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Letter to the Editor

CAR-T Cells Microscopic and Phenotypic Identification in the Peripheral Blood

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To the editor.

The infusion of T cells with a chimeric antigenic receptor (CAR-T cells) represents a recent therapeutic innovation in managing hemopathies. As an indication for large B–cell lymphoma, CAR-T cells are used for patients in relapse after several conventional treatment lines.[1] Nowadays, clinical trials propose this therapeutic approach as second-line therapy (ZUMA-7 clinical trial)[2] and even as first-line therapy in high-risk patients (ZUMA-12 trial).[3] In practice, the collection of patients' T cells is performed by leukapheresis: these T cells will then be genetically modified using a viral vector to make them express a chimeric receptor able to recognize a surface antigen expressed by tumor cells.[4] After being transduced, the CAR-T cells are reinjected by intravenous infusion.[5] The morphologic and immunophenotypic description of CAR-T cells observed in residual cells of CAR-T cells infusion bag has just been described by Galli et al. in this journal.[6] Here we confirm their findings in the peripheral blood (PB) and report about our experience of CAR-T cells automated and microscopic detection and immune monitoring.

A 54-year-old woman was diagnosed in October 2017 with stage III large cell B-cell lymphoma. She was first treated with chemotherapy until complete remission but relapsed in December 2018, and the second line of chemotherapy was undertaken to allow the achievement of a new complete remission. Then, an autologous transplant was performed in April 2019 to intensify the treatment. Unfortunately, a second relapse occurred in May 2020, and a CAR-T-cells therapy project was validated in July. The anti-CD19 CAR-T cells (Axicabtagene Ciloleucel, Yescarta, Kite Gilead) were infused in September after usual conditioning by cyclophosphamide and fludarabine. Blood counts (XN 9000 automated counter, Sysmex, Japan) after CAR-T cells infusion revealed expected pancytopenia due to the depletion conditioning regimen. However, the follow-up of the white differential fluorescence (WDF) channel from the automated counter during the first four days was eventless.

On the fifth day, dots were observed in the area corresponding to high fluorescence cells, flagged as "atypical lymphocytes" (**Figure 1**). Seven days after infusion, the automated counter detected unusual cells in another area (white dots) where abnormal lymphocytes and blasts are usually observed, leading to rejection of the automated leucocytes differential analysis. The WDF channel becomes without peculiarity again from the tenth day, and no more dots were observed in the window above the normal lymphocytes. In the blood smear, several unusually large cells were observed (day 5 post-infusion of CAR-T cells in **Figure 2**). Their sizes were about 15 to 20 µm. These cells were composed of atypical lymphocytes, some with a nucleole, others with immature blastic-like appearance (corresponding to undifferentiated cells at J7), and often

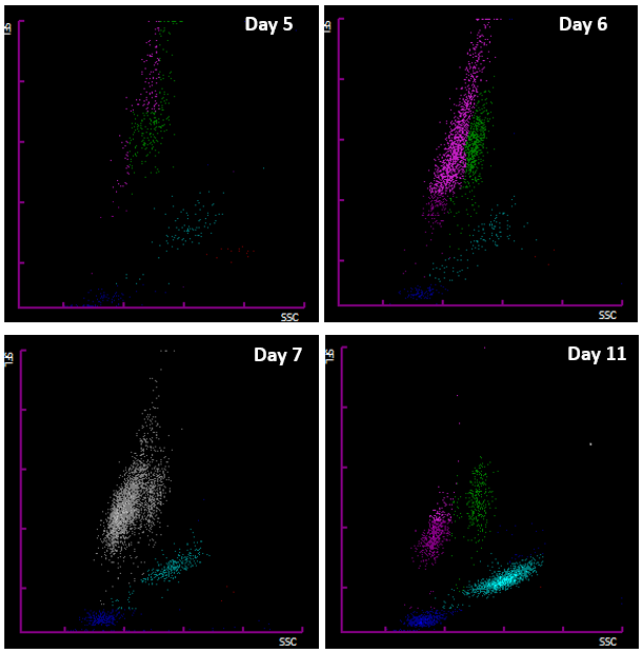

Figure 1. WDF channel (Sysmex-XN counter). Dots corresponding to lymphocytes are pink-colored (days 5 to 11).

