THROMBOSIS AND HEMOSTASIS

Critical role of CD4 T cells in PF4/heparin antibody production in mice

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Key Points

 CD4 T cells play a critical role in controlling production of PF4/heparin-specific antibodies. Antibodies specific for platelet factor 4 (PF4)/heparin complexes are central to the pathogenesis of heparin-induced thrombocytopenia. Marginal zone B cells appear to be the source of such antibodies, but whether T-cell help is required is unclear. Here, we showed that induction of PF4/heparin-specific antibodies by PF4/heparin complexes was markedly impaired in mice depleted of CD4 T cells by anti-CD4 antibodies. Furthermore, Rag1deficient recipient mice produced PF4/heparin-specific antibodies upon PF4/heparin challenge when reconstituted with a mixture of wild-type splenic B cells and splenocytes

from B-cell–deficient (μ MT) mice but not splenocytes from T- and B-cell–deficient (Rag1 knockout) mice. Lastly, mice with B cells lacking CD40, a B-cell costimulatory molecule that helps T-cell–dependent B-cell responses, displayed a marked reduction of PF4/ heparin-specific antibody production following PF4/heparin challenge. Together, these findings show that helper T cells play a critical role in production of PF4/heparin-specific antibodies. (*Blood*. 2015;125(11):1826-1829)

Introduction

Heparin-induced thrombocytopenia (HIT) is the most common druginduced, antibody-mediated thrombocytopenia and occurs 3 to 6 days following heparin treatment.^{1,2} HIT patients develop antibodies quickly, however, which are typically undetectable in a few months.¹ Platelet factor 4 (PF4)/heparin-specific antibodies, central to the pathogenesis of HIT, are predominantly of the immunoglobulin G1 (IgG1) isotype with some IgG2 in humans.²⁻⁴ IgG/PF4/heparin immune complexes bind Fc γ RIIA on the platelet surface and induce platelet activation, leading to thrombocytopenia and a high risk of arterial and/or venous thrombosis/thromboembolism.^{5,6}

Long-lived mature B cells comprise 3 subsets: marginal zone (MZ), B1, and follicular B cells.^{7.8} The MZ subset has been shown to be critical for production of PF4/heparin-specific antibodies.⁹ Typically, MZ B cells produce IgM or IgG antibodies independent of T-cell help.¹⁰⁻¹² Indeed, HIT patients have features of a T-cell–independent humoral immune response, characterized by rapid onset and decline of antibodies and apparent absence of immunologic memory.¹ However, patients with severe HIT possess T cells that have a T-cell receptor with highly restricted complementarity determining region 3 regions and are responsive to PF4/heparin, suggesting a role of T cells in HIT pathogenesis.^{13,14} Nonetheless, direct evidence for a role of T cells in HIT pathogenesis has not been reported. Here, we describe studies to define the role of T-cell help in regulating production of PF4/heparin-specific antibodies.

Study design

Mice

Eight- to 10-week-old Rag1-deficient, CD40-deficient, μ MT, and wild-type C57BL/6 mice from The Jackson Laboratory were maintained in the Biological

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Resource Center at the Medical College of Wisconsin (MCW). Animal protocols were approved by the MCW Institutional Animal Care and Use Committee.

In vivo depletion of CD4 T cells

Wild-type C57BL/6 mice were injected intraperitoneally with anti-mouse CD4 antibodies (clone GK1.5, 250 μ g per mouse; BioXCell) or with isotype control antibodies (rat IgG2b; BioXCell) or phosphate-buffered saline (PBS) on day 0 and day 2. The efficiency of depletion was examined by flow cytometry at day 7 after the first injection, and >99% of CD4 T cells were depleted in the spleen and lymph nodes. To maintain this state, mice were further injected with GK1.5 (250 μ g per mouse) on day 7 and day 14.

Immunization

PF4/heparin immunization was performed as described.⁹ G. Arepally (Duke University) provided mouse PF4. T-cell–dependent and –independent antigen immunizations were performed as described.⁹ The T-cell–dependent antigen was nitrophenyl-chicken γ globulin (NP-CGG; Biosearch Technologies) and the T-cell–independent antigen was trinitrophenyl-Ficoll (TNP-Ficoll; Biosearch Technologies).

Adoptive transfer experiment

Splenic B cells were isolated from wild-type mice by magnetic cell sorting using anti-B220–coated magnetic-activated cell sorting magnetic microbeads (Miltenyi Biotec) and then mixed 1:1 with splenocytes from μ MT or Rag1-deficient mice in PBS supplemented with 2% fetal bovine serum. The mixed cells were transplanted into partially irradiated (300 rad) 8- to 10-week-old Rag1-deficient mice by IV injection (8~10 × 10⁶ cells per recipient). One hour after adoptive transfer, the recipients were immunized with the indicated antigens. Sera were collected at the indicated time points, and antigen-specific antibodies were measured.

