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Research paper

A minimum number of autoimmune T cells to induce autoimmunity?

Angela J.T. Bosch [a], Beatrice Bolinger [a], Simone Keck [a,1], Ondrej Stepanek [a,c], Aleksandra J. Ozga [b,2], Virginie Galati-Fournier [a,1], Jens V. Stein [b], Ed Palmer [a,*]

[a] Departments of Biomedicine and Nephrology, University Hospital Basel and University of Basel, 4031 Basel, Switzerland
[b] Theodor Kocher Institute, University of Bern, 3012 Bern, Switzerland
[c] Institute of Molecular Genetics, Czech Academy of Sciences, 142 20 Prague, Czech Republic

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ABSTRACT

While autoimmune T cells are present in most individuals, only a minority of the population suffers from an autoimmune disease. To better appreciate the limits of T cell tolerance, we carried out experiments to determine how many autoimmune T cells are required to initiate an experimental autoimmune disease. Variable numbers of autoimmune OT-I T cells were transferred into RIP-OVA mice, which were injected with antigen-loaded DCs in a single footpad; this restricted T cell priming to a few OT-I T cells that are present in the draining popliteal lymph node. Using selective plane illumination microscopy (SPIM) we counted the number of OT-I T cells present in the popliteal lymph node at the time of priming. Analysis of our data suggests that a single autoimmune T cell cannot induce an experimental autoimmune disease, but a "quorum" of 2–5 autoimmune T cells clearly has this capacity.

1. Introduction

T cell tolerance first develops in the thymus where emerging T lymphocytes undergo negative selection. Negative selection is critical to eliminate the majority of self-reactive T cells allowing the individual to develop a self-tolerant T cell repertoire. Despite its efficiency, T cell tolerance is not perfect; a small number of T cells with the potential to cause an autoimmune disease escape negative selection and are found in the periphery [1–4]. T cells escaping central deletion as well as peripheral tolerance mechanisms are capable of initiating autoimmunity after infection with recombinant *Listeria monocytogenes* expressing a low-affinity (below-threshold) antigen; these cells even have the ability to respond to self-antigen during bystander infection [5]. Furthermore, it was shown that increased frequency of such lower affinity, self-reactive T cells correlates with augmented susceptibility to develop autoimmune disease [6,7]. Recently, using RIP-OVA mice expressing OVA variants with lower affinity for the OT-I TCR, we reported that T cells expressing a T cell receptor (TCR) just above the affinity threshold for negative selection have the highest potential to mediate an autoimmune disease [8]. During their development, these T cells have an increased risk of avoiding clonal deletion, but still have sufficient self-reactivity to initiate an autoimmune disease when sufficiently primed.

To compensate for the inefficiency of clonal deletion, self-reactive T cells are additionally removed in the periphery by immature dendritic and stromal cells in the lymph node presenting self-antigen derived from the tissues [9,10]. This form of peripheral deletion is triggered by immature and non-professional antigen presenting cells (APCs), which present self-antigens and express low levels of co-stimulatory molecules in the absence of inflammation. Furthermore, regulatory T cells specific for self-antigens belonging to the normal T cell repertoire, provide supplementary control of self-reactive T cells [11]. Despite these tolerance mechanisms, T cell-mediated autoimmune diseases such as type 1-diabetes, multiple sclerosis and rheumatoid arthritis are present in the human population. This suggests that central/peripheral tolerance is defective in affected individuals or that a small number of high affinity, self-reactive T cells, present in most individuals, can occasionally initiate an autoimmune disease.

Whether a single, high affinity, self-reactive T cell is able to initiate autoimmunity is not known. However, using two different techniques for cell fate mapping to study the individual response of naïve OT-I T cells, two different groups independently exhibited that CD8 T cells do not respond homogenously. Most single cells produced small numbers of descendants on day 12 after infection with LM-OVA; these are the so-called 'Dwarfs'. Interestingly, very

* Corresponding author.
E-mail address: ed.palmer@unibas.ch (E. Palmer).
[1] Current address: Department Pediatric Surgery, University Children's Hospital Basel, Spitalstrasse 33, CH-4031 Basel, Switzerland.
[2] Current address: Division of Rheumatology, Allergy and Immunology, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA.

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few of the single cells expanded immensely, generating up to 70,000 descendants per spleen; these are the so-called 'Giants'. Taken together, the data suggested that only activation of ≥50 naïve monoclonal CD8 T cells reliably induce a robust CD8 T cell response in the context of infection with *L. monocytogenes* because of the existing stochastic processes [12,13].

Intriguingly, one of the groups has further examined the number of foreign-reactive T cells required to initiate a robust T cell response [14]. Using single cell adoptive transfer they found that progeny of 28% of the transferred T cells could be recovered after they were activated with *L. monocytogenes* expressing their cognate antigen. In the cases where the responding cells could be recovered, immune responses derived from a single cell protected immunodeficient animals against a lethal *L. monocytogenes* infection.

In contrast, Butler et al. [15] using a mathematical approach suggested that reliable T cell responses require a finite (i.e. a quorum) number of antigen specific T cell precursors. They propose that if the host contains fewer than the "quorum number" of antigen specific T cell precursors, then it cannot generate a productive T cell response. The authors argue that for every self-antigen, a small number of self-reactive T cells will inevitably escape central and peripheral tolerance mechanisms. As long as the number of escaping self-reactive T cells for a given self-antigen is below the "quorum number", the chance of an autoimmune T cell response is unlikely. Assuming that similar principles apply to foreign-reactive T cells, then the generation of a functional T cell response requires a population of T cell precursors that is above the "quorum number". This is likely to be the case for foreign-reactive T cells, since their numbers are not reduced by negative selection. Taken together, it's more likely the collective decisions made by T cell populations, rather than the responses of an individual T cell that determine biological outcomes. We found the quorum idea to be appealing and decided to test it experimentally.

