Plexin-A4 negatively regulates T lymphocyte responses

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Abstract

Semaphorins and their receptors play crucial roles not only in axon guidance during neuronal development but also in the regulation of immune responses. Plexin-A4, a member of the plexin-A subfamily, forms a receptor complex with neuropilins and transduces signals for class III semaphorins in the nervous system. Although plexin-A4 is also expressed in the lymphoid tissues, the involvement of plexin-A4 in immune responses remains unknown. To explore the role of plexin-A4 in the immune system, we analyzed immune responses in plexin-A4-deficient (plexin-A4−/−) mice. Among immune cells, plexin-A4 mRNA was detected in T cells, dendritic cells and macrophages but not in B cells and NK cells. Plexin-A4−/− mice had normal numbers and cell surface markers for each lymphocyte subset, suggesting that plexin-A4 is not essential for lymphocyte development. However, plexin-A4−/− mice exhibited enhanced antigen-specific T cell responses and heightened sensitivity to experimental autoimmune encephalomyelitis. Plexin-A4−/− T cells exhibited hyperproliferative responses to anti-CD3 stimulation and to allogeneic dendritic cells in vitro. Furthermore, this hyperproliferation was also observed in both T cells from neuropilin-1 mutant (npn-1Sema2−/−) mice, in which the binding site of class III semaphorins is disrupted, and T cells from Sema3A-deficient (Sema3A−/−) mice. Collectively, these results suggest that plexin-A4, as a component of the receptor complex for class III semaphorins, negatively regulates T cell-mediated immune responses.

Introduction

Semaphorins are a family of secreted and transmembrane proteins characterized by a conserved amino terminal 'Sema domain' (1). Although they were originally identified as axon guidance factors during neuronal development (2–4), semaphorins have also been shown to have diverse and important physiological and pathological roles in cardiovascular development, tumor progression and immune regulation (5–9). A number of studies with gene-targeted mice have shown that some of membrane-bound semaphorins such as Sema4A, Sema4D, Sema6D and Sema7A are critically involved in immune regulation (10–15). In addition, it was recently reported that a secreted semaphorin, Sema3A, is involved in T cell regulation (16, 17).

In the nervous system, neuropilin and plexin molecules serve as the major semaphorins receptors (18, 19). In particular, plexins are critical for the transduction of semaphorins signals. Plexins can be divided structurally into four classes: plexin-A1–A4, plexin-B1–B3, plexin-C and plexin-D. Plexin-A
not only forms a receptor complex with neuropilins for se-
creted class III semaphorins but also binds directly to trans-
membrane class VI semaphorins in a neuropilin-independent
manner (20–22). Plexin-B1 directly binds to a class IV sema-
phorin, Sema4D (23). Plexin-C1 has been reported to inter-
act with the class VII semaphorin, Sema7A (23). Plexin-D1
has been shown to bind class III semaphorins in both a
plexin-independent and -dependent manner (24, 25). In
the immune system, plexin-A1 has been reported to be
critically involved in dendritic cell (DC) functions (14).
Indeed, plexin-A1−/− mice display severely impaired T cell
responses because of defective DC functions. Although
Sema6D, a class VI semaphorin, can bind to wild-type DCs
and induce IL-12 production, both Sema6D binding and
Sema6D-induced IL-12 production are severely impaired in
plexin-A1−/− DCs, indicating that plexin-A1 is a Sema6D re-
ceptor in the immune system. In addition to plexin-A1, other
plexin molecules are expressed on various cells in the im-
une system. However, aside from plexin-A1, the immuno-
logical functions of other plexins are largely unknown.

In the present study, we analyzed immune responses in
plexin-A4−/− mice. Plexin-A4−/− mice exhibit significantly
elevated T cell responses in vivo and develop exacerbated
experimental autoimmune encephalomyelitis (EAE) when
immunized with myelin oligodendrocyte glycoprotein (MOG)-
derived peptides. T cells from plexin-A4−/− mice as well as
mice lacking class III semaphorins signaling show enhanced
responses when stimulated with anti-CD3 or allogeneic DCs.
Thus, plexin-A4 appears to function as a negative regulator of
T cell responses.

