



Development of a functional cDNA array for evaluation of the Th1/Th2 balance

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Abstract

The immune balance controlled by CD4⁺ helper T cell subsets (T helper 1 (Th1) and T helper 2 (Th2)) is crucial for immunoregulation and its imbalance causes various immune diseases including infections, allergic disorders and autoimmune diseases. Therefore, it is of great importance to develop a system of diagnosing Th1/Th2 imbalances for curing immune diseases. Here we developed a functional cDNA array filter useful for assessing the Th1/Th2 balance in mice. To overcome the disadvantages of conventional microarrays carrying thousands of genes, we prepared an array filter containing 40 Th1-specific and 32 Th2-specific genes, which were selected from over 8700 genes based on (i) the specificity of expression in Th1 or Th2 cells and (ii) an expression level which is high enough for detection using a DNA array. This array filter provided a prompt and precise evaluation for the skewing of the Th1/Th2 balance combined with our calculation algorithm. The bias toward Th1 or Th2 was evaluated visually at a glance by aligning the genes on the filter. Moreover, we succeeded in evaluating the skewing of the Th1/Th2 balance in vivo during acute graft versus host disease (GVHD). Thus, this array filter will provide a novel tool for evaluation of the Th1/Th2 balance in a variety of immune diseases.

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1. Introduction

CD4⁺ T helper cells are classified into two subsets, T helper 1 (Th1) and T helper 2 (Th2) cells, according to their cytokine production profile [1]. Th1 cells play a critical role for the regulation of cellular immunity through the secretion of IL-2, IFN- γ and TNF- β . On the other hand, Th2 cells regulate humoral immunity by producing IL-4, IL-5, IL-6, IL-10 and IL-13. It is now widely accepted that the immune balance regulated by these Th1- and Th2-derived cytokines (Th1/Th2 balance) is critically important in immunoregulation, and its imbalance becomes the cause of the immune diseases [2–5]. The critical role of Th1 or

Th2 cells for inducing immune diseases has been demonstrated by many animal experimental models. For instance, an excessive acceleration of Th1 immunity causes the onset of liver injury [6,7], GVHD [8], diabetes [9] and multiple sclerosis [10], whereas allergic diseases such as asthma [11] are derived from dysregulated Th2 responses. Recently, it became possible to modulate immune balance through controlling the function of innate immune effector cells such as dendritic cells (DC) and NKT using CpG oligonucleotides [12] or α -galactosylceramide (α -GalCer) [13]. Especially, CpG, a ligand of toll-like receptor 9 (TLR9) has been applied to clinical trial of Th2-dependent allergic diseases because it can induce a strong type1-immunity in vivo [12]. Such immunomodulator with a capability of controlling Th1/Th2 balance will become a novel strategy for therapeutic approach to immune diseases. However, we also have to take care about

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the adverse effect of such immunomodulating drugs because Th1/Th2 balance is genetically controlled. In the case of IL-12 clinical trial, the responsiveness to IL-12 was different in each patient and some patients suffered from lethal toxic effect [14]. Therefore, diagnosis of Th1/Th2 balance in each patient is essential for the application of immune balance regulating drugs. The diagnosis of Th1/Th2 balance is also useful for early detection of recurrence or cure of immune diseases. For this purpose, we developed a DNA array for evaluation of the Th1/Th2 balance.

DNA arrays are useful for determining the expression levels of a number of genes at once. Different methods have been developed which use either solid glass/plastic or a nylon membrane to support DNA. Furthermore, the DNA to be mounted can be different. Some arrays are mounted with oligodeoxynucleotides specific to each gene, while others use cDNA fragments. We chose a cDNA array based on a nylon membrane for the following reasons: (1) the number of genes to be mounted, i.e. the number of genes which show Th1/Th2 specificity is limited and (2) results with membrane filters are more reproducible than those with solid bases. We also chose a chemiluminescence-based method of detection, as it is broadly applicable and does not require any specific facilities or equipment. To overcome the low sensitivity of the method, we amplified the cDNA probe using a PCR-based approach. In this way, we are able to detect signals at a sensitivity equivalent to that of the methods employing radioisotopes.

There are several commercial DNA arrays containing immunologically important genes, and some researchers have reported differences in gene expression between Th1 and Th2 cells using the DNA array method [15–18]. The goal of this study is to develop a DNA array that can be used to measure the expression level of Th1- or Th2-specific genes, and apply it for the evaluation of the Th1/Th2 balance in vivo. By choosing a limited number of genes and developing a scoring method, we could easily and reliably evaluate the Th1/Th2 balance in a mouse immune disease model.

