are not central for rejection of skin grafts, CII-specific antibodies directed to joint cartilage probably explain why the reversed tolerance against the non-glycosylated peptide resulted in an exclusive immune attack against the joint.

How then can one explain the failure of disease protection in mice immunized 8 weeks after transplantation? Persistence of antigen has been shown to be required to maintain established tolerance *in vivo* [30–33] and also recently in an autoimmune animal model [34]. It is quite easy to envision that CII-specific T cells will encounter the CII256–270 epitope following transplantation of TSC skin. The surgical procedure of transplantation and the healing involved will induce an inflammatory response and the formation of new tissue. Inflammation will lead to recruitment of inflammatory leukocytes to the site of transplantation and activation of APC resident in the skin. Healing will involve activation and proliferation of both host and donor fibroblasts, which are the main producers of CI, and thus form deposits of extracellular CI, which enable attachment of the graft to the host. In this situation it is likely that mutated CI will become available for APC that process and present it to antigen-specific T cells. Once the graft has healed, however, availability of mutated CI for continuous tolerogenic presentation might decrease as *de novo* synthesis of collagen by fibroblasts would eventually decline and the turnover rate of CI should be low at the transplantation site. Naive T cells have been shown to have limited access to antigens expressed in the epidermis of adult, but not of neonatal, tissue [35]. However, as CI is expressed in the dermis, it could be argued that the grafted epitope would be readily accessible for immune recognition even after the complete healing of the graft. It is also possible that the graft will allow donor fibroblast to migrate out of the graft and into the host, as has been reported for donor leukocytes [36], and there temporarily continue to produce mutated CI. One logical explanation for the ignorance of the transplanted epitope, under physiological conditions once the graft has healed, is the fact that this particular epitope can not, or only poorly, be presented by dendritic cells [37]. It is clear that mutated CI is available for T cell recognition in grafted hosts during the first weeks after transplantation, as the antigen-specific T cell response is modified 4 weeks after transplantation and recipients are protected from arthritis. In contrast, when immunized 8 weeks after transplantation, TSC skin recipients develop severe arthritis, with a B cell response and a non-glycosylated CII-specific T cell response of the same magnitude as control-grafted mice. Hence, it is likely that the period between 4 and 8 weeks after transplantation constitutes a critical time window, where tolerance is lost due to reduced or ceased availability of the grafted epitope owing to complete healing.

4 Materials and methods

4.1 Mice

All experiments were performed in C3H.Q male mice ($H\text{-}2^q$), originally from Dr. D. C. Shreffler, Washington University, St. Louis. The TSC transgenic mouse has been described earlier [14]. Briefly, TSC mice express the CII256–270 rat sequence on CI, which is "systemically" expressed in fibrous connective tissues, *e.g.* in dermis. The transgene was founded in the C3H.Q mouse and subsequently bred as a heterozygote in the same strain. All animals were bred and kept in our animal facility (http://net.inflam.lu.se/).

4.2 Antigen

Rat CII was prepared from the SWARM chondrosarcoma by pepsin digestion [38] and further purified as described earlier [39]. The peptides were synthesized as previously described [8] and the glycosylated CII256–270 peptide contained a β-D-galactopyranose residue on L-hydroxylysine at position 264 [40]. Both collagen and collagen peptides were dissolved and stored in 0.1 M acetic acid. Crude preparation of type I collagen from skin grafts was performed by pepsin digestion as above, followed by pepsin inactivation but with no further purification.

4.3 Immunization

For arthritis experiments, mice were immunized intradermally in the tailbase with 100 μg rat CII emulsified 1:1 in complete Freund's adjuvant (CFA; Difco, Detroit). Five weeks later they were boosted with 50 μg rat CII emulsified 1:1 in incomplete Freund's adjuvant (IFA; Difco). For long-term arthritis experiments, mice were also given a second boost injection 10 weeks after the first immunization. For *in vitro* experiments, mice were immunized in the hind footpads with 50 μg of rat CII in CFA in each foot.