Methods

Mice

C57BL/6 and BALB/c mice were purchased from Clea. Plexin-A4−/− (21), neuropilin-1 mutant (npn-1Sema) (26),
Sema3A−/− (27) and Sema6A−/− (28) mice on a C57BL/6
background were generated as previously described. All
mice used in this study were maintained in a specific pathogen-
free environment. All animal experimental procedures were
consistent with our institutional guidelines.

Antibodies

For flow cytometry, mouse thymocytes and splenocytes were
stained in the presence of Fc block (anti-CD16/32, 2.4G2)
with the following antibodies: anti-CD4 ( GK1.5), anti-CD8α
(53-6.7), anti-B220 (RA-8B2), anti-CD3 (2C11), anti-CD11c
(HL3), anti-CD11b (M1/70), anti-F4/80 (Cl:A3-1), anti-Pan
NK (DX5), anti-CD40 (3/23), anti-CD80 (16-10A1), anti-CD86
(GL1) and anti-I-Ab (24.9-17) conjugated with FITC, PE or
allophycocyanine in the presence of Fc block. All these anti-
 bodies, except for anti-F4/80 (Serotec), were purchased from
BD PharMingen.

Reverse transcription–PCR

Reverse transcription (RT)–PCR was performed with 35
cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s us-
ing the primers 5′-GATGATTCTGGCCTGTGACATCCTC-3′
and 5′-AAGGCCAGTGAGTGGTTCTTGTAG-3′. For re-
time RT-PCR analysis, RNA was isolated from B cells, T
cells, DCs, macrophages and NK cells using RNeasy kits
(Qiagen, Hilden, Germany) and treated with DNase I (Invitro-
gen, Carlsbad, CA, USA) to eliminate genomic DNA. cDNA
was synthesized using a SuperScript II cDNA synthesis kit
(Invitrogen).

In vivo T cell priming

For keyhole limpet hemocyanin (KLH)-specific T cell priming,
mice were immunized in the hind footpad with 10 μg of KLH
emulsified in CFA (Sigma). Five days after immunization,
CD4+ T cells were isolated from the draining lymph nodes
by magnetic cell sorting (MACS; Miltenyi Biotech, Bergisch
Gladbach, Germany), and 5 × 104 cells were stimulated for
72 h with various concentrations of KLH in the presence of
syngeneic irradiated (3000 rad) splenocytes (2.5 × 105 cells
per well). For MOG-specific T cell priming, mice were immu-
 nized with MOG35–55 peptide (Sigma Genosys) emulsified
in CFA. Ten days later after immunization, CD4+ cells were
isolated from the draining lymph nodes by MACS, and 1 × 105
cells were stimulated for 72 h with various concentrations of
MOG35–55 peptide in the presence of irradiated (3000 rad)
syngeneic splenocytes (5 × 105 cells per well). For prolifera-
tion assays, cells were pulsed with 2 μCi [3H]thymidine
([3H]TdR) for the last 16 h of the culture periods. Cytokine
production was measured in the culture supernatants using a
Bio-Plex suspension array system.

Induction of EAE

On day 0, mice were injected subcutaneously in both flanks
with the 100 μg of MOG35–55 peptide emulsified in CFA.
The mice received 100 ng of pertussis toxin intravenously (List
Laboratories) on days 0 and 2. All mice were monitored daily
for clinical signs and were scored using a scale of 0–4 as
follows: 0, no overt signs of diseases; 1, limp tail; 2, com-
plete hind limb paralysis; 3, complete forelimb paralysis and
4, moribund state or death. For passive EAE experiments,
donor mice were immunized with MOG/CFA in the same
fashion as when inducing EAE, but no pertussis toxin was
administered. Splenocytes and draining lymph nodes were
collected 10 days later, single-cell suspensions were prepared,
and RBCs were lysed. Cells (6 × 106 cells per ml) were cul-
tured in RPMI1640 medium with 40 μg ml−1 of MOG35–55
peptide and 10 ng ml−1 of recombinant mouse IL-12 (R&D
Systems). After 3 days of culture, cells were harvested and
T cells were isolated by negative selection using Dynabeads
(Invitrogen). Recipient mice were irradiated sublethally (500
cGy) and received 5 × 106 cells intravenously.