2. Materials and methods

2.1. Generation of Th1 and Th2 cells

Th1 and Th2 cells were induced from DO11.10 TCR transgenic mice (maintained on the BALB/c background, donated by Dr. K.M. Murphy). CD4⁺CD45RB⁺ naive T cells were isolated from nylon-passed spleen cells of DO11.10 TCR-transgenic mice by cell sorting using a FACS Vantage instrument (BD Bioscience, Mountain View, CA). Purified CD4⁺CD45RB⁺ cells were stimulated with 10 µg/ml OVA₃₂₃₋₃₃₉ peptide (donated by Fujiya, Hadano, Japan) in the presence of mitomycin C-treated BALB/c spleen cells, 20 U/ml IL-12 (donated by Genetics Institute, MA), 1 ng/ml IFN-γ (BD PharMingen, San Diego, CA), 50 µg/ml anti-IL-4 mAb (11B11, ATCC, Rockville, MD) and 20 U/ml IL-2

(donated by Shionogi Pharmaceutical, Osaka, Japan) for Th1 development. Th2 cells were induced from the same naive Th cells in the presence of 1 ng/ml IL-4 (PEPROTECH Inc., Rockyhill, NJ), 50 µg/ml anti-IFN-γ mAb (R4-6A2, BD Pharmingen), 50 µg/ml anti-IL-12 mAb (C15.1 and C15.6, provided by Dr. G. Trinchieri, Wistar Institute of Anatomy and Biology, Philadelphia, PA), and 20 U/ml IL-2. After 48 h, cells were restimulated with OVA₃₂₃₋₃₃₉ under the same conditions, and used at 12 days of culture. Before RNA extraction, some cells were stimulated with immobilized anti-CD3 mAb for 2 h at 37 °C.

2.2. GEM microarray analysis and semi-quantitative RT-PCR

Total RNA was isolated from Th1 or Th2 cells and mouse spleen cells by the acid guanidinium-phenol-chloroform (AGPC) method using ISOGEN (Nippon gene, Toyama, Japan). The poly(A)⁺ mRNA was purified from total RNA by incubation with oligo-dT magnetic beads (Toyobo Co., Osaka, Japan). Microarray analyses were performed using the GEM microarray (Incyte Genomics, Palo Alto, CA) with poly(A)⁺ RNAs from anti-CD3 mAb-stimulated or unstimulated Th1 and Th2 cells. The microarray was spotted with about 8700 EST clones.

For semi-quantitative RT-PCR, cDNAs were synthesized by reverse transcription of 1 µg of total RNA at 42 °C for 50 min, using 200 U of Superscript II (Life Technologies, Rockville, MD) and oligo(dT)₁₂₋₁₈. PCRs were performed to amplify each cDNA and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA as a control using Taq DNA polymerase (Promega, Madison, WI) or KOD dash (Toyobo Co.). Reactions with Taq DNA polymerase were performed in a thermal cycler for 25–35 cycles (denaturation: 30 s, 94 °C; annealing: 30 s, 60 °C; extension: 1 min, 72 °C). Reactions with KOD dash were performed for 25–35 cycles (denaturation: 20 s, 98 °C; annealing: 2 s, 60 °C; extension: 30 s, 72 °C). The efficiency of each reverse transcription reaction was confirmed by the PCR for GAPDH. For quantification, PCR products of different cycles were electrophoresed on agarose gels and visualized by ethidium bromide staining. The gel images were incorporated by Densitograph (ATTO, Tokyo, Japan) and each band was quantified using Lane & Spot Analyzer (ATTO). The quantification for each gene was done at a point at which no saturation of the PCR was evident.

2.3. Production of cDNA array filters

A cDNA fragment of about 500 bp was amplified by PCR using an unique primer set for each gene. Primers were selected so that the amplified cDNA fragment does not have high homology to other genes in the database. Each PCR fragment was cloned into pGEM-T Easy vector (Promega) and verified by DNA sequencing with a BigDyeTM Terminator DNA sequencing kit (Applied Biosystems, Foster City, CA) and ABI310 Genetic Analyzer (Applied Biosystems). Each

