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Differential analysis of CD4$^+$ Th memory clones with identical T-cell receptor (TCR)-$\alpha\beta$ rearrangement (non-transgenic), but distinct lymphokine phenotype, reveals diverse and novel gene expression

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SUMMARY

This study describes a subtractive hybridization analysis to identify differences in gene expression between sibling Th memory clones, elicited by virus infection and expressing identical T-cell receptor (TCR)-$\alpha\beta$ rearrangements but distinct lymphokine phenotype: clone Bpp9 secretes interleukin (IL)-4, IL-5 and IL-10; clone Bpp19 secretes interferon (IFN)-γ, low levels of IL-4, and IL-5 on TCR ligation. cDNA sequencing of difference products (DP) identified both novel and known regulatory (DNA: RNA-binding) or signalling proteins (kinases: phosphatases). Of the 10 novel genes identified, three were putative membrane proteins, one a predicted nuclear protein containing a PEST sequence motif, one a predicted transporter fragment and one contained a zinc-finger motif. One of the membrane proteins was found only in RNA from the activated IFN-γ-producing clone, i.e. not in other tissues. In addition, a high frequency of granzyme A, B, C and G transcripts (for clone Bpp9) or transcripts for CD94 and NKG2A (for clone Bpp19) were expressed differentially, together with transcripts that mapped to, so far, unassigned regions of the mouse genome that may be further novel genes. The transcriptional profiles presented here may therefore include candidate regulators of Th diversity and effector function.

Keywords cytokines; murine; T lymphocytes; transcriptional profiling

INTRODUCTION

The Th1/Th2 division of CD4$^+$ T cells, made according to lymphokine secretion phenotypes,[1] is now a central paradigm of cell-mediated immunity. This model provides a rational basis for differences in host responses elicited by virus, bacteria and parasite infections, and accounts for the qualitative and quantitative differences seen in immune memory to natural infection as opposed to immunization. Moreover, from a developmental viewpoint, the Th1/Th2 divergence provides a relevant system for posing questions relating to cell-fate decision, namely how a naive Th0 cell develops and maintains a Th1 or Th2 phenotype on receiving environmental cues: are there distinct signalling pathways that recruit different effector cell types and what are the epigenetic mechanisms that establish commitment?

It is acknowledged that, for the most part, the effects of environmental factors influencing Th1/Th2 development are mediated by cytokines acting in concert with events following antigen-mediated T-cell receptor (TCR) ligation and costimulation. These events lead to chromatin remodelling at the lymphokine loci mediated by key transcriptional factors: the T-box transcription factor T-bet being pivotal for Th1 development and the zinc-finger transcription factor GATA-3 for Th2. Although there is an established role for the innate immune response in early Th1 development, mediated by interleukin (IL)-12 and interferon (IFN)-γ, there has been no such requirement demonstrable for Th2.[2–6]

TCR Tg mice have proved the model system of choice in investigating the cellular and molecular basis of the Th1/Th2 divergence, as evidenced by our cited references. Naive, antigen-specific Tg Th0 cells are cultured, *in vitro*,

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Abbreviation: DP, difference product.

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under conditions that favour Th1 or Th2 expansion for several days prior to comparative analysis and have confirmed and extended earlier findings of *in vivo* Th-cell responses to infection. Here, we describe a non-transgenic example of lymphokine phenotype diversity within the clonal progeny of a virus-specific Th0 cell and our attempts at establishing the molecular basis for this variation in a Th memory population.

We have previously reported clonal analyses of the Th1/Th2 repertoire for influenza virus haemagglutinin following natural infection.[7] Th clones from an individual C57BL/10 donor (described here) and specific for HA1186–205 peptide, released either (IFN-γ and IL-5) or (IFN-γ, low IL-4 and IL-5) or (IL-4, IL-5 and IL-10) on stimulation. This was independent of the type of stimulation, i.e. antigen (either peptide or whole virus) or mitogen [either concanavalin (Con) A or anti-CD3]. TCR-$\alpha\beta$ sequence analysis established that all the Th clones from this one C57BL/10 donor expressed identical (VDJ)β and (VJ)α rearranged sequences despite their lymphokine phenotype diversity, and were therefore deemed to be derived from a common Th0 progenitor cell following virus infection *in vivo*. This multipotentiality has been seen previously in *in vitro* cultures.[8]

The genetic identity of these Th clones and their phenotypic differences made them suitable candidates for investigating the molecular basis of intraclonal diversity in the Th memory repertoire. In this study, two sibling Th clones from the C57BL/10 donor, clone Bpp9 (secreting IL-4, IL-5 and IL-10) and clone Bpp19 (secreting IFN-γ, lower levels of IL-4 and IL-5) were stimulated for a brief period (2 hr) with viral peptide in order to optimize cDNA difference analysis of lymphokine-related events at an early phase of T-cell activation. Analyses of the resulting cDNA sequences using current databases have identified both novel genes and the differential expression of gene products that are known to function in cell signalling or transcriptional and translational control, and may therefore be implicated in Th activity.

