Reduced Immunoregulatory CD31⁺ T Cells in the Blood of Atherosclerotic Mice With Plaque Thrombosis

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Objective—Lymphocyte activation is thought to play a major role in the pathogenesis of atherosclerotic complications such as plaque thrombosis. Circulating CD31⁺ T cells have been shown to regulate human T cell activation. Aim of this study was to evaluate whether the proportion of circulating immunoregulatory CD31⁺ T cells is correlated to the occurrence of plaque thrombosis in aged apolipoprotein (apo) E knockout (KO) mice.

Methods and Results—CD3¹⁺ T cell depletion of spleen T cells enhanced proliferation (P<0.05) and interferon-γ production (P<0.01) while reducing interleukin (IL)-4 (P<0.001) and IL-10 (P=0.001) secretion in response to minimally modified low-density lipoprotein. CD3¹⁺ T cells were counted in 65 apoE KO mice (46-week-old) by flow cytometry. Organizing thrombi could be documented in 28 of 195 (14%) lesions and in at least one of the aorta root lesions in 23 of 65 mice (35%). CD3¹⁺ T cell count was significantly reduced in mice showing plaque thrombosis (72.3±1.5% versus 84.1±1.2%; P<0.0001), but such reduction did not follow induced plaque rupture or experimentally controlled thrombosis.

Conclusions—Reduced CD3¹⁺ T cells in circulating blood is a hallmark of atherosclerotic plaque thrombosis. Our data suggest that CD3¹⁺ T cells may play an important regulatory role in the development of plaque thrombosis. (Arterioscler Thromb Vasc Biol. 2005;25:1659-1664.)

Key Words: atherosclerosis • lymphocytes • thrombosis

Cell-mediated specific immune responses take place in patients with coronary plaque thrombosis¹⁻² resulting in perturbations of the autoreactive T cell and B cell immune repertoires,³⁻⁴ suggesting defective control mechanisms of immune tolerance in these patients. Interestingly, CD3¹⁺ T cells have been shown to regulate ex vivo human T lymphocyte activation,⁵⁻⁶ and CD3 deficiency is associated with perturbations of the autoreactive humoral immune compartment in mice.⁶

CD31, known also as platelet endothelial cell adhesion molecule-1, is a member of the immunoglobulin superfamily. It is expressed by platelets, monocytes, neutrophils, B cells, NK cells, and certain T cell subsets. Thanks to its ability to self-interact, CD31 might be critical for leukocyte–vessel and leukocyte–leukocyte crosstalk.

Interestingly, among human adult blood nucleated cells, T lymphocytes are the sole circulating leukocytes to lose partially (CD8) or consistently (CD4) the expression of CD31 at their surface.⁷ Furthermore, the number of CD3¹⁺ T cells decrease with aging,⁸ whereas the risk of thrombo-atherosclerotic events increases. In striking contrast, the majority of T cells from adult and aged mice remain CD3¹⁺. Therefore, an inadequate CD3¹⁺ T cell-mediated control might contribute to explain the difference in frequency of plaque thrombosis in humans as compared with mice.

Indeed, spontaneous arterial plaque rupture/thrombosis has been described as occurring only incidentally in the hypercholesterolemic apolipoprotein (apo) E knockout (KO) mouse model.⁹ Consequently, we hypothesized that thrombotic complications of atherosclerosis might be linked to an altered CD3¹⁺ T cell-mediated immune control.

We report that the reduction of the number of circulating CD3¹⁺ T cells is a hallmark of plaque thrombosis in atherosclerotic mice.

Methods

Mice and Sampling
A total of 126 apoE KO mice (C57BL/6–apoEtm1Unc, both genders) and 6 C57Bl6 female mice from our internal breeding were maintained on regular chow diet for 40 to 50 weeks in conventional animal facility. Anesthetized mice were euthanized by exsanguination. Blood was collected from the right ventricle in a heparinized syringe and stored on ice. After rinsing the arterial tree with saline, the heart and ascending aorta were excised and snap-frozen in liquid nitrogen. Mononucleated blood cells obtained by density gradient centrifugation were used for flow cytometry. Spleens were individ-
ually meshed through 100-μm nylon filters, repeatedly washed, and erythrocyte lysed by the ACK buffer. Mononuclear spleen cell suspensions were washed, counted, and used as unsorted or CD4+ T cell-enriched preparations for T cell stimulation in culture. Cryo-mounted aorta roots and carotid arteries were used for atherosclerotic lesion analysis. The investigations conform with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication 85-23, revised 1996).