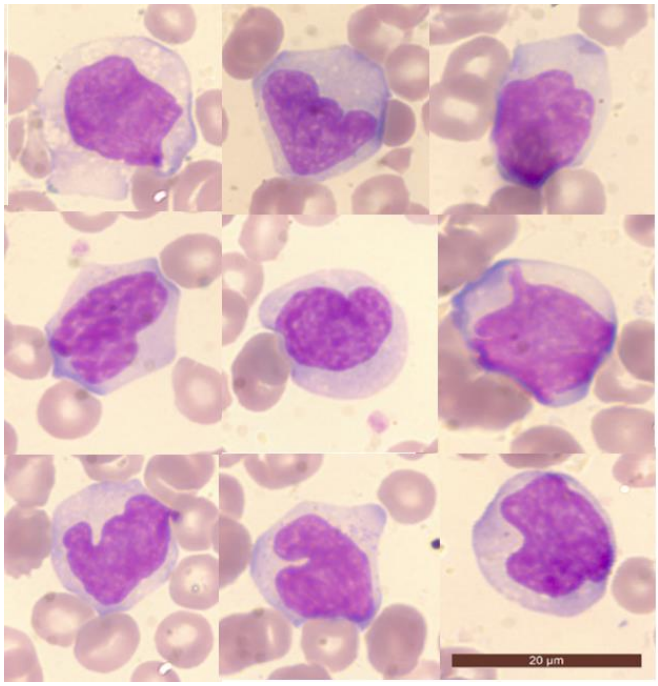

Figure 2. Peripheral blood atypical lymphocytes observed after CAR-T cells infusion (day 5; May-Grunwald-Giemsa stain; x1000).

with a lobulated nucleus. The cytoplasm of these cells was basophil and sometimes with peripheral strengthening. Very frequently, small vacuoles and small azure granulations were observed, both at the limit of visibility with an x50 microscopic average objective. All these lymphoid cells were observed in the first few days when no monocytes and only a few neutrophils were observed, stressing their potential relationship with CAR-T. As mentioned by Galli et al., a possible relapse of the malignant disorder might have been suspected in the absence of context knowledge.[3] From day seven post-infusion, immuno-phenotyping of whole blood by flow cytometry demonstrated the existence of a strong CD45 lymphocyte population, composed of CD3+ T lymphocytes, with a CD4+ (56%) and CD8+ (36%) distribution, a CD3-CD16/56+ NK component (8%). There was a complete lack of CD19 expression by lymphoid cells (**Figure 3**). Repeated flow cytometry analysis confirms initial data, and a full decrease of CAR-T cells was confirmed within 60 days (**Table 1** and **Figure 4**).

It seemed likely to predict the potential presence of atypical lymphoid cells by observing the WDF channel of the Sysmex XN counter. We confirmed in the PB the heterogeneous cytomorphology described by Galli et al. in the leftovers, including few large granular lymphocytes but notable medium-sized basophilic lymphocytes, large promonocytic-like cells, and giant blast-like cells.[3] Large basophilic cells with convoluted nuclei were very easily detected under the microscope during the expansion of CAR-T (focused on **Figure 2**).