In vitro assay

CD4+ T cells and splenic DCs were isolated from the spleen
using MACS, with >95% purity in each experiment. Bone
marrow-derived dendritic cells (BMDCs) were generated from
colonies using granulocyte macrophage colony-stimulating
factor as previously described (29). For T cell proliferation assays, CD4+ cells (5 × 104 cells per well) were cultured with or without immobilized anti-
CD3 (2C11) plus anti-CD28 (37.51) for 72 h. For mixed lym-
phocyte reactions (MLRs), CD4+ T cells (5 × 104 cells per well)
were cultured with irradiated (3000 rad) allogeneic
BMDCs for 72 h. To measure cell proliferation, cells were pulsed with 2 μCi of [3H]TdR for the last 16 h of the culture period. For IL-12 production assays, IL-12 concentrations in the culture supernatants were measured after culturing DCs (1 × 10^6 cells per ml) for 48 h with or without anti-CD40 (3/23) or LPS. The IL-12 was detected by using a mouse IL-12p40 ELISA kit (R&D Systems).

Immunoblotting and Ca2+ mobilization

For immunoblotting analysis, Thy1.2+ cells (2 × 10^6 cells) were seeded into 24-well plates coated with anti-CD3 (1 μg ml^-1) for the indicated times. Cells were solubilized in buffer containing 1% Nonidet-P40, 10 mM Tris–HCl, 150 mM NaCl, 1 mM EDTA, 10 mM Na3VO4, 0.5 mM phenylmethylsulphonylfluoride and a phosphatase inhibitor cocktail (Calbiochem). Whole-cell lysates were separated by SDS-PAGE and immunblotted with anti-phospho-Zap-70, anti-Zap-70, anti-Lat, anti-phospho-extracellular signal-regulated kinase (ERK), anti-ERK, anti-phospho-c-Jun N-terminal kinase (Jnk), anti-phospho-p38, anti-p38, anti-phospho-PLCγ1 or anti-PLCγ1 purchased from Cell Signaling Technology. For Ca2+ mobilization analysis, purified T cells were loaded with 2 μM Fura-2 (Molecular Probes) for 15 min at 37°C. Cells were then washed with loading buffer and stimulated with biotinylated anti-CD3, and then cross-linked with streptavidin.

Results and discussion

Normal lymphoid development in plexin-A4−/− mice

The plexin-A4 expression profile in various mouse tissues was examined by RT-PCR. Plexin-A4 was found to be expressed in a broad range of tissues, including the brain, kidney, lung, heart, liver and spleen of adult mice and embryos (Supplementary Figure 1A, available at International Immunology online). Among various immune cells, T cells, DCs and macrophages, but not B cells and NK cells expressed highly plexin-A4 mRNA (Supplementary Figure 1B, available at International Immunology online).

Because plexin-A4 expression in particular immune cells subsets implied a role for plexin-A4 in the immune system, we analyzed the immunological phenotype of plexin-A4−/− mice. First, we analyzed thymocytes and splenocytes by flow cytometry to determine if the absence of plexin-A4-expression affected lymphocyte development. The absence of plexin-A4 did not alter ratios of CD4+ and CD8+ thymocytes (Supplementary Figure 2A, available at International Immunology online). There were no differences in the expression of cell surface phenotype markers, and the ratios of T cells, B cells, macrophages, DCs and NK cells in the spleen were comparable between wild-type and plexin-A4−/− mice (Supplementary Figure 2B, available at International Immunology online). Additionally, regulatory T cells (CD4+CD25+ or CD4+Foxp3+) and memory T cells (CD4+CD44+) populations were normal in plexin-A4−/− mice (Supplementary Figure 2C, available at International Immunology online). These findings indicate that plexin-A4 is not essential for lymphocyte development.