MATERIALS AND METHODS

T-cell clones

A summary of cytokine specificities and TCR sequences is given in Table 1. T-cell clones Bpp9 and Bpp19 were maintained *in vitro* by stimulation with UV light-inactivated X31 virus (100 HAU/ml) and irradiated (30 Gy) syngeneic spleen cells, as antigen-presenting cells (APCs), every 10–12 days. Interleukin-2-containing supernatant from Con A-stimulated rat splenocyte cultures was added at day 3.

All assays of antigen specificity and lymphokine secretion were carried out at the end of the 10–12-day feed cycle, at which stage the majority of T cells were in the resting G_1/G_0 state.

T-cell purification and stimulation for subtractive hybridization

Viable cells were separated on a miniMACS column (Miltenyi Biotech, Bergisch, Gladbach, Germany) using a MACS Dead Cell Removal Kit (Miltenyi Biotech). Cells were then purified further by incubation with FITC rat antimouse CD4 antibody (BD Biosciences, Franklin Lakes, NJ, USA) and FACS Vantage (BD Biosciences) sorting for cells expressing CD4. Purified T cells were incubated at a concentration of 5×10^5 cells per ml for 2 h with the haemagglutinin peptide p186–205 (10 μg/ml) before harvesting.

cDNA synthesis and subtraction

cDNA was synthesized from total RNA and amplified by polymerase chain reaction (PCR) using the SMART™ cDNA Synthesis Kit (BD Biosciences). dsDNA was purified on a Clontech CHROMA SPIN-1000 column, digested with *Rsa*I restriction enzyme and purified with the Clontech NucleoTrap® PCR Kit (BD Biosciences).

Subtractive hybridization was carried out in both directions (i.e. Bpp19–Bpp9; Bpp9–Bpp19) using the Clontech PCR-Select™ cDNA Subtraction Kit (BD Biosciences). Briefly, adapters were ligated to the tester cDNA. Tester DNA was mixed with an excess of the driver DNA. Differentially expressed DNAs were then enriched by two rounds of hybridization followed by two rounds of PCR amplification.

Subtracted cDNA libraries and sequencing of cloned products

Subtracted PCR products were cloned into the pT-Adv plasmid (Clontech Advantage PCR Cloning, BD Biosciences). TOP10F′ *Escherichia coli* competent cells were transformed and spread onto LB/agar plates. Following incubation at 37°, 96 colonies from each of the Bpp19- or Bpp9-plated libraries were picked arbitrarily for analysis. Plasmids were isolated using Wizard Plus Minipreps (Promega, Madison, WI, USA). The presence of plasmid

Table 1. Summary of Bpp19 and Bpp9 T-cell clones phenotype

(a) Lymphokine secretion of clones*

Clone	IFN-γ	IL-4	IL-5	IL-10
Bpp19	22–70	0·01–0·06	0·8–15	< 0·01
Bpp9	< 0·01	0·2–0·4	17–24	1·9–6·0

(b) Nucleotide and deduced amino acid sequence of TCRβ- and α-chain junctional regions

← Vβ8·2				Jβ2·6 →		← Vα5					Jα24 →
G	R	G	W	E	Q	V	S	N	N	R	I
GGG	CGA	GGG	TGG	GAA	CAG	GTC	AGC	AAT	AAC	AGA	ATC

*Range of response with either virus, peptide or concanavalin A stimulation by ELISA, ng/ml; < 0·01, below detectable levels.
Ref. Graham *et al.* [7].

Table 2. Primers used for RNA tissue panel

Primer	Sequence (5′–3′)
NTOG-1F	ACTATTCTTCTGTCGGTCATGCAG
NTOG-1R	CAACAGGAGTTGGTTTCAAATGAC
NTOG-2F	CTCAAGATGTACCGAGACTCTCAC
NTOG-2R	CAAGTGCTGTCAAATCTAGCA
NTOG-3F	TGGGTCCTCTCATCCTGAAG
NTOG-3R	TCCAAAGACTTTTCTATGCACAA
NTOG-4F	GTGTGCGTCTGTTGGAAGAAT
NTOG-4R	CCTGTTTCCCATGGACTCTG
NTOG-5F	CTAAATTCCCAGCACCCACACT
NTOG-5R	GGAGTGTTTAGATGCCTTTGGA
NTOG-6F	ATCGGAAAAGCCATGCTTCCC
NTOG-6R	AGCTGAAGACGAAGCTGCAGACCT
NTOG-7F	GTAATGGGCATTGTTCATGG
NTOG-7R	TTCCCAGAATCTCCATTTTCA
NTOG-8F	GAGACAGCGGCTACTCCTTTGT
NTOG-8R	AGACCTTGTGTCCTTGCATTG
NTOG-9F	GCTTCACTGGGACAGGCATT
NTOG-9R	CAGCCCCTAGGATGGCATT
NTOG-10F	GGGAGGGTTCATCACGACTGACTGACTGAGA
NTOG-10R	GTTCCTTTCTCTCAGACTGGTCTCCTCT