**Morphometry**

Aorta roots were carefully dissected out of the heart, cryo-embedded in Tissue-Tek, and sectioned as previously described.10 Slides collected at 200, 400, 600, and 800 μm were used for oil red-O staining and quantification of atherosclerotic lesions as previously described.10 Lipid components in lesions were identified as brilliant red droplets. Slides at 250 μm were used for quantification of collagen content by picrosirius red staining and polarized light microscopy analysis. Slides at 300 μm were used for evaluation of cellular/extracellular components of plaques by Masson’s trichrome staining. Organizing thrombi were identified by the presence of discrete gray/cellular areas as opposed to the dense green staining by Masson’s trichrome using a blindfold approach. These gray/cellular structures were confirmed to be organizing thrombi by fluorescence fibrin and platelet immunohistochemistry. Analysis of lesions was performed by computer-assisted image analysis. Captured images of each of the 3 aortic cusps (magnification ×100) were analyzed using a custom program written in Quips language and computer-assisted image analysis (QWIN LEICA).

**Flow Cytometry**

Immunostaining was performed with a cocktail of fluorescent monoclonal antibodies directed toward CD3 (activated protein C) and CD31 (fluorescein isothiocyanate), CD4 (PerCP), CD6 (phycoerythrin [PE]), or CD19 (PE), or CD25 (fluorescein isothiocyanate) and CD45RB (PE). All antibodies were from BD-Biosciences, France. Acquisition was run on 2000 total lymphocytes identified in the FSC-SSC dot-plot using a FACSCalibur and the CellQuest Pro software (BD-Biosciences).

**Immunohistochemistry**

Immunohistochemistry for platelet glycoprotein Ia/IIb (xia C3 and xia G5; Emfret, Germany), MAC3, CD4, vascular cell adhesion molecule-1, platelet endothelial cell adhesion molecule-1 (BD Biosciences), and fibrinogen (DAKOCTymation) was performed on consecutive sections taken between 350 and 400 μm from the appearance of the first aortic cusp. Positive stained cells were revealed by avidin-biotin-peroxidase complexes (ABC elite kit; VECTOR, France) and diaminobenzidine (Dakocytomation) and revealed by avidin-biotin-peroxidase complexes (ABC elite kit; VECTOR, France) and diaminobenzidine (Dakocytomation) and counterstained with hematoxylin. MAC3 cells (macrophages) were counted using the Leica Qwin image analysis system. Fibrin(ogen)/platelets, CD31/CD4, and CD31/MAC3 double stained was performed on consecutive sections between 450 and 500 μm and revealed by combinations of fluorescent secondary antibodies (Alexa 488 goat–anti-rabbit IgG for fibrin, Alexa 546 goat–anti-rat IgG for glycoprotein Ia/IIb, CD4, and MAC3) and biotinylated rabbit–anti-rat IgG/avidin–fluorescein for CD31. CD4+/CD31+ and CD4+/CD31− cells were counted by computer-assisted image analysis.

**CD31+ T Cell Regulation of T Cell Activation in Response to Minimally Modified Low-Density Lipoprotein**

Minimally modified (MM) low-density lipoprotein (LDL) was obtained as previously described for human serum.14 Briefly, LDL was isolated by density gradient centrifugation of pooled serum from 40- to 50-week-old apoE KO mice and was left in plastic tubes at 4°C, in the absence of EDTA, for 4 to 8 weeks. Such MM-LDL are recognized by the LDL receptor by contrast with copper-oxidized LDL, which enter the cell through the scavenger receptors.11 MM-LDL used in T cell cultures contained 2 nanomoles thiobarbituric acid-reactive substances TBARS as malondialdehyde equivalents per milligram of cholesterol.

