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Patterns in donor-specific mRNA and protein production of Th1 and Th2 cytokines by graft-infiltrating lymphocytes and PBMC after heart transplantation

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Abstract We used RT-PCR and ELISA to study the kinetics of IL-2 (Th1) and IL-4 (Th2) both on mRNA and protein level from "naive" PBMC and "primed" graft-infiltrating lymphocytes (GIL) obtained from a heart transplant recipient. For this purpose, these cells were stimulated for 1–48 h with donor and control third-party antigens. Only stimulation of GIL with donor-specific antigen resulted in early detectable IL-2 and IL-4 mRNA and protein levels. Maximal relative IL-2 mRNA levels were significantly higher than maximal relative IL-4 mRNA levels (100-fold) in both GIL and PBMC after donor-specific stimulation. This was accompanied by a maximum protein production of 908 pg/ml IL-2 and 19 pg/ml IL-4 by GIL, and of 82 pg/ml IL-2 and undetectable IL-4 production by PBMC. These results suggest that, after stimulation donor-specific "primed" GIL, and not "naive" PBMC, rapidly produce abundant levels of IL-2 (Th1) and IL-4 (Th2) at both the transcriptional and protein level.

Key words Graft-infiltrating · lymphocytes · PBMC · IL-2 · IL-4 · mRNA

Introduction

Distinct functional T helper (Th) subsets, differing in patterns of cytokine production (Th1: IL-2 and Th2: IL-4), regulate and mediate the immune response after transplantation. We and others found that, in particular, expression of IL-2 mRNA and protein production by graft-infiltrating lymphocytes (GIL) is associated with cardiac graft rejection [1–3]. Furthermore, these GIL produce donor-specific cytokine mRNA and protein patterns in response to in vitro allogeneic stimulation [3, 4]. Also the frequency of IL-2 producing helper T-cells with specificity for donor antigens in the peripheral blood of heart transplant recipients was associated with rejection [5]. In recent studies, we have shown that in vivo maturated, primed, T-cells with specificity for donor antigens home in the allograft, while naive, precursor T-cells are primarily found in the peripheral blood [6]. Nevertheless, the kinetics of the induction of cytokine production by donor-specific in vivo primed T-cells and naive precursor T-cells is unknown. Moreover, differences in the kinetics between these different cell populations might be helpful in distinguishing precursors from primed T-cells. Therefore, we studied the time course of T-cell activation (i.e., mRNA expression) and subsequent release of IL-2 (Th1) and IL-4 (Th2) in GIL and in simultaneously taken PBMC during cardiac rejection both obtained from the same patient. Gene expression and protein production were measured after stimulation with donor and third-party antigens by semiquantitative RT-PCR and ELISA, respectively.

Material and methods

Patient

Rejection was diagnosed in endomyocardial biopsies by histological criteria according to ISHLT [7]. We analyzed GIL and simultaneously taken PBMC from one heart transplant patient during the first rejection episode at day 15 posttransplant.

