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In primed allo-tolerance, TIM-3-Ig rapidly suppresses TGF β , but has no immediate effect on Foxp3

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Summary

T-cell immunoglobulin mucin-3 (TIM-3) is only expressed by differentiated TH1 cells following their proliferative response to antigen, functioning to terminate TH1-mediated immunity upon binding to the TIM-3 ligand, galectin-9. This critical regulatory process involves Treg cells *via* their stable expression of galectin-9. Soluble TIM-3-Ig blocks galectin-9 and prevents induction of peripheral tolerance. Here we have looked for evidence that TIM-3-Ig might also break established regulatory tolerance. Using allo-primed spleen cells cultured *ex vivo* and challenged with irradiated donor-type stimulator cells either alone or together with 20 μ g/ml TIM-3-Ig, we measured daily cytokine release [IL2, interferon gamma (INF γ), transforming growth factor beta (TGF β), IL6, IL10] and cellular Foxp3 protein. In allo-tolerance, a specific effect of TIM-3-Ig was some fourfold reduction in TGF β . Foxp3 was induced in the allo-tolerant response to donor and this was not altered by TIM-3-Ig over the 5-day culture period. No Foxp3 was detected in either rejected or donor stimulator cells at any time. Thus, in an *ex vivo* model of *in vivo* tolerance to heart allografts, TIM-3-Ig therapy appears to reduce the stable tolerogenic environment by a rapid and specific repression of TGF β release.

Keywords

Foxp3; TGF β ; TIM-3

Introduction

Homeostasis in biological systems is mediated by feedback control mechanisms that allow biological functions to be up- or down-regulated according to need. In the immune system, homeostasis can accommodate many 1000-fold amplification of specific lymphocyte subsets because mechanisms are in place for subsequent deletion of redundant cells once their antigenic target has been cleared. The T-cell immunoglobulin mucin (TIM) receptors are involved in the regulation of immune responses and TIM-3 functions in the clearance of redundant TH1 cells, acting as a suicide tag expressed on the surface of differentiated TH1 cells following their proliferative response to antigen [1–3]. The delivery of the apoptotic signal to the TIM-3+ ve T cell is mediated by the lectin, galectin-9 [4]. In the immune

system galectin-9 is constitutively expressed on Treg and on certain antigen presenting cells (APC), creating a microenvironment continuously sensing and targeting redundant TH1 cells for death by apoptosis.

Galectin-9 is a developmentally regulated β -galactoside-binding mammalian lectin [5,6]. The galectin-9 protein has a conserved carbohydrate recognition domain of around 130 amino acids that confers strict carbohydrate recognition [7] and, in the case of TH1 cells, galectin-9 interacts with carbohydrates covalently attached to the surface of TIM-3 [4]. The blockade of galectin-9 results in failure to clear redundant TH1 cells. Such blockade can be achieved by soluble TIM-3, such as the genetically engineered TIM-3-Ig [4]. Soluble TIM-3-Ig interferes with homeostatic mechanisms required for induction of self-tolerant Treg cells, with perturbation of the cytokine milieu and skewing towards an aggressive immune signalling environment [8]. Notably, TH17 cells express only low levels of TIM-3 [9] and are thus relatively resistant to clearance by galectin-9, and – given the link between TH17 and chronic autoimmune conditions – this illustrates how differential sensitivity to the TIM-3/galectin-9 regulatory mechanism may be associated with autoimmune diseases such as multiple sclerosis.

Here we asked, what happens to an established state of *in vivo*-primed allo-tolerance when galectin-9 is blocked by TIM-3-Ig. Using the *in vivo/ex vivo* model, which provides a tightly controlled means to probe for phenotype-linked requirements for the TIM-3/galectin-9 pathway, we discovered that TIM-3-Ig suppresses allo-tolerance at the level of TGF β .

Materials and methods

Generation of *BALB/c-primed* CBA mice

All mice used were male and were obtained from Harlan UK. CBA mice (H2^k) of 10–12 weeks of age received a fully mismatched, vascularized BALB/c (H2^d) heart graft to the neck. Tolerance was generated by a 21-day course of alternate day therapy using blocking monoclonal antibodies to CD4 and CD8 as previously described [10]; after 40 day, levels of therapeutic antibody in the peripheral circulation become undetectable. *BALB/c-tolerant* CBA spleen cells from tolerant recipients were isolated at least 100 day after grafting for *ex vivo* analyses. For comparison, untreated CBA mice were grafted with a BALB/c heart which rejected on day 7. The *BALB/c-rejected* CBA spleen cells were collected at 14 day for *ex vivo* analyses. All procedures were carried out according to Home Office licence under the Animals (Scientific Procedures) Act 1986, UK.

Ex vivo model

The *ex vivo* model uses whole spleen cell populations to mimic the *in vivo* state of transplantable tolerance. There are important differences between the *ex vivo* model and a conventional mixed lymphocyte response (MLR) as the responder 'tolerant' and 'rejector' spleen populations are each derived from mice primed *in vivo* against donor antigen, characterized by second order response kinetics against donor including polarized gene expression of Foxp3 in tolerance, and of IFN γ in rejection.