Chimeric mice

Bone marrow (BM) cells from CD40-deficient or wild-type mice were mixed 1:4 with BM cells from μ MT mice in PBS supplemented with 2% fetal bovine

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Figure 1. Requirement of T cells for production of PF4/heparin-specific antibodies. (A) Impaired production of PF4/heparin-specific antibodies in CD4 T-cell-depleted mice. Wild-type mice were treated with anti-mouse CD4 antibody GK1.5 or PBS, followed by immunization with PF4/heparin complexes. Sera were collected at the indicated time points after immunization and total PF4/heparin-specific IgG levels were measured by enzyme-linked immunosorbent assay (ELISA). Each dot represents a mouse and the horizontal lines indicate the mean values. Data shown are obtained from 10 mice in each group. (B) Normal T-cell-independent responses in CD4 T-cell-depleted mice. GK1.5- or PBStreated mice were immunized with the T-cell-independent antigen TNP-Ficoll. Sera were collected at the indicated time points after immunization, and TNP-specific IgM (left) and IgG3 (right) were measured by ELISA. Data shown are obtained from 6 mice in each group. (C) Impaired T-cell-dependent responses in CD4 T-cell-depleted mice. GK1.5- or PBS-treated mice were immunized with the T-cell-dependent antigen NP-CGG. Sera were collected at the indicated time points after immunization, and NP-specific IgM (left) and IgG1 (right) were measured by ELISA. Data shown are obtained from 6 mice in each group. (D) Failure of adoptively transferred splenic B cells to produce PF4/heparin-specific antibodies in the absence of T cells. Splenic B cells isolated from wild-type mice were mixed with splenocytes isolated from µMT or Rag1-deficient mice at a 1:1 ratio, and then adoptively transferred into partially irradiated Rag1-deficient mice. The recipients were immunized with PF4/heparin complexes. Sera were collected at the indicated time points, and the levels of total PF4/heparin-specific IgG were measured by ELISA. Data shown are obtained from 10 recipients in each group. Each dot represents a mouse and the horizontal lines indicate the mean values.



serum. The mixed cells were transplanted into lethally irradiated (1000 rad) 8- to 10-week-old μ MT mice by IV injection (5 \times 10⁶ cells per recipient). Eight weeks later, the recipients were immunized with the indicated antigens. Sera were collected at the indicated time points, and antigen-specific antibodies were measured.

Statistical analysis

Statistical analysis was performed with the 2-tailed unpaired Student t test.

Results and discussion

MZ B cells play a major role in producing PF4/heparin-specific antibodies.⁹ Typically, MZ B cells participate in T-cell–independent antibody responses.¹⁰⁻¹² However, human patients with severe HIT possess T cells that are responsive to PF4/heparin.^{13,14} Here, we systematically investigated the role of T cells in production of PF4/heparin-specific antibodies in vivo. First, we examined the effect of CD4 T-cell depletion on production of PF4/heparin-specific antibodies. Wild-type mice were depleted of T cells with anti-mouse CD4 antibody GK1.5. Flow cytometry analysis demonstrated >99% deletion of CD4 T cells in spleens, lymph nodes, and blood during the entire duration of the experiment (supplemental Figure 1, available on the *Blood* Web site; data not shown). Following PF4/heparin challenge, production of PF4/heparin-specific antibodies was markedly reduced in these mice relative to controls (Figure 1A, supplemental Figure 2A). In the absence of CD4 T cells, B cells failed to produce any isotypes of PF4/heparin-specific IgG, including IgG2b and IgG3 (supplemental Figure 3). Of note, anti-CD4 antibody-treated mice responded normally to T-cell–independent antigen TNP-Ficoll (Figure 1B) but not T-cell–dependent antigen NP-CGG (Figure 1C), in agreement with the lack of CD4 T cells. Thus, antibody-induced depletion of CD4 T cells markedly impairs PF4/heparin-specific antibody production.

Next, we examined PF4/heparin-specific antibody production in immunodeficient mice reconstituted with B cells but not T cells. Wild-type splenic B cells were mixed with splenocytes from T- and B-cell–deficient Rag1-deficient mice and adoptively transferred into partially irradiated Rag1-deficient mice. The recipients were then challenged with PF4/heparin. Controls were Rag1-deficient mice that received a mixture of wild-type splenic B cells and splenocytes from B-cell–deficient μ MT mice. As shown in Figure 1D, control mice responded to PF4/heparin challenge by producing PF4/heparin-specific antibodies. In contrast, mice given a mixture of wild-type splenic B cells and Rag1-deficient splenocytes barely produced PF4/heparin-specific antibodies upon PF4/heparin complex challenge (Figure 1D), but responded normally to T-cell–independent antigen TNP-Ficoll



(supplemental Figure 2B). These data also show that T cells are required for PF4/heparin-specific antibody production.