Plexin-A4−/− mice displayed enhanced in vivo T cell priming and exacerbated T cell-mediated autoimmunity

Since higher plexin-A4 expression in T cells and DCs suggests that plexin-A4 plays a role in T cell activation, we immunized wild-type and plexin-A4−/− mice with KLH in CFA. Five days after immunization, CD4+ T cells were purified from the draining lymph nodes and restimulated with KLH in the presence of wild-type antigen-presenting cells (APCs). Both CD4+ T cells proliferation and cytokine production were enhanced in plexin-A4−/− mice compared with wild-type mice (Fig. 1). We also immunized plexin-A4−/− mice with MOG peptide to induce EAE, a mouse model of multiple sclerosis. Clinical analysis revealed that plexin-A4−/− mice developed much more severe forelimbs and hind limbs paralysis than wild-type mice (Fig. 2A). Histological analyses showed a higher number of infiltrating mononuclear cells in the spinal cords of diseased plexin-A4−/− mice than wild-type mice (Fig. 2B). To determine the contribution of plexin-A4 to MOG-specific T cell generation, T cells were isolated from the draining lymph nodes of immunized mice and restimulated with MOG peptide presented by syngeneic splenocytes. Plexin-A4−/− T cells had augmented proliferative

![Fig. 1. Plexin-A4−/− mice displayed enhanced T cell priming. Wild-type (closed circles) and plexin-A4−/− mice (open circles) were immunized with KLH in CFA. Five days after immunization, CD4+ T cells were purified from the draining lymph nodes and restimulated with KLH in the presence of splenic APCs. For proliferation assays, cells were pulsed with 2 μCi of [3H]TdR for the last 16 h of the culture period. Cytokines in the culture supernatants were measured using Bio-Plex suspension array systems (Bio-Rad). Data are the mean ± SD of triplicate wells and representative of five independent experiments. *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001; each value was analyzed by a Student’s t-test.](https://academic.oup.com/intimm/article-abstract/20/3/413/652766/fig1)
responses and secreted larger amounts of IFN-γ and IL-17 compared with wild-type T cells (Fig. 2C).

Plexin-A4 is known to function as an axon guidance factor in the nervous system (20, 21, 28). Indeed, plexin-A4−/− mice display abnormal projection of hippocampal mossy fibers (21). We then transferred T cells from MOG-immunized wild-type or plexin-A4−/− mice into wild-type recipients. Mice that received wild-type T cells developed a mild, transient EAE and recovered shortly after the acute episode (Fig. 2D). In contrast, mice that received plexin-A4−/− T cells developed early onset, severer EAE with chronic and persistent disease (Fig. 2D). These findings suggest that hyperactivation of MOG-specific T cells caused by a plexin-A4 deficiency in immune cells is primarily responsible for exacerbated EAE in plexin-A4−/− mice.

**Plexin-A4−/− T cells were hyperproliferative in vitro**

To elucidate the mechanism underlying the in vivo hyperactivation of plexin-A4−/− T cells, we next examined whether plexin-A4 deficiency affected in vitro T cell proliferation. Plexin-A4−/− T cells displayed higher proliferative responses than wild-type T cells after stimulation with a low concentration of anti-CD3 (0.1 μg ml−1) (Fig. 3A, left). When examined kinetically, plexin-A4−/− T cells had enhanced proliferation at all time points (Fig. 3A, right). We also examined the effect of plexin-A4 deficiency on proliferative
concentrations in culture supernatants were measured by ELISA. Data are the mean representative of five independent experiments.

