Acute myeloid leukaemia (AML) is a heterogeneous group of malignant haemopathies involving the defective differentiation of myeloid progenitors (Estey & Dohner, 2006). At diagnosis, AML patients show severe defects in controlling tumour growth. This is partly due to the immunosuppressive environment created by leukaemic blasts (Buggins et al., 2001). Several mechanisms have been implicated in this process, including the expansion of CD25highFoxP3+ (Forkhead-box-P3) regulatory T-cells (Tregs) (Shenghui et al., 2013; Szczepanski et al., 2009). Indeed, a recent report suggested that the T-cell mediated immune response against AML could be predicted based on the composition of Treg subsets (Schick et al., 2013).

Among the mechanisms used by Tregs to inhibit immune responses against cancers, increasing evidence points to a role for extracellular adenosine produced by the ectonucleotidases CD39 and CD73 (Beavis et al., 2012). This extracellular adenosine can bind to adenosine receptors, including the A2A receptor (A2AR), which are expressed on various immune cells. Adenosine suppresses T and Natural Killer (NK) lymphocyte anti-tumour functions (Hausler et al., 2011).

To assess the role of Treg subpopulations in creating an immunosuppressive environment in AML, we examined the CD25high and FoxP3+ regulatory CD4+ T-cell populations in the peripheral blood from 46 AML patients at diagnosis and 21 healthy controls (HCs) by flow-cytometry (Fig S1, Tables SI, SII). The percentage of CD4+ cells among CD3+ T-cells was comparable in patients and HCs (medians: 77.0% vs. 73.3%, respectively; NS). As in previous studies (Schick et al., 2013; Shenghui et al., 2011; Szczepanski et al., 2009), patients had an increased frequency of circulating CD25high cells (medians: 7.7% vs. 4.3%, respectively; P = 2.10−5) and FoxP3+ CD4+ T-cells (medians: 5.4% vs. 2.9%, P = 1.10−2) (Fig 1A). Compared to HC samples, all AML categories expressed CD73 at very low levels. Only a minority of Tregs in patients and HC expressed CD73 at very low levels.

The increase in FoxP3+CD4+ Tregs prompted further analysis of the naïve CD45RAFoxP3low and memory CD45RA−FoxP3high Treg subsets (Miyara et al., 2009) in 10 representative patients and 11 HC (Fig S2A and Table SII). These samples showed no imbalance in percentages between naïve and memory Treg subsets, and both were CD25High/C-D127Low compared to conventional CD4+ T-cells (Fig S2B). However, CD25 expression on FoxP3+ Treg subsets was strongly reduced on naïve and memory Tregs in patients compared to controls (Fig 1B), although this expression remained higher than that of the conventional CD4+ T-cells from the same patient (Fig S2 and Table SIII).

The expression of homing receptors specific for skin (CLA and CCR4), intestine (CD49d, CD103), lymph nodes (CCR7) and inflamed tissues (CCR6) was then analysed on Treg subpopulations. Only CCR4, CCR6, CCR7 and CD49d were detectable on Tregs from both patients and HC (Figs 1C, S2C, Table SIII), and expression was significantly decreased on patients’ T-cells (both conventional and Treg populations). This suggests that all T-cell subpopulations in AML patients at diagnosis have an altered capacity to migrate into tissues.

Because the phenotype of all T-cell populations was significantly affected in AML patients compared to HC, we investigated whether blast cells were involved in this remodelling. We examined CD39 and CD73 expression on Tregs and the corresponding AML cells, hypothesizing that both cells could generate an adenosine-enriched environment potentially affecting patient immunity. CD39 was detectable in all T-cell subsets in patients and HC, with higher expression levels on Tregs (Table SIII and Fig S2C). However, CD39 expression levels, but not CD39+ Treg percentages, were decreased in patients compared to HC (Fig 2A). This suggests that CD39 function in Tregs could be maintained in AML patients. Finally, only a minority of Tregs in patients and HC expressed CD73 at very low levels.

CD39 and CD73 expression was also analysed on CD45low/CD43low/Lin− tumour cells (Figs 2B and S3). CD39 was frequently detected on AML blasts, with high inter-patient variability. CD39 expression was equivalent on these cells compared to mature CD45high/CD34low/Lin− T-cell populations. In contrast, CD73 expression was generally lower on blasts than on mature populations (medians: 1.7% vs. 19.0%, respectively; P = 3.10−2), except in two patients who had a vast majority of CD73+ leukaemic cells resulting in about 50% of tumour cells being positive for both ectonucleotidases.