Interactions between CD40 on B cells and its ligand on CD4 T cells play a critical role in T-cell-dependent B-cell responses.15,16 To define the role of helper T cells in PF4/heparin-specific antibody response, we studied the effect of B-cell-specific CD40 deficiency. BM cells from CD40-deficient mice were mixed with BM cells from μ MT mice, and transplanted into lethally irradiated µMT mice. The resulting BM chimeric mice possessing B cells derived from CD40-deficient BM cells and thus lacking CD40 failed to produce PF4/heparin-specific antibodies following PF4/heparin challenge (Figure 2A). In contrast, control BM chimeric mice that received a mixture of wild-type and µMT BM cells and thus possessed wild-type B cells responded normally to PF4/heparin challenge (Figure 2A). As expected, BM chimeric mice possessing CD40-deficient B cells responded to the T-cellindependent antigen TNP-Ficoll (Figure 2B) but not T-cell-dependent antigen NP-CGG (Figure 2C), consistent with a lack of T-cell help in these mice. Therefore, T cells provide critical help to B cells in producing PF4/heparin-specific antibodies through the CD40-CD40 ligand interaction.

MZ B cells recognize bacterial antigens, including those with repetitive epitopes, and produce antibodies, mainly IgM and some IgG, independent of T-cell help.^{10-12,17} A previous study reported that athymic nude mice fail to response to PF4/heparin challenge, indicating the involvement of T cells in HIT antibody production.¹⁸ However, Figure 2. Requirement of the CD40-CD40 ligand interaction between B and T cells for production of PF4/heparin-specific antibodies. BM cells from wildtype (CD40^{+/+}) or CD40-deficient (CD40^{-/-}) mice were mixed with BM cells from μMT mice at a 1:4 ratio, and then transplanted into lethally irradiated μ MT mice. Eight weeks after transplantation, the chimeric mice were immunized with the indicated antigens. (A) Failure of chimeric mice with B-cell-specific deficiency of CD40 to produce PF4/heparin-specific antibodies. The chimeric mice were immunized with PF4/heparin complexes. Sera were collected at the indicated time points after immunization, and the levels of total PF4/ heparin-specific IgG were measured by ELISA. Data shown are obtained from 10 chimeric mice at each group. (B) Normal T-cell-independent responses in chimeric mice with B-cell-specific deficiency of CD40. The chimeric mice were immunized with the T-cellindependent antigen TNP-Ficoll. Sera were collected at the indicated time points after immunization, and TNPspecific IgM (left) and IgG3 (right) were measured by ELISA. Data shown are obtained from 5 mice in each group. (C) Impaired T-cell-dependent responses in chimeric mice with B-cell-specific deficiency of CD40. The chimeric mice were immunized with the T-celldependent antigen NP-CGG. Sera were collected at the indicated time points after immunization, and NPspecific IgM (left) and IgG1 (right) were measured by ELISA. Data shown are obtained from 5 mice in each group. Each dot represents a mouse and the horizontal lines indicate the mean values

athymic nude mice possess many immune defects, including impaired development of B cells.¹⁹ Thus, inability of these mice to produce PF4/heparin-specific antibodies could be due to something other than T-cell deficiency per se, including defective B cells. Here we have shown that mice fully intact except for T-cell deficiency display a severely impaired ability to generate PF4/heparin-specific antibodies and that production of such antibodies by B cells requires T-cell help mediated through the CD40-CD40 ligand interaction. Previous studies have shown that CD40-CD40 ligand interaction appears not to be required for B-cell development.^{16,20} However, a recent study has shown that this interaction can broadly alter the B-cell receptor repertoire during B-cell development.²¹ Thus, it is possible that CD40 might be required for positive selection of PF4/ heparin-specific B cells during development and further research is warranted. Our previous study has shown MZ B cells are critical for production of PF4/heparin-specific antibodies.⁹ Although MZ B cells typically participate in T-cell-independent responses, there are exceptions to this general rule because previous studies have consistently shown that, under some circumstances, MZ B cells can interact with T cells and produce high-affinity antibodies possessing somatic hypermutation utilizing a T-cell-dependent pathway.^{22,23} Thus, MZ cells are functionally heterogeneous in their requirement for T-cell help. The antibody response to PF4/heparin-specific antigens represents another example of a T-cell-dependent response by this B-cell subset.

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Authorship

Contribution: Y.Z. contributed to research design, performed the research, and analyzed the results; M.Y. performed some initial

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experiments; A.P. critically reviewed the manuscript; R.H.A. provided intellectual input and critically reviewed the manuscript; L.Y. provided intellectual input; R.W. provided intellectual input, supervised the study, performed the research, and critically reviewed the manuscript; and D.W. conceived and supervised the study, analyzed the results, and wrote the manuscript.

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