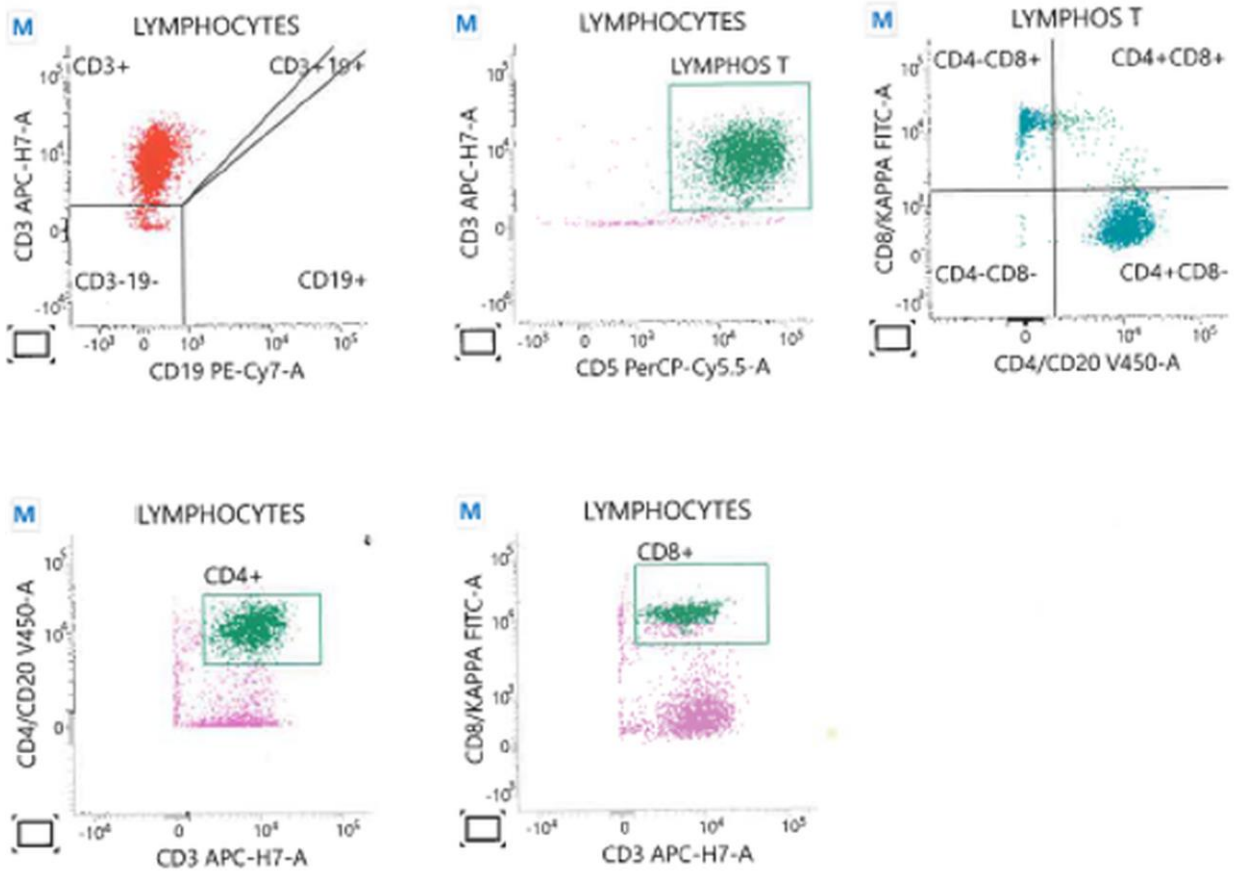

Figure 3. Lymphocytes immunophenotypic assessment at day 5.