Table 1
List of the genes spotted on the filter

Grid	Gene	Accession no.	CV ^a	Grid	Gene	Accession no.	CV ^a
B01	IFN- γ	M28621	80.4	I01			
B02	MIP-1 α	NM.011337	124.8	I02	CD8 α	M12825	−2.9
B03	C/EBP α	NM.007678	6.3	I03	CD69	L23638	−3.3
B04	GATA4	NM.008092	6.5	I04	Integrin β 7	NM.013566	−4.5
B05	TNF α	NM.013693	38.6	I05	Pcsk3	NM.011046	−24.1
B06	Notch4	NM.010929	6.4	I06	c-maf	S74567	−4.9
C01	MIP-1 β	NM.013652	40.0	J01			
C02	IRS4	NM.010572	43.4	J02			
C03	CD40 ligand	NM.011616	18.6	J03	IRF-1	NM.008390	−4.0
C04	LTX β	NM.008518	29.9	J04	Glycoprotein 49A	NM.008147	−9.5
C05	TNF β	NM.010735	107.9	J05	Metallothionein	NM.013602	−15.9
C06	TA-GAP	BC039780	29.3	J06	Ctla2 α	NM.007796	−39.4
D01	Placental calcium-binding protein	X05835	20.2	K01			
D02	GM-CSF	X03019	20.2	K02			
D03	RANTES	NM.013653	30.9	K03	IIGP	NM.021792	−2.0
D04	CD6	NM.009852	15.4	K04	Superoxide dismutase 2	NM.013671	−4.0
D05	Galectin-3	X16834	7.2	K05	NF κ B	NM.009045	−8.9
D06	CD97	NM.011925	26.5	K06	IL-2 receptor α	NM.008367	−14.6
E01	CD9	NM.007657	15.1	L01			
E02	CXCR3	NM.009910	11.8	L02			
E03	Chandra	NM.021718	28.9	L03	IL-10 receptor	NM.001558	−7.3
E04	Stra13	NM.011498	23.0	L04	TDAG51	NM.009344	−6.3
E05	Onzin	AF269458	6.1	L05	IL-4 receptor α	NM.010557	−4.7
E06	IL-12 receptor β 2	NM.008354	1.9	L06	CD53	NM.007651	−25.5
F01	GBP3	NM.018734	3.1	M01	IL-9	NM.008373	−8.5
F02	PIP3-E	AK038257	9.7	M02	Laminin A5	U37501	−2.9
F03	STAT4	NM.011487	8.9	M03			
F04	PDE4B	NM.019840	6.1	M04	NFAT1	NM.010899	−2.9
F05	EST	AA138966	0.9	M05	PPAR γ	NM.011146	−3.6
F06	Purine—nucleoside phosphorylase	NM.013632	7.6	M06	CCR2	NM.009915	−3.1
G01				N01	ECM1	NM.007899	−31.1
G02	Annexin A6	NM.013472	8.8	N02	IL-5	NM.010558	−29.1
G03	PKC η	NM.008856	15.0	N03	GATA3	NM.008091	−10.3
G04	CDK (p21)	NM.007669	43.3	N04	CRABP2	M35523	−6.8
G05	T-bet	NM.019507	7.7	N05	IL-10	NM.010548	−13.0
G06	E2F transcription factor 4	AI509945	10.9	N06			
H01				O01	IL-4	NM.021283	−72.5
H02	CD4	NM.013488	13.4	O02	CYP11A	NM.019779	−338.7
H03	Osteopontin	NM.009263	18.5	O03	Preproenkephalin	M13227	−158.7
H04	Annexin A2	NM.007585	40.3	O04	IL-13	NM.008355	−93.5
H05	IL-2	NM.008366	51.7	O05	20 α -HSD	AB059565	−43.4
H06	Rab7	NM.009005	15.0	O06			

Th1-specific genes were spotted on grids A–H, and Th2-specific genes on grids I–O.

^a CV, compensation value.

cDNA fragment (10 ng) amplified from the cloned plasmid was spotted on nylon membranes by Genetic-Lab (Sapporo, Japan) or Toyobo Co.

Each gene listed in Table 1 was spotted in duplicate on the left half of the filter, so that B01–B06 lie from left to right in the second line. Control β -actin and GAPDH were spotted on the first line (A01 and A02) and the last line (P01 and P02) of the left half, respectively. All of the genes B01–O06 and the control genes were spotted on the left half of the filter, and the same DNAs were spotted in radial symmetry on the right half. As a result, Th1-specific genes lie in the upper left quarter and lower right quarter, and Th2-specific genes lie in the lower left quarter and upper right quarter except for the corners spotted with control DNA.

2.4. cDNA array analysis

Probes for hybridization were made using a cDNA Amplification System (Toyobo Co.). cDNA was synthesized from 1.5 μ g of poly(A)⁺ mRNA using a primer containing poly(dT), followed by poly(dC) tailing and amplification by PCR with biotinylated dUTP. The array filters were pre-incubated in 5 ml of PerfectHyb (Toyobo Co.) at 68 °C for 20 min. Then, biotin-labeled probes were denatured and added to the pre-hybridization mixture. After incubating overnight at 68 °C, the filters were washed three times in 2 $\times$ SSC and 0.1% SDS for 5 min at 68 °C and three times in 0.1 $\times$ SSC and 0.1% SDS for 5 min at 68 °C. After washing, specific signals on the filters were detected with an Imaging High

Chemifluorescence Detection kit (Toyobo Co.) according to the manufacturer's instructions. Using this system the biotin-labeled probes are detected by incubation with streptavidin, followed by biotinylated alkaline phosphatase, and finally a substrate, CDP-Star. Chemiluminescence images were taken with a Fluor-S MultiImager System (Bio-Rad Laboratories, Hercules, CA). Signal intensities of the cDNA array spots were measured using ImaGene software (BioDiscovery Inc., Los Angeles, CA). The amount of gene expression was normalized based on the intensity of the housekeeping genes (β -actin and GAPDH).