Individual spleen cell suspensions were obtained from 37 female 40-week-old apoE KO mice and 6 matched C57Bl6 mice. CD4 (total and CD31+) T cell sorting was performed by using the Dynal mouse CD4+ negative selection kit. Purified rat anti-mouse CD31 antibodies were added to the in-house antibody cocktail, at 1 μg/105 target cells, for CD4/CD31− T cell selection. Whole cell suspensions (n=27 apoE KO mice, 2×105 cells/well) or sorted (n=10 apoE KO and 6 C57Bl6 mice) total or CD31-depleted CD4+ cells (1.5×105 cells/well) plus antigen presenting cells (irradiated pooled whole spleen cells, strain-matched, 1.5×105 cells/well) were cultured in triplicates in complete RPMI 1640 medium containing 10% fetal calf serum with or without 10 μg/mL MM-LDL. After 5 days of culture, 50 μL of supernatant were collected and immediately used for cytokine measurement, and replaced with an equal volume of complete medium containing 10 μC/mL (final dose: 0.5 μC/well). Cytokines (interferon-γ, IL-4, and IL-10) were measured by enzyme-linked immunosorbent assay (DuoSet, R&D Systems). Cells were harvested the next day for proliferation (cpm) analysis in a β-counter (background proliferation in duplicate plates without LDL preparation was subtracted from the experimental data obtained in the presence of MM-LDL). Proliferation and cytokine production were compared in sorted CD31+ or CD31−/CD4+ T cell cultures, whereas proliferation data from cultures of unsorted spleen cells were correlated with the percentage of autologous circulating CD31+ T cells.

**Temporal Relationship Between CD31+ T Cell Reduction and Thrombosis or Experimentally Induced Plaque Rupture/Thrombosis**

To evaluate the influence of spontaneous thrombosis, 10 40-week-old female mice were treated with an antithrombotic agent (abciximab, REOPRO, intraperitoneally, 2 mg/kg per day) for 10 weeks to block spontaneous thrombosis. The time course of CD31+ T cell percentage in the blood was evaluated starting from 4 weeks before treatment and was repeated 3 times during REOPRO treatment and again 1 week after treatment discontinuation, when mice were euthanized. Spontaneous fresh thrombi that could have occurred after drug wash-out were looked for by Masson’s trichrome staining of aorta root cross-sections.

To evaluate the effect of plaque rupture/thrombosis, 14 50-week-old female mice were subjected to mechanical plaque rupture by repeated passage of a guide wire in the lumen of the innominate carotid artery. Blood CD31+ T cells were counted 1 day before wire injury and again at the time of euthanization 2 days later. Carotid arteries were carefully dissected and snap-frozen. Cross-sections from the proximal 400 μm were analyzed by Masson’s trichrome staining for the presence of carotid plaque rupture/thrombosis.

**Statistical Analysis**

Results are expressed as mean±SEM. Comparisons were performed by 1-factor ANOVA and correlations by simple regression (StatView). P<0.05 was considered statistically significant.

**Results**

**Histological Features of Plaque Thrombosis**

Samples from 65 apoE KO mice (46-week-old, 35 male, 30 female) were used for correlation between lesion morphometry and flow cytometry data. Organizing thrombi on atherosclerotic plaques were identified as discrete gray/cellular structures by Masson’s trichrome staining of aorta root sections (Figure 1). An organizing thrombus could be identified in 28 of 195 lesions (14%) and in at least 1 of the 3 aortic cusp lesions in 23 of 65 mice (35%). Collagen fibers were reduced around and within the thrombus but a thin layer of extracellular matrix often separated the thrombus from the
lumen (Figure 1). The fibrin/platelet and lipid components were significantly increased in the lesions with a thrombus (Table), whereas the mean lesion size was not different in plaques with or without thrombi (Table). Enriched macrophages (Table) and massive vascular cell adhesion molecule-1 positive areas (data not shown) could also be observed more frequently in the thrombus group. Gray/cellular areas contained only few lipid deposits as detected by oil red O staining (data not shown) and were confirmed to be organizing thrombi by fluorescent immunohistochemistry (Figure 2); they were composed essentially of fibrin, various amounts of platelets, and included several MAC3+ monocyte–macrophages that were mostly CD31− and a variable number of T cells (CD4+), few of which (28±5%) were positive for CD31 (Figure 2). The percentage of CD4+/CD31− cells in atherosclerotic lesions was significantly lower in the thrombus group as compared with the “no thrombus” group (Table) (P=0.001), whereas the number of total plaque-infiltrated CD4+ T cells tended to be higher in the thrombus group (Table) (P=0.2049, not significant).