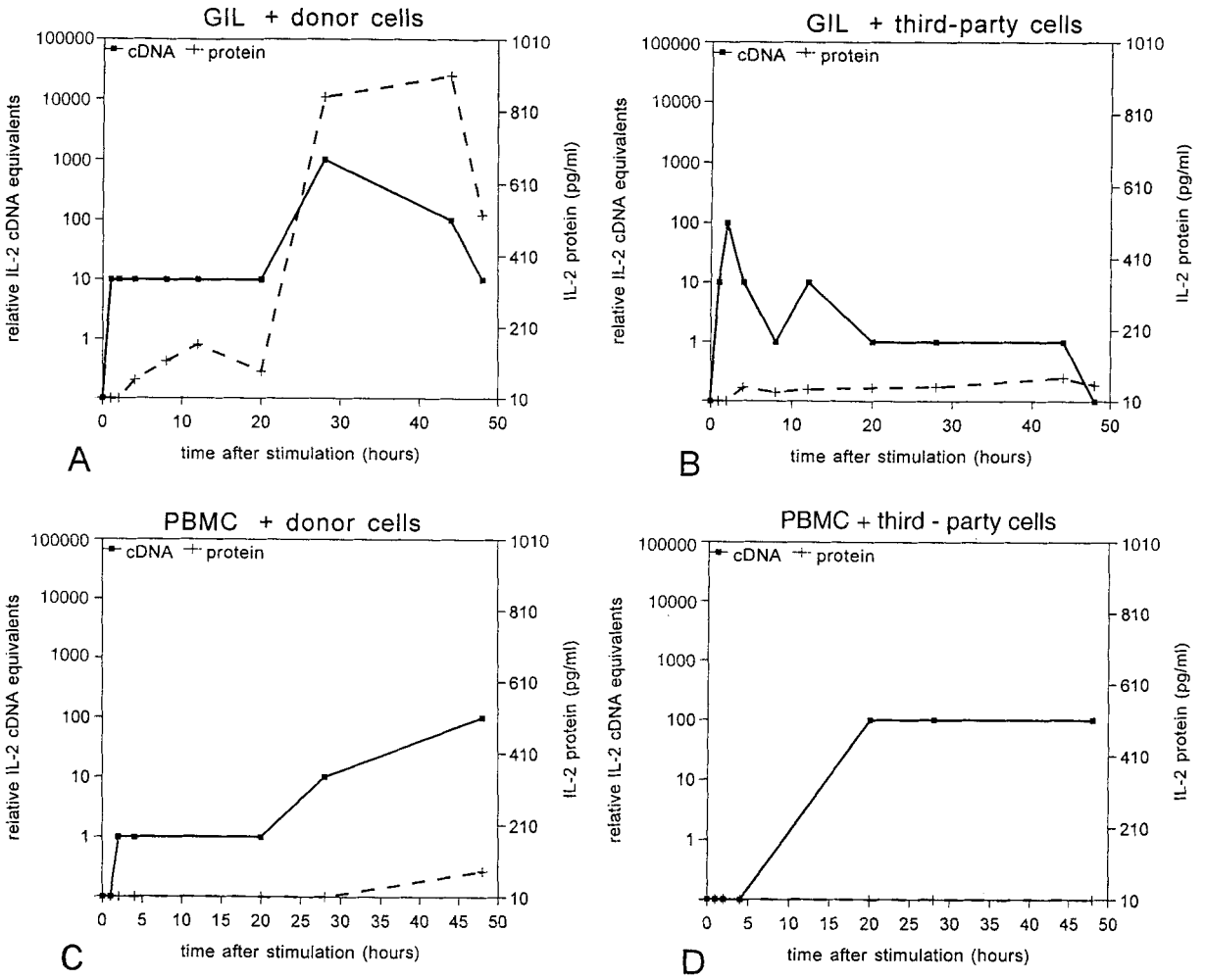

Fig. 1 A–D Time course for induction in "primed" graft-infiltrating lymphocytes (GIL) and "naive" PBMC of IL-2 mRNA and protein by semiquantitative RT-PCR and ELISA, respectively. Kinetics of IL-2 mRNA expression and protein production after stimulation with donor and third-party cells by GIL (**A, B**) and PBMC (**C, D**). After titration, the relative amount of IL-2 cDNA was determined by the highest dilution showing a positive signal. No significant difference in the amount of cDNA for keratin was found between different samples (data not shown)

Cultures

GIL were grown from the endomyocardial biopsy, taken on the day of histological rejection, and expanded in IL-2-containing medium [3]. PBMC were isolated by Ficoll Isopaque ($\delta = 1.077$) density-gradient centrifugation. Before testing, the GIL and PBMC were washed and 5×10^4 cells/well were incubated in IL-2-free medium for 24 h. Thereafter, 5×10^4 irradiated (60 Gy) and washed Epstein-Barr virus-transformed B-cell lines of donor or third-party origin were added. Supernatants and cell pellets were harvested after 1, 2, 4, 8, 12, 20, 28, and 48 h of co-culture and stored at –80 °C before analysis of cytokine production.

Total RNA and cDNA preparation

Cell pellets were homogenized in guanidinium isothiocyanate and total RNA was extracted by the phenol/chloroform method in the presence of carrier DNA to improve yields [1]. cDNA was synthesized from the isolated mRNA with random primers. Aliquots were used directly for PCR amplification.