For calculation of the "score" of the Th1/Th2 balance, we employed a compensation value to reflect the importance of each gene in determining the bias of the balance. These values are calculated from the signal intensities of anti-CD3 mAb-stimulated Th1 and Th2 cells, namely $(\text{Th1CD3})^{1.5}/(\text{Th2CD3})^{1.3}$ for Th1-specific genes and $-(\text{Th2CD3})^{1.5}/(\text{Th1CD3})^{1.3}$ for Th2-specific genes. The absolute compensation values are high for those genes with high expression levels and with high specificity for Th1 or Th2 cells. The final score is the sum of (signal intensity) $\times$ (compensation value) calculated for each gene.

2.5. Induction and analysis of acute GVHD in mice

Th1-dependent acute GVHD mice were generated by injecting C57BL/6 (B6) [H-2^b] spleen cells into BDF1 [H-2^{b/d}] mice. A single-cell suspension of spleen cells from B6 mice was prepared in RPMI 1640 medium and passed through a sterile nylon mesh. Then cells were washed and diluted to 5×10^8 cells/ml. Acute GVHD was induced by injecting 1×10^8 B6 spleen cells into BDF1 mice. Age-matched BDF1 mice free of injections were used as controls. Sera were recovered from the mice on day 7 and levels of IFN- γ were measured. Spleen cells from model mice were used for intracellular cytokine staining, cell surface marker staining, cytotoxicity assay, and RNA extraction for array analysis. The amount of remaining host cells was also analyzed by flow cytometry on day 14.

Serum IFN- γ levels were measured using OptEIA mouse IFN- γ set (BD Pharmingen, San Diego, CA), which is based on the sandwich ELISA method.

For the detection of cytoplasmic cytokine expression, cells were treated with brefeldin A (Sigma–Aldrich, St. Louis, MD) and stimulated with immobilized anti-CD3 antibody (2 μ g/ml) for 6 h. Then, cells were stained with FITC-conjugated anti-H-2^d antibody (BD Pharmingen), fixed with 4% paraformaldehyde, and lysed in permeabilizing solution (50 mM NaCl, 5 mM EDTA, 0.02% NaN₃ and 0.5% Triton X-100, pH 7.5). Finally, cells were stained with PE-conjugated anti-IFN- γ antibody (BD Pharmingen) for 45 min and analyzed by flow cytometry.

For cell surface staining, spleen cells were stained with FITC-conjugated anti-H-2^d antibody and PE-conjugated anti-B220 antibody (BD Pharmingen), then analyzed by flow cytometry.

The cytotoxicity was determined by conducting 4 h ⁵¹Cr-release assays as described previously [19]. P815 cells, a mastocytoma cell line of H-2^d, and MBL2 cells, a T cell lymphoma cell line of H-2^b, were used as target cells. The cytotoxicity (expressed as a percentage) was calculated using the following equation [19]. Percent cytotoxicity = $(^{51}\text{Cr release with effector cells} - \text{spontaneous } ^{51}\text{Cr release})/(\text{maximal } ^{51}\text{Cr release with 0.1 M HCl} - \text{spontaneous } ^{51}\text{Cr release}) \times 100$.

3. Results

3.1. Selection of Th1- or Th2-specific genes

Gene expression profiles of anti-CD3 mAb-stimulated or unstimulated Th1 and Th2 cells were analyzed by GEM microarray to find genes specific to Th1 or Th2 cells. Among about 8700 genes on this array, 53 genes were judged as Th1-specific (more than two-fold increased expression compared to Th2 cells), and 21 genes were Th2-specific without anti-CD3 mAb stimulation (Fig. 1A). When anti-CD3-stimulated Th1 and Th2 cells were compared, 56 Th1-specific and 44 Th2-specific genes were identified (Fig. 1B). In total, 81 and 52 genes were identified as Th1- and Th2-specific, respectively, either before or after anti-CD3 stimulation. To search for specific genes that were not represented on the GEM microarray, we also performed suppression subtractive hybridization (SSH) [20]. This analysis allowed us to identify eight Th1-specific and 11 Th2-specific genes. From these results and from the previous reports [15–18], we chose 153 specific genes (or ESTs) for further study.