### Reduced Circulating CD31+ T Lymphocytes Is a Hallmark of Plaque Thrombosis

The proportion of CD4+/CD25+/CD45RB+ natural regulatory T cells13 was very low in the mice used for the present study

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**Characteristics of Plaques With or Without a Thrombus**

<table>
<thead>
<tr>
<th></th>
<th>Thrombus (n=28)</th>
<th>No Thrombus (n=167)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Masson’s trichrome</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Fibrin/platelets (grey)*</td>
<td>9.9±0.3</td>
<td>5.1±0.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Fibrous tissue (green)*</td>
<td>42.3±1.6</td>
<td>37.7±1.4</td>
<td>NS</td>
</tr>
<tr>
<td>Picrosirius red</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Collagen (birefringent)*</td>
<td>12.7±1.2</td>
<td>34.5±1.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Oil red O</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipids (red)*</td>
<td>27.3±1.2</td>
<td>18.7±0.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lesion size†</td>
<td>21.5±0.8</td>
<td>22.3±0.6</td>
<td>NS</td>
</tr>
<tr>
<td>Immunohistochemistry</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Macrophages (DAB)‡</td>
<td>49.7±1.2</td>
<td>39.7±1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total CD4+ cells (IF)‡</td>
<td>22.0±7.8</td>
<td>12.5±3.3</td>
<td>NS</td>
</tr>
<tr>
<td>CD4/CD31+ cells (IF)‡</td>
<td>13.6±4.1</td>
<td>35.6±6.4</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Staining in the aorta root cross-sections was evaluated by computer-assisted image analysis.

*Percentage of positively stained areas within lesion surface area.
†Percentage of total vessel surface area occupied by lesions.
‡Percentage of immunolabeled positive cells among total cells within lesions.

DAB indicates diaminobenzidine; IF, immunofluorescence; NS, not significant. Data are expressed as mean±SEM.
and did not allow reliable statistical analysis. Preliminary studies in younger mice (8- to 24-week-old) showed that virtually all circulating leukocytes were CD31+. By contrast, in 46-week-old apoE KO mice, the percentage of CD31+ cells among circulating CD3+ T lymphocytes was between 61% and 94%. Interestingly, the percentage of blood CD31+ T cells was significantly reduced in the presence of a thrombus (72.3±1.5% of CD3+ lymphocytes) as compared with mice without thrombosis (84.1±1.2% of CD3+ lymphocytes; P<0.0001; Figure 3). The area under the receiver-operating characteristic curve was 0.865 (Figure 3). For values below the cutoff point of 81% of total CD3+ blood lymphocytes (sixtieth percentile), the sensitivity was 87.5% and the specificity was 73.2% (Figure 3). Therefore, reduced blood CD31+ T cell count has a good predictive value for thrombosis in mice. CD19+/CD31+ cells and total granulocytes, monocytes, CD19+, CD3+, CD4+, and CD8+ were similar in the 2 groups of mice (data not shown). Thus, the only CD31+ circulating leukocyte population that decreased in apoE KO mice with plaque thrombosis was the T cell population. No difference was observed between male and female mice in thrombus occurrence or peripheral CD31+ T cell count (data not shown); therefore, data from the 2 genders were pooled.