Cell culture

Responder spleen cells were obtained from either *BALB/c-tolerant* CBA or *BALB/c-rejected* CBA, mice. Each culture flask contained 4×10^7 responder cells (sufficient to adoptively transfer donor-specific tolerance *in vivo*) and 6×10^7 irradiated (20Gy) donor-type stimulators in a total of 10 ml serum-free growth medium (GM, Iscoves + 100 U/ml each of penicillin and streptomycin). Irradiated spleen cells do not contribute to the cytokine milieu as their DNA is inactivated by cross-linking. After 120 h, 500 μ l of GM containing a further 6×10^7

irradiated donor-type stimulator cells was added to each flask to re-boost the immune response. Replicate flasks were removed at 0, 48, 120 (immediately after re-boosting) and 123 h. A 500- μ l sample of culture supernatant was collected daily, spun free of cells and stored at -80°C . Two parallel series of flasks were set up, one without additions, the second receiving 20 $\mu\text{g}/\text{ml}$ TIM-3-Ig at time zero, this dose being known to increase aggressive immunity [8].

Cell lysis

At harvest, cells were held on ice, with any adherent cells being included following brief treatment with 0.25% trypsin. After washing in ice cold 0.1% BSA/PBS, the cells were separated into cytoplasmic, nuclear and DNA fractions using standard methods. All fractions were stored at -80°C .

ELISA

Culture supernatant was analysed for cytokines using ELISA DuoSet kits from R&D Systems as follows: IFN- γ (DY485), IL-2 (DY402), IL-4 (DY404), IL6 (DY406), IL-10 (DY417), TGF- β (DY240). All samples were measured in triplicate and quantified against external standards as per the manufacturer's instructions. The spread of results between replicates was less than 5%. The same supernatant was assayed for each of the cytokines measured allowing specific effects on a given cytokine to be detected. All cytokine measurements are given in picogram/millilitre.

Western blot

Rabbit anti-Foxp3 serum was kindly donated by Dr Fred Ramsdell (Celltech). Anti-actin (MAB1501) was from Chemicon International; goat polyclonal anti-STAT4 was from R&D (PAF-St4). The Pharmacia Phast system was used for SDS-PAGE and Western blotting of lysates using standard procedures. The optical density (O.D.) of each protein was measured and compared to the O.D of the actin signal in the same sample lane.

Results

Treatment with TIM-3-Ig inhibits TGF β release in allo-tolerance

In allo-tolerance, there was a marked reduction in TGF β in the presence of TIM-3-Ig, as shown in Table 1 and Fig. 1. Active TGF β began to increase by 96 h with untreated controls reaching 400 pg/ml, but falling to below 100 pg/ml in the presence of TIM-3-Ig. As TIM-3-Ig-treated cultures showed sustained low TGF β levels, the effect was not because of delayed release kinetics. Importantly, the suppression of TGF β by TIM-3-Ig was highly selective in that release of other cytokines including IL6, IL2, IL10 and IFN γ was not suppressed. This implies a direct relationship between TGF β release and the TIM-3/galectin-9 pathway, rather than some secondary effect upon TGF β . In allo-rejection, significant levels of TGF β were detected by 96 h but these were fivefold less than in allo-tolerance and in accord with the known positive correlation between TGF β and tolerance. The presence of TIM-3-Ig further reduced TGF β levels roughly twofold in rejection.

The hallmark of the primed *ex vivo* allo-tolerance versus allo-rejection model is a massive release of IFN γ specific for rejection, as found in the current experiment (Table 1). Notably, the presence of TIM-3-Ig had no effect on IFN γ in either rejection or tolerance, further supporting the notion of a specific and primary relationship between TGF β and the TIM-3/galectin-9 pathway.

TIM-3-Ig does not prevent the rapid Foxp3 response to donor-stimulation

We asked if TIM-3-Ig alters the rapid, allo-primed Foxp3 response and found that it did not (Fig. 2a,b). STAT4 protein was present in both tolerance and rejection, emphasizing the specificity of Foxp3 for tolerance in our *in vivo/ex vivo* model. Thus, although the tolerogenic micro-environment had been reduced by TIM-3-Ig, the primed epigenetic programme for Foxp3 expression in response to donor was not blocked within the 5-day experimental period. Given the rapid Foxp3 recall response, with Foxp3 protein expression being detected within 48 h and prior to detectable release of TGF β , we anticipate that any effect of TIM-3-Ig upon Foxp3 gene expression would require more prolonged treatment of allo-activated cells.

Discussion

We explored the potential role of galectin-9 in the maintenance of immune tolerance by asking, does blockade of galectin-9 perturb an established state of regulatory allo-tolerance? Our finding of a requirement for galectin-9 in the normal maintenance of TGF β levels within the responsive tolerogenic micro-environment is compatible with a model wherein the complex immune response repertoire associated with allo-tolerance is being continuously sensed and reinforced by mechanisms that include galectin-9-linked pathways to regulate TGF β and to maintain the stable tolerogenic micro-environment.