4.4 Skin grafting

At 4 or 8 weeks prior to immunization, mice were engrafted with (2–3 cm^2) skin from either adult TSC transgenic mice or age-matched littermate controls onto the back of non-transgenic recipients. The grafts were covered with gauze that was removed 1 week later. Skin grafting was performed under anesthesia of chloral hydrate and barbiturate. Graft survival was followed visually during the experiment and at the end of experiments the grafts were removed and used for transgenic CI preparation, as described above, to ensure graft acceptance.

4.5 Arthritis development

Development of clinical arthritis was followed through visual scoring of the mice starting 2 weeks after immunization and continuing until the end of the experiment. Arthritis was evaluated using an extended scoring protocol [41] ranging from 1 to 15 for each paw with a maximum score of 60 per mouse. Each arthritic toe and knuckle was scored as 1, with a maximum of 10 per paw. An arthritic ankle or midpaw were given a score of 5.

4.6 CII antibody response

For arthritis experiments, blood samples were taken at the time of boost immunization(s) as well as at the termination of experiment for analyzes of CII antibody response. The amounts of total anti-CII IgG as well as the CII-specific IgG1 and IgG2a isotypes were determined through quantitative ELISA [42].

4.7 Lymphocyte assays

Ten days after immunization cells from draining lymph nodes were prepared and restimulated *in vitro* for determination of antigen-specific proliferation and IFN-γ production as described [2, 15]. In some experiments, the number of antigen-specific cell in spleen or lymphnodes was determined by an IFN-γ ELISPOT assay as described by Heeger et al. [43] using ImmunoSpot M200 plates (Cellular Technology, Cleveland, OH).

T cell hybridoma HCQ.4 and HCQ.10, specific for CII256–270 without post-translational modification and for CII256–270 with glucose on hydroxylysine at position 264, respectively, were used to detect transgenic type I collagen, prepared from naive TSC mice or recipients grafts at the end of arthritis experiments, as described earlier [9].

4.8 MHC class II + peptide multimers

Soluble recombinant I-A^q molecules with CII256–270 covalently tethered to the I-$A\beta^q$ chain were made using essentially the same methods as described previously [44]. Recombinant protein was purified from HIGH-FIVE™ cells infected with recombinant virus stock using the methods of Radu et al. [44]. Following purification, protein was biotinylated using biotin ligase (Avidity, Denver, CO). Multimeric complexes were formed by incubating the biotinylated I-A^q molecules with PE-labeled streptavidin (PharMingen, San Diego, CA) at a molar ratio of 4:1 for 10 min on ice. Bulk lymph node cells were incubated with multimer at 37°C for 30 min. An antibody specific for the CD3ε chain (145–2C11, from our hybridoma collection) was added together with the multimer since this has been shown to improve multimer staining [45], this was also confirmed by us (data not shown). To reduce unspecific multimer staining cells were incubated on ice prior to multimer incubation with RGD-peptide (Life Technologies, Gaithersburg, MD) at 10 μg/5×10^5 cells for 15 min (to inhibit integrin-dependent unspecific staining) and then with purified anti-FcγRII/III antibody for 5 min (24.G2, from our hybridoma collection). Cells were then washed and stained with anti-CD4 antibody (cytochrome-conjugated H129.19, PharMingen) and anti-CD11c antibody (FITC-conjugated N418, from our hybridoma collection) before analyzed by flow cytometry (FACScan; Becton Dickinson, Mountain View, CA). For each analyzes 50,000 $CD4^+$ events were collected. Multimer staining of T cell hybridoma clones was performed similarly to lymph node cells as above, altough RGD-peptide was not used, dead cells were excluded using propidium iodide (PI) and only 10,000 events were collected.

4.9 Statistics

Frequency of arthritis was analyzed by the χ^2 test, while antibody levels, multimer staining, *in vitro* responses of lymphocytes and other analyses of severity and onset of arthritis were analyzed with Mann-Whitney U test.

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