Selected genes were evaluated by semi-quantitative RT-PCR to confirm the difference in gene expression between Th1 and Th2 cells (Fig. 1C). By taking a more than two-fold difference as "specific", 22 genes were identified as specific to stimulated and unstimulated Th1 cells and 15 genes as specific to Th2 cells. Also we found 22 genes that show specificity to Th1 cells only after stimulation, and 17 genes specific to stimulated Th2 cells. Another 37 genes showed less than two-fold difference in RT-PCR. We then cloned amplified cDNA fragments of the specific genes in a plasmid vector, and used them as templates to amplify cDNA fragments. By spotting amplified DNA fragments onto nylon membrane filters, we made pilot array filters and performed array analyses using stimulated or unstimulated Th1 and Th2 cells (data not shown). In these analyses, we found that the expression of 32 genes was not detected using the cDNA array, even though differences in gene expression between Th1 and Th2 were evident in RT-PCR. These genes, being uninformative in the array analysis, were removed from the selection list. Finally, we selected 72 genes (40 Th1-specific and 32 Th2-specific) to make the final cDNA array filter (Table 1). In order to make the skewing of the Th1/Th2 balance visible at a glance, genes were aligned so that Th1-specific genes lie from the upper left corner to lower right corner, and Th2-specific genes lie from

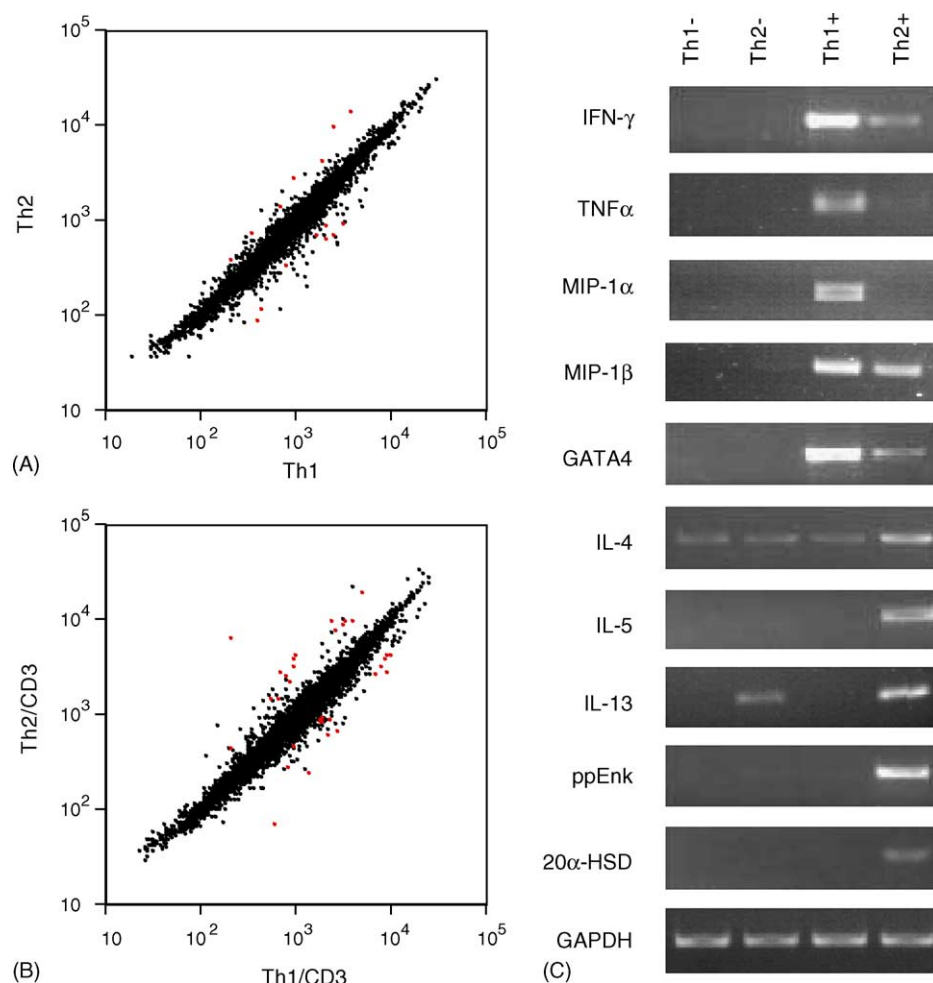


Fig. 1. Selection of Th1- or Th2-specific genes. (A and B) GEM microarray analysis of Th1 and Th2 cells. Poly(A)⁺ RNA (1 μ g) purified from unstimulated (A) or anti-CD3 mAb-stimulated (B) Th1 and Th2 cells was used for the analysis. About 8700 genes and ESTs were analyzed for a difference in gene expression between Th1 and Th2 cells. The expression level of each gene in arbitrary units is shown on the graph. Genes selected for our filter are spotted in red. (C) Examples of semi-quantitative RT-PCR for genes that were differentially expressed between Th1 and Th2 cells. Total RNA (1 μ g) from anti-CD3-stimulated (+) or unstimulated (–) Th1 and Th2 cells was reverse transcribed and used as a template for PCR. Bands of amplified DNAs were visualized by ethidium bromide staining after agarose gel electrophoresis. Results are representative of at least three different PCRs using different preparations of Th1 and Th2 cells.

the upper right to lower left. As controls, GAPDH and β -actin genes were located at the four corners.