Ectonucleotidase function was then examined in AML cells by stimulating carboxyfluorescein succinimidyl ester-labelled peripheral blood lymphocytes from HC with a CD39+CD73− HL60 AML cell line. The CD39-specific inhibitor, ARL67156 (Hausler et al., 2011), was added to some cultures. After a 5 day incubation, the proliferation index showed that proliferation of CD8+ but not CD4+ T-cells was weakly but consistently inhibited by AML cells (Fig 2C). This difference was

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Fig 1. Increased percentage and altered phenotype of Treg subsets in AML patients at diagnosis. (A) Increased percentages of CD25<sup>high</sup> and FoxP3<sup>+</sup> Treg cells among CD4<sup>+</sup> T lymphocytes in all acute myeloid leukaemia (AML) patients at diagnosis (n = 46) or according to the French-American-British (FAB) classification compared to healthy controls (HC, n = 21). Individual values are shown as open circles; the black bar marks the median value for each group. (B-C) Naïve CD45<sup>RA</sup>+</sup>FoxP3<sup>+</sup>CD4<sup>+</sup> and memory CD45<sup>RA</sup>-FoxP3<sup>hi</sup>CD4<sup>+</sup> Treg subsets in patients (n = 10) and HC (n = 11). Expression of (B) the CD25 and CD127 cytokine receptors and (C) the CCR4, CCR6 and CCR7 chemokine receptors and CD49d integrin. Expression levels were assessed as the ratio of the mean of fluorescent intensity (Exp MFI) for each marker divided by the MFI for the isotype control (Ctrl MFI). *P values indicated above plots were calculated using a Mann-Whitney test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns: not significant.
not observed in the presence of ARL67156, indicating a role for CD39 in this effect. Both CD4+ and CD8+ subsets express the A2AR (Beavis et al., 2012; Nikolova et al., 2011), but CD8+ T-cells were more often CD73+ (up to 10-0% of CD8+ T-cells in patients and 16-8% in HC, Table SIII). This suggests that CD39+ AML-cell-mediated inhibition could depend on CD73 expression on CD8+ T-cells.

Altogether, the increase in the proportion of circulating Treg cells in AML patients at diagnosis, with the altered expression of cytokine and homing receptors on Treg subsets, suggest delocalization of these cells from tissues to peripheral blood. Although Treg percentage or phenotype has not been linked to disease outcome in this cohort, peripheral blood. Although Treg percentage or phenotype has not been linked to disease outcome in this cohort, increased Treg subsets could indicate an overall immunosuppressive environment in tissues including the bone marrow. This environment would be supported by expression of the ectonucleotidases CD39 and CD73 observed in some patients, not only on Treg subsets but also on AML blasts. Adenosine production by AML cells, supported by Tregs expressing ectonucleotidases, could prevent an anti-tumour CD8+ T-cell response, which is reminiscent of previous observations (Schick et al., 2013). This adenosine production could also contribute to resistance to treatments, as observed in chronic lymphocytic leukaemia where extracellular adenosine protects tumour cells from apoptosis induced by chemotherapeutic agents (Serra et al., 2011). Altogether, this work indicates that Treg can collaborate with leukaemic cells to produce extracellular adenosine, contributing to the immune escape of AML.

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Author contributions

ND, AB and AT designed the study; ND, GH and PH performed experiments; ND, GH, PH, ZK, AB and AT analysed data; HD and NB collected samples; ND wrote the manuscript; ZK, HD, NB, AB and AT critically reviewed the manuscript.

Disclosure and competing interests statement

Dr A. Bensussan is cofounder and shareholder of OREGA Biotech. The other authors have no competing interests.

Nicolas Dulphy1,2,3
Guylaine Henry2
Patrice Hemon4,5
Zena Khaznadar1,3
Hervé Dombret6,7
Nicolas Boissel6,7
Armand Bensussan4,5*
Antoine Toubert1,2,3*

1Institut Universitaire d’Hématologie, Sorbonne Paris Cité, Univ Paris Diderot, 2Department of Immunology and Histocompatibility, Assistance Publique–Hôpitaux de Paris (AP–HP), Hôpital Saint-Louis, 3UMR-S940, Institut National de la Santé et de la Recherche Médicale (INSERM), 4UMR-S976, INSERM, 5Laboratory of Immunology, Dermatology and Oncology, Sorbonne Paris Cité, Univ Paris Diderot, 6Department of Adult Haematology, AP–HP, Hôpital Saint-Louis, and 7EA-3518, Sorbonne Paris Cité, Univ Paris Diderot, Paris, France.
E-mail: nicolas.dulphy@univ-paris-diderot.fr

*AB and AT contributed equally to this manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Materials and methods.
Table S1. AML patients and HC recruited in the study.
Table SII. Antibody panels and clones used in the study.
Table SIII. Levels of markers on T-cell subsets in AML patients at diagnosis and in HC.

Fig S1. Gating strategy used to identify CD25HI and FoxP3HI CD4+ T-cells in blood.

Fig S2. Treg subset phenotypes in AML patients.

Fig S3. AML blasts from AML patients at diagnosis express heterogeneous levels of CD39 and CD73.
References


