

Design and methods

Antibodies

Primary antibodies: Details of the antibodies used as primary reagents for immunostaining germinal center T cells are given in Table 1. The anti-SAP antibody used in this study was a commercial polyclonal reagent. A monoclonal antibody of the same specificity (anti-SH2D1A, Cell Signaling Technology, Inc, Boston, MA, Cat. No. 2805) was tested on tonsil sections and a limited number of diffuse large B cell lymphoma cases, and showed the same reactivity pattern as the polyclonal antibody.

The anti-PD-1 antibody was a monoclonal reagent produced following immunization of a BALB/c mouse with the natural killer cell line YT (DSMZ, Braunschweig, Germany). Splenic cells were fused with a mouse myeloma cell line as previously described (1). A hybridoma secreting an antibody designated NAT, that gave selective immunolabeling of T cells in germinal centers, was chosen for cloning and further study. The antibody is of IgG1 subtype and was used as undiluted tissue culture supernatant. Western blotting against protein lysates from the YT cell line showed that it reacted with a molecule of approximately 47 kDa molecular weight (Figure 5 in the main text). This is similar to the molecular weight reported for the PD-1 molecule, and the immunohistologic and flow cytometric reactivity of antibody NAT was also similar to that observed with a known anti-PD-1 antibody (data not shown).

To confirm that the monoclonal antibody NAT recognized human PD-1 protein, a pCMV6-XL5-PD-1 vector (Homo sapiens programmed cell death 1 (NM-005018.1), Origene, USA) expressing human PD-1 was transfected into HEK293T cells (Human embryonal kidney cell line, ATCC, Manassas, Va, USA). Cytospin preparations were prepared and immunostained with

antibody NAT or with a goat anti-PD-1 monoclonal antibody (R&D System, USA) following a conventional immunoperoxidase protocol (2). Both antibodies gave positive reactions, confirming the specificity of antibody NAT for the PD-1 molecule (Figure 6). Furthermore, a 47 kDa band was observed when these cells were tested by Western blotting using the same antibodies.

Tissue samples

Biopsy samples (fresh and formalin-fixed paraffin-embedded tissues) were obtained from the tissue archives of the CNIO Tumor Bank Network, from the files of the Histopathology Department, John Radcliffe Hospital, from the Institute of Pathology, University of Kiel and from the Department of Pathology, University Clinic Frankfurt am Main. The material investigated comprised sections of either whole tissue blocks or tissue-arrays, prepared in the institutions referred to above. All samples were reviewed by at least two pathologists, and in each case diagnosis had been made on the basis of conventional histologic and immunohistologic examination according to the criteria of the World Health Organization (WHO) classification (3). The diagnosis of angioimmunoblastic T cell lymphoma was based on a combination of morphologic features (e.g. "clear cell" neoplastic cell morphology, expanded follicular dendritic cell meshworks, vascular proliferation) and phenotype (e.g. expression by tumor cells of CD3 and CD4 accompanied by CD10 and/or BCL6).

Immunostaining techniques

Single and double immunoenzymatic staining: Prior to immunostaining, cryostat sections were fixed in acetone for 10 minutes and immunostained using a conventional immunoperoxidase technique (2). Paraffin tissue sections were first de-waxed, subjected to an antigen retrieval treatment and subsequently stained by immunoperoxidase technique following a protocol previously described (4). Incubations omitting the specific antibody or with unrelated antibodies were performed as controls. Double immunoenzymatic labeling of paraffin sections was performed using a protocol previously described (4). In the first reaction, immunostaining was performed using the EnVision peroxidase kit and diaminobenzidine (DAB) chromogen-substrate (Dako K5507, Dako, Denmark). In the second reaction, immunostaining was performed using the alkaline phosphatase kit (Dako K5355, Dako, Denmark) and chromogen provided with the kit. The evaluation and scoring of PD-1 and SAP immunostaining was performed by at least two pathologists.

