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Commentary on" The FLAMSA Concept Past and Future"

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The FLAMSA concept was designed as a conditioning regimen for acute leukemia separating leukemia therapy from reduced intensity conditioning. Leukemia treatment consists of a short and intensive chemotherapy followed after a 3-day rest by conditioning with intensive immune suppression and reduced intensity of myeloablative (stem cell toxic) treatment preferably total body irradiation (TBI). Furthermore, it included T cell depletion "*in vivo*" with antithymocyte globulin (ATG) for prophylaxis of graft-versus-host disease followed by restitution of immunity by the transfusion of donor lymphocytes [1-3]. The first experiences in patients with high-risk acute myeloid leukemia were encouraging; survival of relapsed and primary refractory patients was improved as compared to previous studies. Duration and intensity of prior chemotherapy was critical; patients with primary refractory disease and no more than 2 cycles of therapy had an excellent chance of continued remission [4,5].

The concept of FLAMSA sequential conditioning was convincing to many groups; reports of single centers and multicenter studies were published [6-19] (Table 1). Several studies used busulfan [20], treosulfan [21-24], or melphalan [16] instead of total body irradiation (TBI), retrospective comparisons found results equivalent of FLAMSA-TBI to myeloablative conditioning [25]. However, there are several reports on retrospective studies [26], only one prospective randomized study of FLAMSA compared to other forms of conditioning (FIGARO trial) described no improvement of relapse rate and survival with FLAMSA [27]. In contrast to the original study most patients in the FIGARO trial were in remission; these patients had less benefit from additional chemotherapy. In the original FLAMSA study chemotherapy with cytarabine (ARA-C), fludarabine and amsacrine was necessary to reducing blasts immediately prior to conditioning with total body irradiation, antithymocyte globulin and cyclophosphamide.

A second finding of the FIGARO trial was that the rate of patients with minimal (measurable) residual disease (MRD) was not reduced despite additional FLAMSA chemotherapy. MRD is characterized as disease surviving standard chemotherapy. In acute myeloid leukemia (AML) residual disease most likely differs qualitatively form the bulk of blasts. Leukemia cells constituting MRD are resistant to most chemotherapy because they are enriched of dormant or slowly proliferating cells, presumably leukemia stem cells. These cells cannot be eliminated by a short intensive chemotherapy, they can only be killed independently of their cell cycle by radiation or stem cell toxic (myeloablative) drugs as busulfan, treosulfan, melphalan, thiotepa and similar. In the FIGARO trial busulfan in reduced doses was given in both arms. The original FLAMSA regimen contained total body irradiation (TBI) with 4 Gy. On an average, 4 Gy radiation inactivates 99 percent of progenitor cells. Ideally TBI was applied as a single dose at very low dose rates (<10cGy/min). This way the inactivation of hematopoietic progenitor cells was improved in relation to non-hematopoietic toxicities [28]. Normal stem cells compete with leukemia stem cells for the niche in the bone marrow [29], the niche being adjusted to the leukemia stem cell. These leukemia stem cells are protected against chemotherapy in the niche. Therefore, it is important to achieve full donor chimerism as soon as possible. Three months after transplantation full donor chimerism was achieved in only 50 percent of patients in the FIGARO trial [27] as compared to virtually all patients in the original FLAMSA trial [1]. The intensity of conditioning may not be important for patients transplanted in remission;

Table 1:

