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Altered distribution and function of splenic innate lymphoid cells in adult chronic immune thrombocytopenia

Sylvain Audia[a,b,*,1], Thomas Moulinet[a,1], Marion Ciudad-Bonté[a], Maxime Samson[a,b], Olivier Facy[c], Pablo Ortega-Deballon[c], Philippe Saas[a], Bernard Bonnotte[a,b]

[a] Université de Bourgogne Franche-Comté, INSERM, EFS BFC, UMR1098, Interactions Hôte-Greffon-Tumeur/Ingénierie Cellulaire et Génique, F-21000 Dijon, France
[b] CHU Dijon Bourgogne, Service de Médecine Interne et Immunologie Clinique, Centre de Référence Constitutif des Cytopénies Auto-immunes de l'adulte, F-21000 Dijon, France
[c] CHU Dijon Bourgogne, Service de Chirurgie Digestive, Cancérologique, Générale et d'Urgence, F-21000 Dijon, France

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ABSTRACT

Innate lymphoid cells (ILCs) have been characterized as innate immune cells capable to modulate the immune response in the mucosae. Human ILCs have been rarely described in secondary lymphoid organs except in tonsils. Moreover, their function and phenotype in human secondary lymphoid organs during autoimmune diseases have never been studied. We took advantage of splenectomy as a treatment of immune thrombocytopenia (ITP) to describe and compare splenic ILC from 18 ITP patients to 11 controls. We first confirmed that ILC3 represented the most abundant ILC subset in human non-inflamed spleens, accounting for 90% of total ILC, and that they were mostly constituted of NKp44$^-$ cells. On the contrary, proportions of ILC1 and ILC2 in spleens were lower than in blood. Splenic IL-2- and IFN-γ-producing ILC1 were increased in ITP. While the frequencies of total splenic ILC3 were similar in the two groups, splenic GM-CSF-producing ILC3 were increased in ITP.

This is the first description of human ILC in a major secondary lymphoid organ during an autoimmune disease, ITP. We observed an expansion of splenic ILC1 that could participate to the Th1 skewing, while the increased production of GM-CSF by splenic ILC3 could stimulate splenic macrophages which play a key role in ITP pathophysiology.

1. Introduction

Innate lymphoid cells (ILC), defined as lymphoid cells that lack the expression of rearranged antigen-specific receptors, are categorized in 3 groups, according to their phenotype, transcription factors and functions. Group 1 ILC includes *Natural Killer* (NK) cells that depend on the transcription factor *Eomes*, and ILC1 that express T-bet and produce IFN-γ in response to IL-12. ILC2 depend on GATA-3 and produce IL-5 and IL-13, while ILC3 are characterized by the expression of RORγT and produce IL-17, IL-22 and GM-CSF [1,2]. ILC are mostly localized in the mucosal tissue (gut, lungs, skin), whereas they represent only a minor part of the immune cells in blood and in secondary lymphoid organs (SLO). They participate to the initiation of the immune response and modulate the adaptive immune response by promoting B cell activation and antibody production, and by activating or inhibiting T cell functions [3–6]. Thus, ILC are innate cells that physiologically participate to tissue homeostasis and orchestrate the first steps of the adaptive immune response. There is also growing evidence for their role in human pathology [7], notably in autoimmune diseases (AID) such as systemic sclerosis [8], psoriasis [9], during the early stages of rheumatoid arthritis [10] and also in graft versus host disease [11].

Immune thrombocytopenia (ITP) is an AID with a complex pathogenesis that is better understood [12]. Briefly, B-cells produce antiplatelet antibodies that facilitate the phagocytosis of platelets by splenic macrophages. The stimulation of B cells and the production of autoantibodies occur in the spleen and are orchestrated by T follicular helper cells [13]. Splenic T lymphocyte polarization is skewed to Th1 and cytotoxic T cells also participate to platelet destruction [12].

Here, we aimed to determine whether splenic ILC frequencies and functions were altered during ITP. We also compared the proportion and phenotype of ILC subsets between spleen and blood of healthy controls.

* Corresponding author. Batiment B3, INSERM 1098, 15 rue Maréchal de Lattre de Tassigny, 21000 Dijon, France.
E-mail address: sylvain.audia@u-bourgogne.fr (S. Audia).
[1] Both authors contribute equally.

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Table 1
Characteristics of splenectomized subjects.