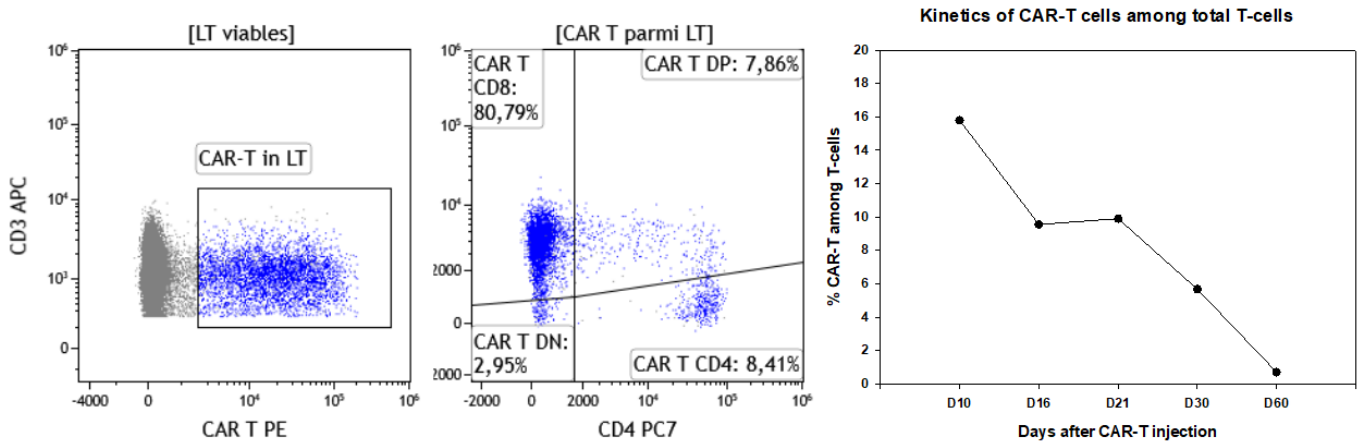

Figure 4. CAR-T cells repartition (day 21) and CAR-T cells among total T cells (follow-up, days 10 to 60).

Table 1. Immuno-phenotypic follow-up (day 5 to 91).

Day	3	4	5	6	7	14	21	91
Leukocytes (x10^9/L)	0.120	0.190	0.440	2.520	4.180	1.550	1.460	4.130
Lymphocytes (%)	0	0	76	89	93	24	29	10
Atypical lymphocytes (%)	0	0	70	74	78	2	0	0
Lymphocytes (x10^9/L)	0	0	0.334	3.532	3.894	0.371	0.432	0.416
CD3 (%)	0	0	91	ND	98.4	97.1	92.3	54.6
including CD4 (%)	0	0	56	ND	29.6	24.9	31.7	25.7
including CD8 (%)	0	0	36	ND	68.2	66.8	56.7	26.6
CD19 (%)	0	0	0	ND	0	0	0	0
CD56 (%)	0	0	4	ND	1.3	2.8	7.4	42

Immunophenotyping of patient PB after CAR-T cells show a mixture of CD4+ and CD8+ T cells, sometimes NK cells. These cells correspond, among others, to the reinjected cells, observed a few days after infusion and linked to the well-documented expansion peak of CAR-T cells generally between 5 to 10 days post-infusion. Our case report allowed us to document that (i) day 5 is the day of detectable CAR-T in whole blood, (ii) day 7 is the peak of expansion, (iii) the total time exposure to CAR-T lasted more than 21 days, (iv) CD8+ cells decrease with time, and (v) that B-cell aplasia persisted for more than 90 days. Pharmacokinetics revealed that the circulation of CAR-T cells in peripheral blood was linked to a better response to the treatment.

Furthermore, according to the ZUMA-2 study of CAR-T cells Tecartus (Brexucabtagene Autoleucel, Kite Gilead), the number of anti-CD19 CAR-T cells in the blood was positively correlated with an objective response (complete or partial response).[7] Concerning prolonged B-cell aplasia, an indirect marker of anti-CD19 CAR-T cells potency, it highlights that immune monitoring using flow cytometry is mandatory. CAR-T cells currently promise hopeful options for curing malignant diseases (nowadays mainly in onco-hematology field) and represent a new challenge for assessment in hematology laboratories from initial to long-term patient follow-up.[8]

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Competing interests: The authors declare no conflict of Interest.

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