Study	No of pats.	Conditioning	Non-relapse Mortality*	Rel inc.*	Survival (time)*	Reference
Original	75	FLAMSA + 4Gy TBI, ATG, CY	33%(1yr)	n.a.	42%	[1] [4]
German multicenter	121 20	FLAMSA-TBI FLAMSA-BUS	18% na	28% na	44% (4yr) na	[10]
British multicenter random. trial	176	FLAMSA -BUS control	20% (1yr) 16% (1yr)	26.7% 29.5%	56% 57%	[27]
French Study refractory or relapsed AML	58 41	FLAMSA/clofarabin myeloablative	17% 15%	57% 50%	33% 39%	[70]
HR MDS upfront Duesseldorf	30	Mel 24pts Mel+TT 6 pts.	20% (4yrs) 66% (4Yrs)	30%	70%	[51]
AML elderly median age 46	72 58	FLAMSA-TBI FLAMSA-Treo	13% 28%	70% 35%	47% 43%	[23]
AML high risk rel/ref poor cytogenetkics	60	FLAMSA-TBI	28%	n.a.	42%	[6]
AML r/R	31 12	FLAMSA-TBI FLAMSA-Bu	20-26%	n.a.	39%	[18]
refr AML	34 10	FLAMSA-TBI FLAMSA-BUS	18%&	69	15%	[11]
AML active dis haplo	21 12	FLAMSA-TBI/Bus Clof-TBI	15%	35%	48%	[19]
EBMT study rel/refr	213 40 13	FLAMSA -TBI 4Gy FLASMA-BU-CY FLAMSA-BU or Mel	25,9%	48,5%	30,4%	[13]
EBMT high risk AML in remission	159 106	FLAMSA-TBI FLAMSA-BUS	19.4% 31.1%	21.2% 25.7%	62% 46.7%	[7]
EBMT CR1/CR2**	203 145 281	FLAMSA-TBI FLAMSA-BUS Treo/Flu	15,2% 25,2% 13,2%	20,4% 27,6% 39%	69% 53,4% 59,9%	[21]
EBMT CR1/CR2	1197 258 141	FLU-BUS FLAMSA-TBI FLAMSA -Bus	16.1% 16,4% 26,7%	30,3% 21,9% 23,1%	60% 69% 49,4%	[20]
EBMT study refr/rel	113 112 631	FLU-Treo TBF (Thiot BU Flu) FLAMSA	5% 13% 6%	46% 54% 53%	37% 24% 34%	[24]
EBMT refr/rel	258 314 318 128	BUS-CY CY-TBI FLAMSA-TBI FLAMSA-Chemo[a]	16% 19% 18% 7%	56% 51% 55% 53%	33% 34% 36% 50%	[14]

*If not otherwise indicated 2 years

**Significant differences in year of transplant, patient age, remission status, FLT3-ITD, CMV seropositivity, in vivo T cell depletion, stem cell source (bone marrow vs. pbsc)

a chemo included busulfan 6,4 mg/kg, or melphalan or CY or treosulfan TBI dose 4 Gy without info on fractionation and dose rate, oral busulfan and *ex vivo* T depletion were excluded; ATG treatment not specified, DLI not given

it is important for patients transplanted with active disease [30]. TBI has the advantage of exact dosing and the inclusion of metabolically restricted sites, it has the disadvantage of the requirement of a large field radiation facility. Therefore, multicenter studies with radiation are difficult to perform. The combination of cyclophosphamide (CY) and anti-thymocyte globulin (ATG) may also contribute to the induction of complete chimerism at 3 months. This combination has been successful to abrogate sensitization by prior transfusions in dogs [31] and in conditioning patients with severe aplastic anemia [32].

Thirdly, the higher incidence of acute GVHD may be caused by insufficient T cell depletion. The use of mobilized blood stem cells instead of marrow comprises a higher content of T cells. T cell antibodies should persist in the patient in significant concentrations to inactivate donor T cells. Dependent on the preparation of ATG 60 mg/kg are enough to inactivate donor T cells "*in vivo*" [33], saturation of CD52 by alemtuzumab was sufficient and GVHD prevented with 30 mg in the HLA-identical situation [34]. Higher doses were complicated by infections and delayed immune restitution [34].