	Controls (n = 11)	ITP (n = 18)	*p*-value
Age, years	47 [28.5–66.5]	48.4 [29.2–54.8]	0.7
Sex ratio (Female/Male)	6/5	9/9	1
Platelet count, x10^9/L	231.5 [138–285]	25 [15–28]	< 0.001
Disease duration, months		24 [16.5–61.8]	
Previous treatments, n (%)			
Steroids		16 (88.8)	
Intravenous immunoglobulins (IVIg)		14 (77.7)	
Rituximab		8 (44.4)	
Thrombopoietin-Receptor Agonists (TPO-RA)		8 (44.4)	
Dapsone		7 (38.8)	
Hydroxychloroquine		2 (11.1)	
Vincristine		1 (5.5)	
Danazol		1 (5.5)	
Treatment within the 2 weeks prior to splenectomy, n (%)			
IVIg		7 (38.8)	
TPO-RA		6 (33.3)	
Steroids		4 (22.2)	
None		1 (5.5)	
Response to splenectomy, n (%)		14 (77.7)	

2. Methods

2.1. Patients

Patients and healthy donors were enrolled after giving an informed consent in accordance with the declaration of Helsinki (Clinicaltrials.gov: NCT02042560). This study was approved by the local ethic committee. Primary ITP were included (platelet count < 100×10^9/L with exclusion of familial, viral, drug-induced thrombocytopenia, and other auto-immune disease-related thrombocytopenia). Splenectomy was performed as second line therapy, at least one year after the onset of the disease, as recommended [14,15]. Spleens of 18 ITP patients were compared with 11 control spleens (10 post-traumatic spleens, 1 organ donor). None of the latter suffered from autoimmune diseases, infections or cancer. Their characteristics are reported in Table 1. Blood was obtained from 9 healthy donors.

2.2. Spleen preparation

Sterile spleen tissues were mechanically disrupted using a syringe plunger. After the cells were dissociated, they were filtered through a 100 µm nylon strainer. Red blood cells were removed by a 10-min incubation in a hemolytic solution (150 mM ammonium chloride, 10 mM potassium bicarbonate, and 0.1 mM EDTA) at room temperature. Cells were washed and filtered again. Splenocytes were stored in liquid nitrogen until needed.

2.3. Flow cytometry

The antibodies used for splenocyte and peripheral blood cell staining are reported in Table 2. Lineage (Lin) cells were defined by the expression of at least one of the following markers: CD3, CD14, CD19, CD20, CD94, FcεRI, TCRαβ, TCRγδ, CD123 and CD34. For membrane staining, 1–2 x 10^6 cells were incubated for 20 min with the appropriate antibodies or control isotypes. Intracellular staining was performed after cells were fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) following the manufacturer's instructions. For cytokine measurement, intracellular staining was

Table 2
Antibodies used for flow cytometry.

	Antibodies	Fluorochrome	Clone	Manufacturer
Lineage cocktail	Lineage cocktail 3 (CD3, CD14, CD19, CD20)	Fluorescein isothiocyanate (FITC)	SK7, MϕP9, SJ25C1, L27	BD Biosciences
	CD16 (FcγRIII)	FITC	eBioCB16	eBioscience
	CD34	FITC	581	BD Biosciences
	CD94	FITC	DX22	eBioscience
	CD123	FITC	7G3	BD Biosciences
	CD303 (BDCA-2)	FITC	201A	Biolegend
	TCRα/β	FITC	IP26	eBioscience
	TCRγ/δ	FITC	B1.1	eBioscience
	FcεRI	FITC	AER-37(CRA1)	eBioscience
ILC markers	CD117 (ckit)	Brilliant violet (BV)421	104D2	Biolegend
	CD127	BV510	A019D5	Biolegend
	CD161	BV605	HP-3G10	Biolegend
	CD294 (CRTH2)	Phycoerythrin-Cyanin7 (PE-Cy7)	BM16	Biolegend
	CD336 (NKp44)	Peridinin-Chlorophyll Protein complex (PerCP)-eFluor710	44.189	eBioscience
Chemokine receptors	CCR6	AlexaFluor (AF)647	G034E3	Biolegend
	CXCR3	PE	1C6	BD Biosciences
Cytokines	BAFF	Allophycocyanin (APC)	1D6	eBioscience
	GM-CSF	APC	BVD2-21C11	Miltenyi
	INF-γ	APC	4S.B3	eBioscience
	IL-2	APC	MQ1-17H12	eBioscience
	IL-4	FITC	8D4-8	eBioscience
	IL-17	PE	eBio64CAP17	eBioscience
	IL-22	PE	22URTI	eBioscience
	TNF-α	PE	Mab11	eBioscience
Lymphocyte markers	CD3	BV510	OKT3	Biolegend
	CD4	PE-Cy7	SK-3	eBioscience
	CD8	Pacific Blue (PB)	RPA-T8	BD Biosciences
Activation and costimulatory molecules	CD86	PE	IT2.2	eBioscience
	HLA-DR	APC	AC122	Miltenyi
Transcription factors	T-bet	PE	eBio4B10	eBioscience
	RORγt	APC	AFKJS-9	eBioscience
Others	Ki67	PE	REA183	Miltenyi