The percentage of blood CD31+ T cells was directly proportional to the collagen content in atherosclerotic lesions (R=0.345, P<0.01) and inversely correlated to the amount of fibrin/platelets (R=-0.460, P<0.001). No significant correlation was found between CD31+ T cells and the plaque content in lipid or fibrous tissue (data not shown), whereas the percentage of infiltrated macrophages in lesions tended to be inversely proportional to the percentage of blood CD31+ T cells without reaching statistical significance (R=-0.197, P=0.1164).

**CD31+ T Cell Reduction Is Not a Consequence of Experimental Plaque Rupture and/or Thrombosis**

Immune artery plaques of 14 50-week-old apoE KO mice were mechanically ruptured by 5 passages of a catheter guide wire through the external carotid artery. Four mice died shortly after wire injury. Plaque rupture and/or thrombosis were confirmed 2 days later by Masson’s trichrome staining (Figure 1) in all surviving mice (n=10). Flow cytometry analysis performed before and after wire injury demonstrated that blood CD31+ T cell percentage does not change after plaque rupture/thrombosis (mean delta=-1.3±7.2%, not significant). Independently of experimentally induced plaque rupture, time course variations of CD31+ T cell proportion were also monitored in a separate group of 10 40-week-old mice that were treated for 10 weeks with the antithrombotic agent REOPRO. One week after discontinuation of the antithrombotic treatment, 2 mice had a fresh thrombus over a plaque of the aortic root. Interestingly, these 2 mice showed a significant reduction of the CD31+ T cell count during the antithrombotic treatment and therefore likely before the occurrence of the thrombotic event (Figure 3).

**Immunoregulatory Function of CD31+ T Cells**

Specific proliferative T cell response to MM-LDL was inversely correlated to the percentage of circulating CD31+ T cells (n=27, R=-0.433, P<0.05; Figure 3). Frequency distribution of circulating CD31+ T cell percentage allowed assigning the mice to the bottom (<85%, n=4), middle (85% to 88%, n=4), and top (>88%, n=19) tertiles. T cell proliferation to MM-LDL was significantly enhanced in the bottom (3113±751 cpm; P<0.05) as compared with the middle (1651±303 cpm) and top (1610±206 cpm) tertiles. CD4+ spleen cell sorting allowed us to document that CD4+/CD31+ spleen T cells from apoE KO mice proliferated twice more than total CD4+ cells (Figure 4). Of note, 90±2% of total CD4+ spleen cells were CD31+. Similarly, significant differences were found in the production of cytokines in response to in vitro stimulation with MM-LDL between CD31+ and CD31- CD4+ T cells (Figure 4). Specifically, CD4+CD31+ T cells produced greater amounts of interferon-γ and less quantities of IL-4 and IL-10 than total CD4+ T cells (Figure 4). Conversely, no difference was detected in the supernatants of CD4+/CD31- T cell cultures as compared with cultures of total CD4+ T cells from C57Bl6 mice. Overall, T cell activation in response to MM-LDL appeared considerably lower in nonatherosclerotic C57Bl6 mice (Figure 4).
Discussion

The formation of a thrombus results from alteration of the vascular wall and is beneficial to arrest bleeding during homeostasis but may become a cause of catastrophic disease when it leads to thrombotic occlusion of atherosclerotic vessels curtailing arterial blood flow to vital organs. An alteration of the integrity of the vascular wall such as plaque rupture or the loss of endothelial cells at the surface of plaques (plaque erosion) is commonly observed in the sites of plaque thrombosis. However, the primary causes of this failure of integrity of the vascular wall (plaque rupture or erosion) remain unclear. A regulatory mechanism, less known, ensures that no thrombus is formed in the absence of the vascular wall damage. This regulation requires a dialog between the actors of thrombosis (platelets and coagulation factors) and the leukocytes present in the blood and the cells of the vascular wall with which they are in constant contact.

During the past years, a new paradigm has emerged: the acute clinical manifestation of atherosclerosis is characterized by a systematic increase of the markers of inflammation and by activation of lymphocytes. We have demonstrated that lymphocytes (T and B) of coronary patients are transiently activated and that their biological actions seem to be directed against the proteins present within the atheromatous plaques complicated by the thrombus. Thus, the loss of integrity of the vascular wall in plaque thrombosis may depend on an immunoinflammatory response directed against it. In other words, plaque thrombosis would be the result of a failure in the regulation of the blood/vessel interface. The key to such regulatory mechanism is the intercellular communication.