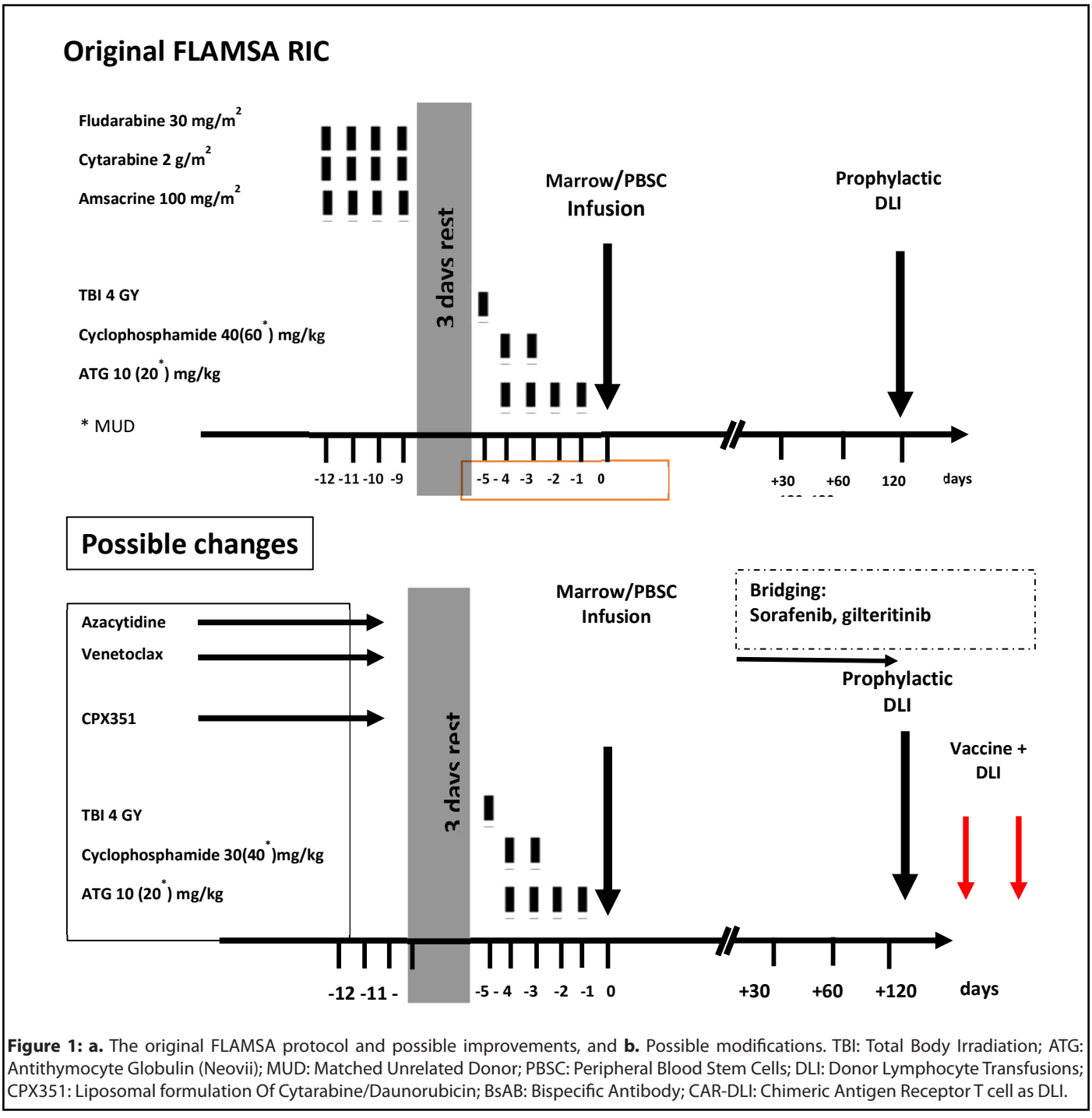

Figure 1: a. The original FLAMSA protocol and possible improvements, and **b.** Possible modifications. TBI: Total Body Irradiation; ATG: Antithymocyte Globulin (Neovii); MUD: Matched Unrelated Donor; PBSC: Peripheral Blood Stem Cells; DLI: Donor Lymphocyte Transfusions; CPX351: Liposomal formulation Of Cytarabine/Daunorubicin; BsAB: Bispecific Antibody; CAR-DLI: Chimeric Antigen Receptor T cell as DLI.