3.2. Verification of the cDNA array using RNAs from Th1 or Th2 cells

DNA array analyses for anti-CD3 mAb-stimulated or unstimulated Th1 cells and anti-CD3-stimulated or unstimulated Th2 cells were performed using this filter. The results of hybridization for stimulated cells are shown in Fig. 2. There are apparent differences in the gene expression pattern between stimulated Th1 and Th2 cells. Strong signals were located from the upper left to lower right in the stimulated Th1 cells, and from the upper right to lower left in the stimulated Th2 cells. The expression level of each gene was quantified by measuring the intensity of the signal. The relative expression level of each gene was calculated by comparing it to the internal standard (GAPDH and β -actin). The

specificity of gene expression in Th1 or Th2 cells analyzed with this cDNA array was consistent with the results of RT-PCR.

3.3. Evaluation of Th1/Th2 balance in acute GVHD model

To test if this DNA array filter can evaluate the skewing of the Th1/Th2 balance in vivo during immune disease, we conducted an analysis of acute GVHD mice, an animal model of Th1-type disease. In these mice, the serum IFN- γ level was elevated on day 7 (Fig. 3A). Analysis of intracellular IFN- γ production by spleen cells indicated that both CD4⁺ and CD8⁺ cells originating from the donor produce IFN- γ (Fig. 3B). Also, the cytotoxicity of spleen cells to P815 cells (H-2^d) was elevated, whereas no cytotoxicity to MBL2 cells (H-2^b) was evident (Fig. 3C and D). Moreover, there was a decrease in the number of host cells in the spleen on day

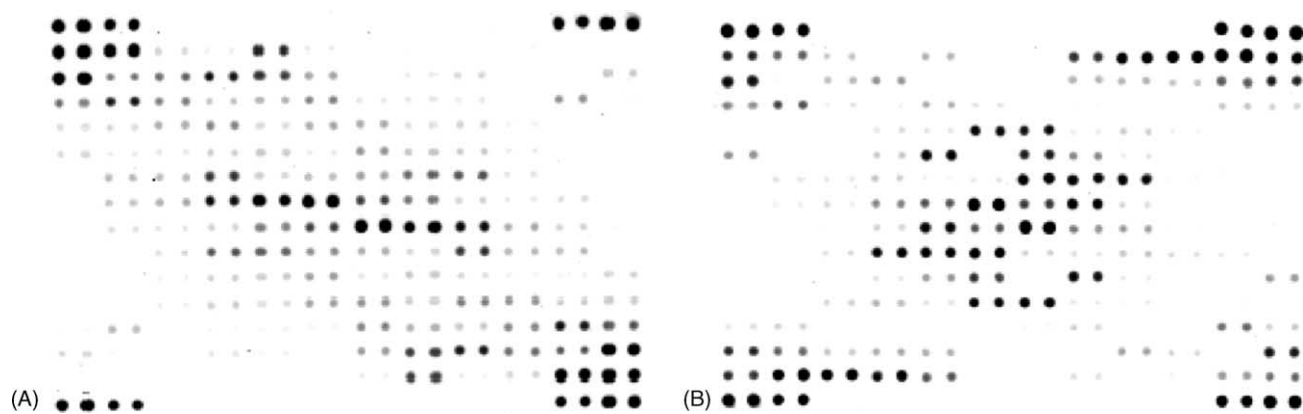


Fig. 2. DNA array analysis of Th1 and Th2 cells. Probes for hybridization were prepared from 1.5 μg of poly(A)⁺ RNA from anti-CD3 mAb-stimulated Th1 (A) or Th2 cells (B). After cDNA synthesis and poly(dC) tailing, the whole mixture of cDNA was amplified and labeled with biotin-dUTP by PCR. After hybridization and washing of the probe, signals on the filter were visualized by chemiluminescence using biotinylated alkaline phosphatase and CDP-star. Th1-specific genes are aligned from the upper left corner to lower right corner of the filter, and Th2-specific genes, from the upper right to lower left corner. GAPDH and β -actin cDNAs are located at the four corners.

14 (Fig. 3E). Thus, this model represents the symptoms of acute GVHD caused by Th1 polarization. The spleen cells from acute GVHD mice were harvested at an early phase (day 7) of GVHD and analyzed using the cDNA array filter (Fig. 3F). The resulting image shows a characteristic Th1-type gene expression pattern, indicating strong signals from the upper left to lower right. The control spleen cells from BDF1 mice of the same age did not show an evident pattern of deviation (Fig. 3G).