Here, we specifically addressed the question of how many autoreactive T cells are required to induce an experimental autoimmune disease. We started with an established model, where limiting numbers of self-reactive OT-I T cells are transferred into RIP-OVA mice, which express the cognate antigen, ovalbumin (OVA) in several tissues including pancreatic β cells. Following priming with cognate antigen, a high frequency of mice develops diabetes. We modified this model in the following way. Limited numbers of OT-I T cells were transferred into RIP-OVA mice; these host mice were immunized with antigen-loaded DCs into a single footpad to restrict T cells priming to a single draining (popliteal) LN; the number of autoimmune OT-Is present in the popliteal LN at the time of priming (24 h after transfer) was determined using selective plane illumination microscopy (SPIM). Furthermore, injection of αCD62L-antibody at the time of priming, avoided further influx of circulating OT-I and other T cells into the draining popliteal LN. By using this modified model we were able to determine the minimum number of autoimmune T cells required to induce this experimental disease. Together with a mathematical approach, our results strongly support the idea that a quorum of at least 2–5 OT-I T cells within a single draining LN must respond to initiate experimental autoimmune diabetes in this model.

2. Materials and methods

2.1. Mice

RIP-sOVA mice, OT-I TCR transgenic mice recognizing $K^b/OVA_{257-264}$, OT-II TCR transgenic mice recognizing $I\text{-}A^b/OVA_{323-339}$, TCR327 transgenic mice recognizing D^b/LCMV gp33-41 and B3K508 T cell receptor transgenic mice recognizing the $I\text{-}A^b$/3K were bred in our facility. UBC-GFP mice were kindly provided by R. Skoda (University of Basel) and crossed to OT-I TCR transgenic mice. Experiments were carried out in accordance with the federal and cantonal laws of Switzerland.

2.2. Adoptive cell transfer and footpad immunization

Mice were i.v. injected with single-cell suspensions of OT-I T cells. T cell counting was performed using AccuCheck counting beads (Invitrogen) as described perviously [16]. The following day BM-derived DCs were stimulated and loaded with LPS 1 μg/ml (Sigma) and 10^{-7} M $OVA_{257-264}$ peptide, 10^{-7} M $OVA_{323-339}$ peptide, 10^{-7} M 3 K peptide or 10^{-6} M gp33 peptide for 90 min, washed and injected s.c. into the footpad; this was followed by i.v. injection of anti-CD62L mAb (Bio X cell).

2.3. Generation of BM-derived DC

BMDC were prepared from tibias and femurs after removing the bone marrow cells by flushing the bones with RPMI medium (Gibco). Cells were counted and cultured in cell culture dishes ($2x10^6$/plate) in complete medium supplemented with GM-CSF for 10 days. Medium was changed on day 3, 6 and 8. DCs were harvested using 0.25% Trypsin-EDTA.

2.4. Urine glucose measurements

Urine glucose was controlled using test strips (Diabur-test 5000; Accu-chek). Mice with urine glucose levels >1,000 mg/dl for 2 consecutive measurements were considered diabetic.

2.5. Flow cytometry

Single-cell suspensions were generated from LNs and cells were incubated with the indicated mAbs at 4 °C for 20 min. Cells were analyzed by flow cytometry using a BD Canto II or Fortessa flow cytometer and FlowJo software (TreeStar), gating on viable leukocytes using the live/dead fixable near-infrared dead cell stain kit from Invitrogen (Life Technologies).

2.6. Antibodies

Brilliant violet (BV) 510 labeled anti-mouse CD8 (53-6.7), BV650 labeled anti-mouse CD45.1 (A20), BV711 labeled anti-mouse CD3 (17A2), Alexa-700 labeled anti-mouse CD25 (PC61) and PerCPCy5.5 labeled anti-mouse CD44 (IM7) were purchased from Biolegend. PECy7 labeled anti-mouse CD8β (H35-17.2) was purchased from ebioscience. Fluorescin (FITC) labeled anti-mouse CD4 (RM4.5), FITC labeled anti-mouse CD19 (ID3), FITC labeled anti-mouse CD11b (M1/70), FITC labeled anti-mouse NK1.1 (PK136) and allophycocyanin (APC) labeled anti-mouse CD45.2 (104) were purchased from BD.

2.7. Adoptive transfer of FACS sorted OT-I T cells and immunization

Defined numbers (1, 10, 500 or 1000) of OT-I T cells were sorted on a BD Aria III sorter as follows. Single-cell suspensions of OT-I T cells were labeled with CD4, CD19, CD11b, NK1.1 and CD44 and sorted for living cells, negative for all markers listed above. Sorting was performed directly into 96 well plates prefilled with 400,000 "carrier" cells isolated from LNs of RIP-sOVA (OVA tolerant) mice. Sorted OT-Is plus carrier cells were immediately adoptively transferred into RIP-OVA recipient mice, followed by injection of BM-derived DCs into the tail vein; just before injection, these DCs had been stimulated with LPS 1 μg/ml (Sigma) and loaded with 10^{-7} M $OVA_{257-264}$ peptide for 90 min.