The fourth component of sequential therapy, donor lymphocyte transfusions (DLI) were planned for restitution of immunity after T cell depletion. Adjuvant DLI were beneficial in a matched case comparison [35], but many centers were reluctant because of the risk of GVHD. A recent time dependent analysis of DLI after FLAMSA conditioning in high-risk AML did not find a benefit of DLI [36]. However, a study of similar patients receiving *ex vivo* T cell depleted transplants showed a significant benefit of DLI [37]. Furthermore, post-transplant relapses could be treated with donor cells in combination with low dose cytarabine [38], or azacytdine [39]. In some patients with more aggressive relapses FLAMSA and donor cells without reduced intensity conditioning were helpful (personal observation). Contrary to CML, AML may be rapidly progressive and unable to differentiate dendritic cells of leukemia origin [40]. We advised DLI after discontinuation of post-grafting immune suppressive treatment and absence of GVHD for 30 days. As a rule, this can be initiated between 120 and 160 days after transplant. DLI should be given in escalating doses starting with low doses between 1 x 10^6/kg and 1 x 10^7 /kg T cells. Even then, infections in particular viral infections can set off GVHD. Therefore, precautions should be taken to prevent infections. In the FIGARO trial, DLI were not regularly given, only in patients with mixed chimerism and impending relapse.

The FLAMSA regimen is conceptual for further steps to improve outcome of allogeneic stem cell transplantation (Figure 1). Active and primary refractory disease was well controlled by fludarabine and cytosine arabinoside (ARA-C) and amsacrine as non-cross-reactive drug for antracyclins. The substitution of fludarabin by clofarabine has been advocated by some colleagues [41], but more severe gastrointestinal toxicity has restricted its use. Today we have several new options for improvement: a liposomal formulation of the fixed combination of ARA-C and daunorubicin (CPX351) gave better responses and less toxicity than the conventional 7+3 regimen of ARA-C and daunorubicine [42], targeted drugs to FLT-3 [43], IDH-1 [44] and IDH-2 [45] as well as BCL-2 [46]. Combinations of the hypomethylating agent azacytidine [47] with the BCL2 inhibitor venetoclax [48] or with the IDH-1 inhibitor ivosidenib [44] or the IDH-2 inhibitor enasidenib [45] have been combined with great success. Histone deacetylase inhibitors Panobinostat [49] and vorinostat [50] have also been studied in AML. These new treatments may substitute the antileukemia part of FLAMSA in elderly and frail patients. They induce remissions or reduce blasts to a level at which conditioning with reduced intensity is sufficient to control rejection, GVHD and permit complete donor chimerism. This way even elderly patients may have a chance for transplantation without intensive chemotherapy [51]. Myeloablation with higher doses of radiation or intensive stem-cell toxic chemotherapy as busulfan, treosulfan, melphalan, thiotepa and others may not be necessary. FLAMSA 4 Gy TBI has been found equivalent to myeloablative conditioning in primary refractory and relapsed AML [25]. Unfortunately, levels of MRD were not reported. Cyclophosphamide combined with ATG enhances the generation of complete chimerism. The dose of cyclophosphamide may be reduced in elderly and unfit patients, the reduction to 50 mg/kg was sufficient for conditioning for transplantation in patients with severe aplastic anemia [52]. Antibody conditioning is still in its preclinical stage [53], but recent personalized targeted radio-immunotherapy trials are promising [54]. In this latter trial chimerism was complete at day 100 in over 90 percent of patients.