3.4. Scoring of Th1/Th2 balance

To evaluate the Th1/Th2 balance more objectively using the results of our cDNA array, we created an algorithm to quantify the skewing from neutral to Th1 or Th2, calculat-

ing from the signal intensities of the spots on the filter. To reflect the importance of genes for evaluation of the Th1/Th2 balance, the signal intensity of each gene was multiplied by a compensation value designated for the gene. These values were set positive for Th1-specific genes and negative for Th2-specific genes. The final score is the sum of the compensated values calculated for all of the genes on the filter (Table 2). This calculation gives a positive score for skewing to Th1, and a negative score for Th2. According to this calculation, the score for anti-CD3-stimulated Th1 cells was 16,012, and the score for anti-CD3-stimulated Th2 cells was –18,859. The score for the GVHD model was 2014, compared to 260 for the control mice. Thus, this scoring method gave a quantitative evaluation of Th1-skewing in a model of acute GVHD.

Table 2
Examples of quantification and values used for scoring

Gene	Signal intensities		Compensation value	Calculated values	
	Th1 (anti-CD3)	Th2 (anti-CD3)		Th1 (anti-CD3)	Th2 (anti-CD3)
IFN- γ	37.9	8.7	80.4	3048.3	701.1
Mip-1 α	42.7	7.1	124.8	5329.8	890.3
Mip-1 β	43.2	17.3	40.0	1725.7	693.1
TNF α	13.7	4.7	38.6	527.3	182.2
TNF β	14.3	2.3	107.9	1546.2	244.1
LTX β	16.7	7.3	29.9	499.7	217.1
...	...	...	...	...	...
CYP11A	1.8	37.0	–338.7	–605.1	–12527.3
ppEnk	2.6	31.3	–158.7	–419.5	–4973.1
IL-13	2.5	20.9	–93.5	–232.4	–1951.9
IL-4	2.4	17.0	–72.5	–172.6	–1229.9
ECM-1	3.2	12.6	–31.1	–100.7	–392.1
20 α -HSD	2.0	10.2	–43.4	–85.0	–442.0

To reflect the importance of each gene to the calculation of the score, a compensation value (Table 1) was designated. These values are based on the signal intensities of anti-CD3 mAb-stimulated Th1 and Th2 cells, set positive for Th1-specific genes and negative for Th2-specific genes, and modified so that their sum equals 0. For scoring, the signal intensity of each gene was multiplied by the compensation value, and the final score is the sum of the compensated values calculated for each gene.