Left) Splenic CD4+ T cells purified from wild-type (black bars) or plexin-A4+/− (white bars) mice were stimulated with anti-CD3 (0.1 µg ml−1) in the presence or absence of anti-CD28 (5 µg ml−1) for 72 h. (Right) Splenic CD4+ T cells purified from wild-type (closed circles) or plexin-A4+/− (open circles) mice were stimulated with anti-CD3 (0.1 µg ml−1). Proliferation was evaluated at different time points. (B) Enhanced proliferative responses to allogeneic BMDCs in plexin-A4+/− T cells. Splenic CD4+ T cells purified from wild-type (closed circles) or plexin-A4+/− (open circles) mice were cultured with irradiated allogeneic BMDCs for 72 h. Cells were pulsed with 2 µCi of [³H]TdR for the last 16 h of the culture period. (C) Enhanced T cell stimulatory activities in plexin-A4+/− DCs. Irradiated BMDCs derived from wild-type (closed circles) or plexin-A4+/− (open circles) mice were cultured with allogeneic CD4+ T cells for 72 h. Cells were pulsed with 2 µCi of [³H]TdR for the last 16 h of the culture period. (D) Normal expression of costimulatory molecules in plexin-A4+/− DCs. BMDCs (upper) or splenic DCs (lower) from wild-type or plexin-A4+/− mice were cultured for 24 h with anti-CD40. Cells were stained with allophycocyanine-anti-CD11c and FITC-anti-CD40, -anti-CD80, -anti-CD86 and -anti-I-A (thick lines). CD11c-positive cells were analyzed. (E) Normal IL-12 production in plexin-A4+/− DCs. BMDCs from wild-type (black bars) or plexin-A4+/− (white bars) were cultured for 48 h in the presence or absence of anti-CD40 (upper) or LPS (lower). IL-12p40 concentrations in culture supernatants were measured by ELISA. Data are the mean ± SD of triplicate wells. The results shown in (A–E) are representative of five independent experiments. *P < 0.05, **P < 0.01, ***P < 0.005, ****P < 0.001; each value was analyzed by a Student’s t-test.

responses of T cells stimulated with antigens presented by APCs in an MLR. The responses of plexin-A4+/− T cells to allogeneic DCs were significantly higher than those of wild-type T cells (Fig. 3B). It is also noteworthy that the ability of plexin-A4+/− DCs to stimulate allogeneic CD4+ T cell proliferation was stronger than that of wild-type DCs when plexin-A4+/− DCs were used as stimulators in an MLR (Fig. 3C). However, we did not observe any differences in MHC class II expression, costimulatory molecule expression or IL-12 production between plexin-A4+/− and wild-type DCs after anti-CD40 or LPS stimulation (Fig. 3D and E), indicating that the enhanced allostimulatory activity of plexin-A4+/− DCs cannot be simply explained by an alteration in the expression of costimulatory molecules or cytokines.

Our findings indicate that a plexin-A4 deficiency in either T cells or DCs can enhance T cell activation, although the absence of T cells appears to have a greater impact. In contrast, there was no difference in in vitro B cell responses to various mitogenic stimulations between wild-type and plexin-A4+/− mice (Supplementary Figure 3, available at International Immunology online), which was consistent with the low plexin-A4 expression in B cells.
These results indicate that plexin-A4 expression in both T cells and DCs plays a role in negatively regulating T cell activation.

**Partially enhanced TCR signals in plexin-A4−/− T cells**

We then examined TCR signaling in plexin-A4−/− T cells. Anti-CD3-stimulated plexin-A4−/− and wild-type T cells were analyzed for phosphorylation of signaling molecules downstream of the TCR. Anti-CD3 stimulation enhanced phosphorylation of both Zap-70 and Lat in plexin-A4−/− T cells (Fig. 4A). However, there were no significant differences in the phosphorylation of other signal molecules, such as ERK, Jnk, p38 and PLC-γ1, between plexin-A4−/− and wild-type T cells (Fig. 4B). Consistent with the observation that the plexin-A4 deficiency did not affect PLC-γ1, the anti-CD3-induced calcium response was comparable between wild-type and plexin-A4−/− T cells (Fig. 4C). Thus, plexin-A4 deficiency appears to affect some TCR signals directly or indirectly, although it remains unclear whether this alteration in TCR signaling is responsible for the hyperreactivity of plexin-A4−/− T cells.