Double immunofluorescence: Double immunofluorescence labeling of tissue sections for SAP in combination with CD3 or PD-1 antigen was performed as previously described (5). Double immunostaining to detect PD-1 with other antigens (e.g. BCL6, PAX-5 and Ki67) was performed by incubating paraffin sections following antigen retrieval for one hour at room temperature in a humid chamber with a mixture of the two primary antibodies diluted in PBS + 10% FCS (Australian Foetal Calf Serum, Life Technologies Inc., USA). Slides were then washed three times in PBS containing 0.5% Tween 20 for periods of 5 minutes. Slides were subsequently incubated for one hour with fluorochrome-conjugated antibodies specific for different Ig isotypes (Molecular Probes, Leiden, The Netherlands) (diluted in PBS 1:200) in a humid chamber in the dark. Slides were washed three times subsequently in PBS containing 0.5% Tween 20 for 5-minute periods. Following washing, sections were mounted in antifading mounting medium

(Qbiogene, Illkirch, France) containing DAPI (Molecular Probes, Leiden, The Netherlands). Slides were examined on a Nikon E800 Eclipse fluorescence microscope (Nikon, Kingston-upon-Thames, UK) equipped for epifluorescence, and images were captured with an AxioCam charge-coupled device (CCD) camera (Zeiss, Jena, Germany) and Axiovision software (Imaging Associates, Bicester, UK), and adjusted using Photoshop software (Adobe, San Jose, CA, USA) (6).

Western Blotting

Western blotting analysis of anti-SAP antibody was performed using cell lysates of the following cell lines: YT (NK lymphoma-derived) and Jurkat, CCRF/CEM, Molt-4 and Karpas 299 (all T cell lymphoma-derived) (Figure 5 in the main text). Cell pellets were lysed with RIPA buffer supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany) run on a 15% SDS-PAGE gel, and subsequently treated following a protocol described elsewhere (7). Western blotting with the anti-PD-1 antibody NAT was performed against proteins extracted from YT cell line pellets by lysis in RIPA buffer (supplemented with protease and phosphatase inhibitors). Proteins were resolved by 12% SDS-PAGE and electrotransferred to nitrocellulose membrane (Hybond-ECL, Amersham Pharmacia Biotech, Arlington Heights, IL) before blocking and probing with anti-PD-1 antibody. After washing, membranes were incubated with HRP-conjugated anti-mouse Ig (Dako, Glostrup, Denmark) (dilution 1:200). Chemiluminescent detection was performed incubating membranes with ECL-reagent (Amersham Pharmacia Biotech). Beta-actin was used as protein loading control.

Table 1. Antibodies used for immunostaining germinal center T cells.

<i>Anti-</i>	<i>Clone/ product name</i>	<i>Source</i>
BCL6	GI191E/A8 (67)	CNIO Monoclonal Antibody Unit, Madrid, Spain.
CD3	A0452	Dako, Glostrup, Denmark.
CD10	NCL-L-CD10-270	Novocastra Laboratories, Newcastle upon Tyne, UK.
CD57	B321 (NK-1)	Abcam, Cambridge, UK.
CXCL13	53610	R&D Systems, Minneapolis, USA.
PD-1 (CD279)	NAT	CNIO Monoclonal Antibody Unit, Madrid, Spain.
SAP (SLAM-associated protein)	sc-8333	Santa Cruz Biotechnology, Santa Cruz, Ca, USA.

Figure 6

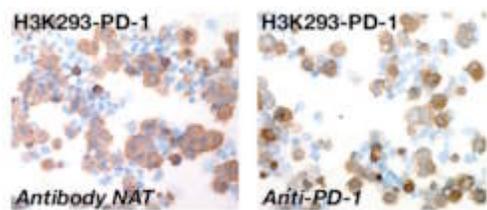


Figure legend

Figure 6. Immunoperoxidase staining of HEK293T-PD-1 transfected cells. Immunostaining of the transfected cell line with antibody NAT (left) and with anti-PD-1 (right) shows same positive reaction indicating the specificity of the antibody NAT for the PD-1 molecule.

References

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