PCR analysis

For semiquantitative analysis, cDNA samples were titrated (tenfold) and aliquots of each dilution were amplified. Samples were amplified using specific primers for IL-2 and IL-4 and were subjected to 40 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 2 min, and extension at 72 °C for 3 min. PCR primers detecting transcripts for the house-keeping gene keratin were used as an internal control to confirm successful RNA extraction and cDNA amplification [1]. Verification of an RT-PCR product was achieved by Southern-blot hybridization. After titration, the relative amount of target cDNA was determined by the highest dilution showing a positive signal. To exclude interassay variability, amplification of titrated cDNA of the positive control, cDNA isolated from 10^6 Hut78 cells (ATCC; T-cells with constitutive cytokine mRNA expression), was included in each analysis.

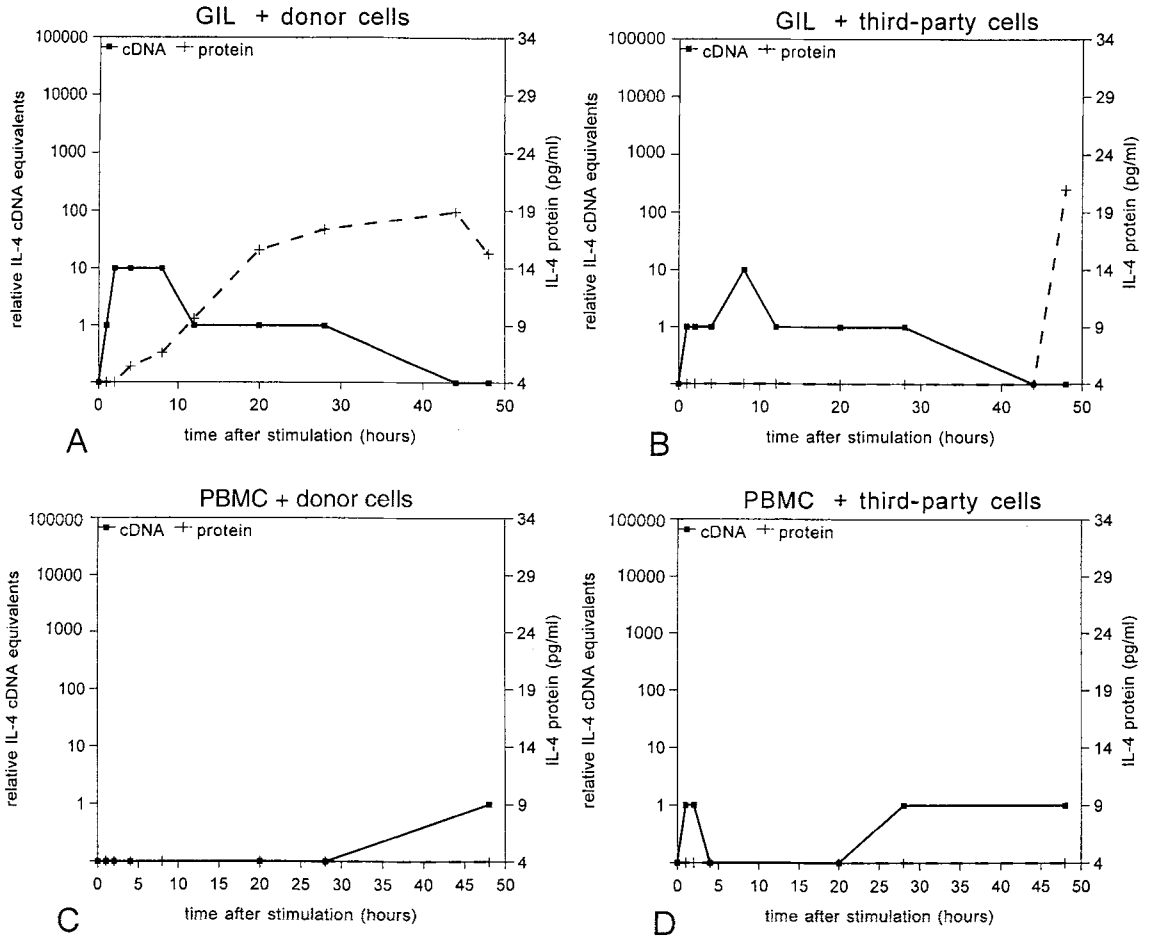

Fig. 2 A–D Time course for induction in "primed" GIL and "naive" PBMC of IL-4 mRNA and protein by semiquantitative RT-PCR and ELISA, respectively. Kinetics of IL-4 mRNA expression and protein production after stimulation with donor and third-party cells by GIL (**A, B**) and PBMC (**C, D**). After titration, the relative amount of IL-4 cDNA was determined by the highest dilution showing a positive signal. No significant difference in the amount of cDNA for keratin was found between different samples (data not shown)