We postulated the hypothesis that CD31, a protein that binds to itself, is one such key element in this regulation because it is expressed on the surface of endothelial cells and of circulating platelets, monocytes, neutrophils, and lymphocytes.

The recent shift in the clinical paradigm of acute coronary syndromes led to a burst of activity in developing animal models related to plaque vulnerability and particular emphasis is set on the apoE KO atherosclerotic mouse model. In this model, spontaneous plaque thrombosis is described as extremely rare and external interventions such as chronic high-fat diet or mechanical injury coupled to p53 gene transfer have been used to increase the rate of plaque rupture/thrombosis.

In the present study, we report that, with aging, spontaneous plaque thrombosis occurs in 35% apoE KO mice. Plaques with a thrombus showed reduced collagen content but increased lipid pool and enhanced inflammation as compared with “stable” unthrombosed plaques, thus supporting the commonly used definition of “vulnerable plaques” in atherosclerotic mice.

Importantly, we report that a reduced percentage of circulating immunoregulatory CD31+ T cells is a hallmark of plaque thrombosis. Of note, we show that CD31+ T cell count does not change after mechanical plaque rupture. Furthermore, spontaneous reduction in CD31+ T cell takes place in the absence of thrombosis, during antithrombotic treatment with abciximab, and precedes the occurrence of spontaneous thrombosis after discontinuation of the drug. These data suggest that CD31+ T cell reduction is likely not a consequence of plaque rupture or thrombosis.

CD31+ T cells represent <20% of human adult peripheral blood lymphocytes and are able to regulate T cell activation in vitro. To the best of our knowledge, no data had confirmed such immunoregulatory property of CD31+ T cells in mice, probably because of the fact that virtually all T cells express CD31 in young (<24-week-old) mice.

Our study shows that, in aged (>40-week-old) atherosclerotic mice, T cell activation in response to the atherosclerosis-related antigen MM-LDL is inversely proportional to the percentage of peripheral T cells expressing CD31. Furthermore, CD31-depleted CD4 spleen cells show enhanced activation in response to MM-LDL as compared with total (≈90% CD31+) CD4 cells. These findings extend the previous data on human T cells and confirm that mouse CD31+ T cells also exert an immune control over specific T cell activation.

Remarkably, like in other experimental models of T cell-mediated inflammatory disease, lesion-infiltrated CD4+ cells are mainly CD31+. A reduced number of...
immunoregulatory CD31⁺ T lymphocytes as compared with CD4/CD31⁺ T cells in the lesion could lead to an increased susceptibility for the development of plaque thrombosis. Infiltrated CD31⁺ T cells, in the virtual absence of regulatory CD31⁺ T cells, might contribute to a greater tissue damage because we found that CD31⁺ T cells produce greater amounts of interferon-γ in response to specific stimulation in vitro and this cytokine appears critical in the immune response linked to plaque complications of patients with acute coronary syndromes.¹²⁶ The pro-inflammatory phenotype of MM-LDL–activated T cells in the absence of the immunoregulatory CD31⁺ T cells was further documented by the decreased production of IL-4 and IL-10.

As compared with cultured spleen CD4⁺ cells devoid of CD31⁺ cells, cultures containing CD4⁺/CD31⁺ cells produced greater amounts of IL-10. This suggests that immunoregulation by CD31⁺ T cells might depend, at least in part, on this cytokine, as is the case for other classes of regulatory T cells. Some of these such regulatory cells, like Th3 and Tr1 T cells, have been shown to modulate the inflammatory response linked to atherogenesis and to modify the extent of the disease.²⁷,²⁸ Our study provides the first evidence that the immunoregulatory CD31⁺ T cells may be implicated in plaque thrombosis. We cannot presently rule out a role for the other natural regulatory CD4⁺/CD25hi/CD45RBlo T cells as a peripheral risk marker of plaque thrombosis that can be easily and repeatedly evaluated in living mice.

Acknowledgments

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References

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