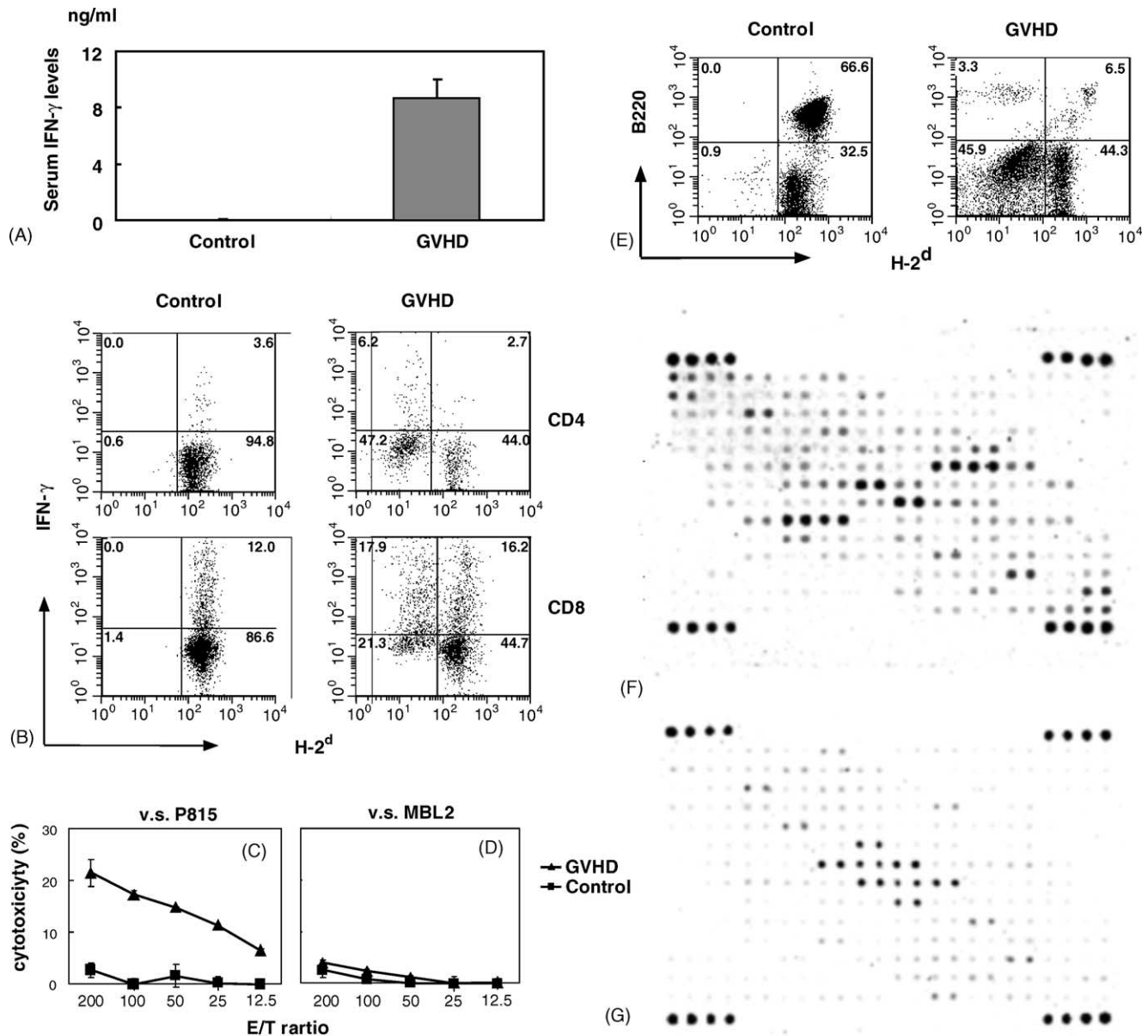


Fig. 3. Analysis of Th1/Th2 balance in an acute GVHD model. Spleen cells (1×10^8) from B6 mice were transferred into BDF1 mice. After 7 days (A–D, F, G) or 14 days (E), serum and spleen cells were investigated. Untreated BDF1 mice were used as controls. (A) Serum IFN- γ levels in acute GVHD and control mice. (B) IFN- γ production in spleen cells. Spleen cells from GVHD or control mice were stimulated with anti-CD3 mAb, and intracellular IFN- γ expression was measured by flow cytometry. (C and D) The cytotoxic activity of spleen cells from GVHD and control mice against P815 cells or MBL2 cells. (E) Flow cytometric analysis of host cell deletion in the spleen of GVHD mice. (F and G) DNA array analysis of spleen cells from GVHD mice and control mice. Poly(A)⁺ RNAs were purified from spleen cells of GVHD (F) or control mice (G), and analyzed as described in Fig. 2 legend. Results are representative of three experiments using different preparations.

4. Discussion

The DNA array is a powerful tool for investigating the gene expression levels of a number of genes at once [21–24]. Examples of success using this technology include the search for genes specifically expressed in diseases like cancer [25–28], viral infection [29], inflammation [30–32] or hematopoietic disorders [33], and the examination of genes specifically expressed in certain lineages of differentiated cells. However, we have experienced some difficulties in

applying this technology. For example, some genes known to be expressed specifically in diseases or cell lineages could not be detected in the cDNA array because of the low expression level and the relative insensitivity of the detection method. Moreover, commercial DNA array filters or microarrays are spotted with a large number of genes, which may complicate the analysis if a very small number of genes show a difference in gene expression. In other words, such DNA arrays are useful to search for specific genes, but are not optimal for analyses like the diagnosis of Th1/Th2 imbalance.

Here, we developed a functional cDNA array filter, which is optimized for the evaluation of the Th1/Th2 balance. To overcome the difficulties in using conventional DNA arrays, we first minimized the number of genes spotted on the filter by removing genes that were not informative because of a low expression level. Furthermore, we arranged the alignment of genes to be spotted on the filter, so that the skewing of the Th1/Th2 balance can be visualized at a glance. Finally, we have developed a calculation algorithm to diagnose Th1/Th2 imbalances from the overall gene expression patterns, rather than the mere analysis of each specific gene on the filter. We have confirmed that this cDNA array analysis is useful for the diagnosis of changes in the Th1/Th2 balance.

In the present paper, we demonstrated that the cDNA array filter is applicable to the diagnosis of an imbalance of mouse Th1/Th2 immunity at an early phase of GVHD. We are now developing a similar filter to be used for human samples. This cDNA array would potentially be useful for the diagnosis and treatment of a number of disorders that show skewing of the Th1/Th2 balance. For instance, patients with multiple sclerosis, a demyelinating disease of the central nervous system, experience repeated relapse and convalescence. When the immune balance skews to Th1, demyelination takes place and clinical symptoms appear. If patients are not treated immediately at this stage with, for example, steroids, the demyelination becomes irreversible and sequelae would remain. With the use of our cDNA array filter, changes in the Th1/Th2 balance would be detected at the stage where changes occur in gene expression, i.e. before clinical symptoms appear. This means that the patients can be treated at a very early stage of aggravation, and as a result, would be able to live normally without sequelae. Alternatively, this filter may also be used for the early diagnosis of GVHD after bone marrow transplantation. Likewise, this cDNA array filter could become a powerful tool for the early diagnosis, early treatment, and prevention of relapse of immune diseases caused by the skewing of the Th1/Th2 balance.

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