**Possible involvement of class III semaphorins in T cell responses as plexin-A4 ligands**

Our observations with plexin-A4−/− mice suggested that plexin-A4 negatively regulates T cell activation; however, the plexin-A4 ligands in the immune system are unknown. Plexin-A4 and neuropilin-1 are known to form a receptor for Sema3A, a class III semaphorin, in the nervous system (21, 30). In addition, plexin-A4 directly interacts with the transmembrane semaphorin, Sema6A (21, 22). Therefore, we examined T cells from mice with defective class III semaphorin signaling. We first examined T cells from npn-1Sema−− mice, in which the binding site of class III semaphorins is disrupted. Npn-1Sema−− T cells showed enhanced responses to anti-CD3 stimulation (Fig. 5A) and allogeneic DCs (Fig. 5B), as observed in plexin-A4−/− T cells (Fig. 3A and B). Sema3A−/− T cells, which have defective Sema3A production, were also slightly hyperproliferative in response to stimulation with anti-CD3 (Fig. 5C) and allogeneic DCs (Fig. 5D). In addition to class III semaphorins, a transmembrane semaphorin, Sema6A, is also known to directly interact with plexin-A4 (21, 22). However, we could not detect any difference in T cell activation between Sema6A−/− mice and wild-type mice (Fig. 5E and F). These results indicate that plexin-A4, as a receptor for class III semaphorins such as Sema3A, plays a role in negatively regulating T cell proliferation and activation. Consistent with these findings, it was recently reported that Sema3A produced by tumor cells or by T cells and DCs suppressed T cell activation (16, 17). However, it remains unclear if Sema3A is the only class III semaphorin that negatively regulates T cells through plexin-A4 deficiency or neuropilin-1 mutation. In addition to plexin-A4, plexin-A1 is also a signal transducer for class III semaphorins (31). However, the phenotype of plexin-A1−/− mice is completely different from those of plexin-A4−/−, npn-1Sema−− and Sema3A−/− mice (14). Furthermore, unlike plexin-A4−/− mice, plexin-A1−/− mice showed defective T cell responses.

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**Fig. 4.** Enhanced TCR signals in plexin-A4−/− T cells. (A, B) Enhanced TCR-induced phosphorylation of Zap-70 and Lat in plexin-A4−/− T cells. Splenic T cells were stimulated with anti-CD3 for the indicated periods. The cells were solubilized and the lysates were immunoblotted with antibodies to (A) phosphorylated-Zap70 (p-Zap70), Zap70, p-Lat or β-actin, (B) p-ERK, ERK, p-Jnk, p-p38, p-PLC-γ1, PLC-γ1 or β-actin. (C) Normal calcium mobilization in plexin-A4−/− T cells. Splenic T cells were loaded with Fura-2, pretreated with biotinylated anti-CD3 and then cross-linked with streptavidin.
Collectively, plexin-A4 appears to be a major signaling transducer of class III semaphorins in T cells, although we cannot completely exclude a possibility that plexin-A4-independent mechanisms may be involved in the enhanced T cell responses in npn-1Sema-/- T cells. We observed enhanced phosphorylation of some signaling molecules such as Zap-70 and Lat in plexin-A4-/- T cells; however, it is still unknown if plexin-A4 directly modulates TCR signals. It is also unclear how the activation of Zap-70 and Lat is selectively affected in the absence of plexin-A4. In the nervous system, class III semaphorins regulate the actin cytoskeleton and integrins to exert chemorepulsive activities on neuronal axons (32, 33). It is possible that a similar mechanism may underlie plexin-A4-mediated T cell regulation. Further studies will clarify not only the detailed molecular mechanism by which plexin-A4 regulates T cells but
also the physiological and pathological significance in T cell immunity.

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**Abbreviations**

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<tr>
<th>Term</th>
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<tr>
<td>APC</td>
<td>Antigen-presenting cell</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony-stimulating factor</td>
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<td>Jnk</td>
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<td>KLH</td>
<td>Keyhole limpet hemocyanin</td>
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<tr>
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<td>Magnetic cell sorting</td>
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<td>Mixed lymphoid reaction</td>
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<td>[3H]Tdr</td>
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**References**