Cytokine production

The concentration of IL-2 and IL-4 in the supernatants was determined by ELISA (IL-2, detection range 10–1000 pg/ml, Immunotech, Marseille, France; and IL-4 range 4–450 pg/ml, CLB, Amsterdam, The Netherlands).

Results

Messenger RNA expression and protein production for IL-2 and IL-4 were not measured at detectable levels in unstimulated control GIL and PBMC nor in the irradiated stimulator cells (data not shown). In GIL, messengers coding for IL-2 and IL-4 genes were already detectable 1 h after stimulation with donor and third-party antigens and reached maximum levels within 30 h, while protein production was measurable from 4 h after stimulation with cytokines accumulating at least 48 h after (Fig. 1 A, B, Fig. 2 A, B). Both the amounts of IL-2 and IL-4 mRNA and protein were higher after stimulation with donor antigens than after stimulation with third-party antigens. In addition, after stimulation with donor antigens, these GIL produced significantly more IL-2 than IL-4 mRNA (100-fold), which was accompanied with a subsequent maximum production of IL-2 protein (908 pg/ml) and IL-4 protein (19 pg/ml). In PBMC, messengers coding for IL-2 were detectable 2 h, and for IL-4 28 h, after stimulation with donor antigens and 20 h and 1 h, respectively, after stimulation with third-party antigens (Fig. 1 C, D, Fig. 2 C, D). Neither the relative IL-2 mRNA level nor the relative IL-4 mRNA level was significantly different between donor and third-party stimulation, but only donor-specific stimulation resulted in measurable IL-2 protein levels at 48 h. Both in GIL and PBMC, the relative maximum donor-specific IL-2 mRNA level was significantly higher compared to the

relative donor-specific maximum IL-4 mRNA level (100-fold).

Discussion

After transplantation, it is thought that allogeneic primed T-cells are responsible for the process leading to graft destruction. After allogeneic stimulation these cells produce cytokines. However, the kinetics of donor-specific IL-2 and IL-4 mRNA and the subsequent protein production by these primed, and also by naive T lymphocytes, is not known. Therefore, we have studied the time course for the induction of mRNA and protein for IL-2 and IL-4 in "naive" PBMC and in vivo maturated "primed" GIL after stimulation with either donor or third-party cells. In time, the induction of both mRNA and protein was quicker by GIL than by PBMC. Moreover, in GIL, on both the transcriptional and the protein level the production of IL-2 (Th1) and IL-4 (Th2) was donor specific. These observations suggest that, especially in vivo, primed T-cells with donor specificity mediate early events of the immune response within the transplanted organ. In addition, these results confirm our previously reported data concerning the correlation between cytokine mRNA transcription and protein production by GIL [4]. In contrast, after third-party antigen stimulation even considerable relative mRNA levels did not result in a subsequent high protein production. This could mean that other presently unknown but probably donor-specific posttranscriptional factors are involved in the translation process or that other stimuli might give an additional donor-specific signal. Using limiting dilution analysis, Bishop et al. [8] showed that primed IL-2 producing T-cells could also be measured in the peripheral blood of sponge matrix allografts, whereas conflicting data were reported after clinical transplantation [9, 10]. However, we could not detect these primed T-cells in PBMC on the basis of significant early donor-specific mRNA transcription and subsequent protein production by the highly sensitive RT-PCR and ELISA, respectively. This suggests that alloantigen primed T-cells are not or are below the detection level present in the peripheral blood.

In conclusion, the kinetics of cytokine mRNA and protein production was significantly different between "naive" PBMC and "primed" GIL. We found that only in vivo maturated primed T-cells are capable of secreting IL-2 (Th1) and IL-4 (Th2) immediately upon antigen stimulation.

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