DLI should restore immune capacity after the depletion of T cells. They could be stored away at the time of transplantation or collected prior to use. The major risk of the transfusion of unmodified donor lymphocytes is GVHD. One precaution is the delay of DLI until tolerance is established and immunosuppressive treatment can be discontinued. The second precaution is repeated transfusions and escalated doses starting at low doses of 1 x 10^6/kg T cells. A third precaution is the prevention of infection, because they can set off GVHD by inflammation, production of interferons and expression of HLA class II in non-hematopoietic cells. As a rule, the time lag after transplantation is 4 to 5 months including a month off immunosuppressive therapy. During this time rapidly proliferative forms of leukemia tend to relapse. Early relapses are a common problem of FLT3 mutated leukemia, maintenance treatment with sorafenib [55] can prevent relapse. Sorafenib could be started 2 to 3 months after transplantation. Patients with persistent MRD positivity may not benefit, DLI were not part of the protocol. Another maintenance treatment with Panobinostat is also beneficial in high risk MDS and AML [49]. Several ways have been explored to improve graft-versus-leukemia effects without increasing GVHD. In any case patients treated with DLI should be protected from infections. Anti-viral and anti-bacterial prophylaxis can be considered, perhaps also precautions as against SARS-COV 2 with masks, disinfection and safe distance.

The graft-versus-leukemia (GVL) effect of DLI is strong in chronic myelogenous leukemia (CML) in patients with relapse in chronic phase, it is less in transformed phase and in AML. GVL effects can be improved by manipulation of the effector cells and/or manipulation of the target cells and their antigen presentation. The selection of NK cells or cytokine induced killer (CIK) cells has been used with some success. HLA-haploidentical donors can be selected avoiding killer receptor inhibitors [56]. Depletion of T cells has been advised and NK cells generated from stem cells. Depletion of naive T cells can also help to prevent acute GVHD. Marrow infiltrating lymphocytes display tumor specificity and can be expanded, they consist mainly of central memory T cells [57]. T cells can be activated and expanded against multiple leukemia associated antigens (WT1, PRAME, NY-ESO-1, survivin), they show good activity in adjuvant treatment of remission patients [58]. However, loss of antigen, loss of HLA-heterozygosity, PD-L1 expression and infiltration of immune privileged sites results

in immune escape. Transduction of EBV reactive T cells with a selected T cell receptor against WT-1 prevented relapse in 12 patients [59]. These approaches are restricted to HLA-A2 and the continued expression of WT1.

We studied improvement of GVL effects by improvement of antigen presentation by differentiation of leukemia blasts to dendritic cells of leukemia origin [60,61]. In contrast to chronic myelogenous leukemia (CML) where antigen expression is stable during chronic phase and long-lasting remissions can be induced, continued differentiation of AML blasts is difficult to achieve. Immune effects of targeted therapies may help. In some cases of azacytidine treatment cytotoxic T cells could be activated against testis antigens [47]. Azacytidine may also initiate the production of endogenous human retrovirus [62]. This way the leukemia cells may become immunogenic. Sorafenib leads to the release of IL 15 [63]. Peptide vaccination may be a way to improve graft- versus-leukemia reactions. Mutated regions of nucleophosmin I have elicited CD4 and CD8 responses in patients with AML [64] and neoepitopes of FLT3 internal tandem duplication (ITD) were recognized by autologous CD8 cells [65]. Recognition of peptides is better by allogeneic T cells than autologous T cells [66]. Therefore, peptide primed DLI may be helpful. Another way to improve the GVL reaction, is treatment with a bispecific antibody that leads to T cell activation on binding with CD123 target antigen [67]. Recent advances in the generation of chimeric antigen receptor positive T cells have also a good chance to induce immunity to AML blasts via binding to CD13 and TIM 3 [68]. Allogeneic CAR-T cells may have advantages in the recognition of neo-antigens, in the effector function and persistence [69].

In summary FLAMSA may be modified by remission induction or blast reduction with targeted therapy. Conditioning should include doses of TBI, melphalan, treosulfan or busulfan sufficient for the elimination of MRD and induction of complete donor chimerism at day 100, *in vivo* depletion of T cells and immune restitution with DLI. Open questions remain the form and duration of maintenance therapy until DLI and the optimal way of applying DLI for improvement of graft-versus-leukemia effects without the risk of severe GVHD.

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