We hypothesize that the naive T cell, when activated by cognate antigen, enters a transition state of 'arrested differentiation' before developing further to a fully differentiated TH1, TH2 or TH17 phenotype. We argue that such an arrested state of differentiation is likely to be associated with a characteristic gene expression profile and we have already demonstrated a link between the stem cell-related gene axotrophin/MARCH-7 both to the expression of Foxp3 and to the regulation of T-cell-derived leukaemia inhibitory factor (LIF) [11,12]. LIF is a pleiotrophic cytokine with a key role in the regulation of stem cells, preventing their differentiation whilst supporting their proliferation. Our hypothesis proposes that the LIF/axotrophin axis regulates Treg cells, maintaining proliferation and the tolerogenic micro-environment that in turn prevents their differentiation [11,13]. As TGF β is a tolerogenic cytokine, the potential role of TGF β in our arrested differentiation model is the subject of further studies.

We previously demonstrated that allo-tolerance must be actively and continuously maintained *in vivo* as absence of donor antigen results in loss of tolerance beyond 7 days [10]. Within the context of the arrested differentiation hypothesis, we argue that donor antigen-driven pathways must be required to ensure maintenance of the tolerogenic micro-environment, actively preventing full differentiation of TH1-type allo-aggressive cells. The finding that TIM-3-Ig reduces the tolerogenic cytokine milieu by lowering TGF β release indicates that galectin-9 is somehow involved. The discovery that a robust form of CD4+ cell-dependent allo-tolerance can be at least partially broken by interception of the TIM-3/galectin-9 pathway identifies a novel approach to modulating antigen-specific tolerance that has relevance to disease states where immune tolerance may protect against immune-mediated attack against a pathogen or cancerous cell.

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TGF β secretion in *ex vivo* allo-tolerance and allo-rejection spleen cell cultures with or without TIM-3-Ig

Figure 1.

TGF β release in *ex vivo* allo-tolerance and allo-rejection spleen cell cultures plus or minus TIM-3-Ig. Levels of TGF β secretion in supernatant samples from spleen cell cultures of (*BALB/c-tolerant*CBA)-responders against BALB/c stimulators (tolerance response) and (*BALB/c-rejected*CBA)-responders against BALB/c stimulators (rejection response) with or without TIM-3-Ig (20 ng/ml) assayed by ELISA. At each time point, the sequence of data is as follows: rejection; rejection plus TIM-3-Ig; tolerance; tolerance plus TIM-3-Ig.

Figure 2.

Foxp3 protein expression by allo-tolerant spleen cells is not immediately blocked by TIM-3-Ig. *In vivo* primed allo-tolerant CBA spleen cells were challenged with donor-type irradiated BALB/c spleen cells at time 0 h, with a second boost at 120 h. (a) Western blots shows a Foxp3 band obtained from protein extracted from the DNA pellets. Each lane is of samples taken at 0, 48, 120 (immediately after addition of booster donor antigen) and 123 h. The GM (controls) sample set is of cultures grown in growth medium; the GM + TIM-3-Ig sample set is identical to the controls with the exception of addition of 20 µg/ml TIM-3-Ig to the growth medium at time 0 h. Track at 0 h is the same for with and without TIM-3-Ig. (b) Normalization of Foxp3 protein to cytoplasmic actin confirmed that TIM-3-Ig did not prevent Foxp3 protein expression over the 5-day experimental period. For each time point the histogram sequence is as follows: rejection (baseline); rejection + Tim-3-Ig (baseline); tolerance (solid); tolerance + TIM-3-Ig (cross-hatched).

Table 1

Allo-tolerance versus allo-rejection: effect of TIM-3-Ig on soluble cytokine release (pg/ml).

Time (h)	TGFB		IL6		IL2		TIM-3-Ig		IL10		IFN γ	
	Control	TIM-3-Ig	Control	TIM-3-Ig	Control	TIM-3-Ig	Control	TIM-3-Ig	Control	TIM-3-Ig	Control	TIM-3-Ig
Tolerance												
0	0	0	0	0	0	0	0	0	0	0	0	0
24	0	0	41	43	22	18	11	32	0	0	0	0
48	0	0	91	97	88	67	41	55	19	16	16	16
72	0	0	110	146	156	152	48	63	69	62	62	62
96	457	92	174	211	228	221	71	72	169	152	152	152
120	374	156	197	227	247	223	83	91	298	314	314	314
123	521	227	145	230	143	150	65	62	152	215	215	215
Rejection												
0	3	0	2	3	2	0	6	5	0	314	0	314
24	19	5	57	61	21	24	61	84	314	838	314	838
48	16	02	69	80	47	45	125	149	4033	3614	4033	3614
72	21	15	106	79	46	57	199	194	8486	7124	8486	7124
96	81	34	106	69	28	34	176	174	8486	9533	8486	9533
120	73	42	80	69	30	32	176	198	12571	10214	12571	10214
123	89	51	159	81	31	35	209	227	16290	12257	16290	12257