inserts was confirmed following digestion with *Eco*RI restriction enzyme and by 1·2% agarose gel. Inserts were then sequenced with a DNA Sequencing Kit (Applied Biosystems, Foster City, CA, USA) using M13 primers and run on an ABI Prism 377 DNA Sequencer (Applied Biosystems). All primers were supplied by GenoSys (Sigma-GenoSys, Pampisford, Cambs, UK).

Data analysis

The resulting sequences were submitted to translated BLAST searches through the European Bioinformatics Institute (EBI) (http://www.ebi.ac.uk) and National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) websites. Sequences were also submitted to sequence search and alignment by hashing algorithm (SSAHA) searches of the mouse genome using the Ensembl Genome Browser (http://www.ensembl.org). For genes where more than one transcript match was found sequences were aligned using the CLUSTALW (http://www.ebi.ac.uk) and AUTOASSEMBLER 2·0 (Perkin Elmer) programs. Functional analysis was carried out using FINGERPRINTSCAN and PPSEARCH programs (http://www.ebi.ac.uk).

RNA panel

Forward and reverse primers of around 20 base pairs (bp) in length were designed for the novel gene transcripts using Sigma's basic oligo calculator (http://www.sigma-genosys.co.uk) and the PRIMER3 program (frodo.wi.mit.edu/cgi-bin-/primer3) (Table 2). Primers were supplied by GenoSys (Sigma-GenoSys, Pampisford, Cambs, UK). cDNAs were synthesized from a mouse total RNA master panel (BD Biosciences Clontech, Palo Alto, CA) using 10 mM dNTPs (Amersham Biosciences UK Ltd., Little Chalfont, UK), primer $p(DT)_{15}$ (Roche Diagnostics Ltd., Lewes, UK), NaPPi and M-MLV-reverse transcriptase (Invitrogen, Gibco BRL, Paisley, UK). The resulting cDNAs were subjected to PCR: 33 cycles of 30 seconds at 95°, 30 seconds at 60° and 2 min at 68° using Advantage 2 polymerase mix and Advantage 2 PCR buffer (BD Clontech), dNTPs (Amersham) and the relevant primers. The resulting products were run on a 1·2% agarose gel against known molecular weight markers to check specificity.

RESULTS

Identification of cDNA difference products (DPs)

Following sequencing on all positive plasmids, 73 Bpp9 and 77 Bpp19 DP sequences, varying between approximately 100–1000 bp in length, were submitted to BLAST or SSAHA searches and the results presented in Table 3 (Bpp19) and Table 4 (Bpp9). Positive matches are divided for clarity into five categories: novel genes (Group A), known genes for regulatory/signalling proteins (Group B), known genes for frequent DP (Group C), a miscellaneous group of known genes with diverse structure/function (Group D) and Group E, where sequences could be mapped to regions of the mouse genome, but had no evident gene assignment. Gene names, EMBL and Ensembl identification number, chromosomal location, protein group and the frequency of DP found are given.

Group A: novel DPs

Several cDNA sequences could not be assigned to known genes but matched predicted novel genes on the mouse genome. These genes are assigned by homology to known genes in other species, or have a predictive algorithm or motif that allowed a structure/function assignment. Predicted

Table 3. Transcriptional profile of T cell clone Bpp19

	Gene name	Chromosome	EMBL	Ensembl	Gene group[(Ref.)]	Repeat frequency
Group A (novel genes)	NTOG-1 (NM_133718)	9	BE854018	G32328	Membrane protein (364 aa)	1
	NTOG-2 (NM_172514)	15	AA254633	AA254633	Membrane protein (287 aa)	1
	NTOG-3 (T5841)	3	AK005184	G28165	Membrane protein (135 aa)	1
	NTOG-4 (1110019I14Rik)	3	AK003820	G37400	Putative amphipathic transporter fragment (293 aa)	1
	NTOG-5 (T27763)	1	BE454285	G26485	Zinc-finger protein (132 or 285 aa)	1
	NTOG-6 (T35738)	16	AC015605	G35301	PEST-containing nuclear protein (178 aa)	1
	NTOG-7 (T36489)	5	AA414454	G39968	No description (260 aa)	1
	NTOG-8 (NM_153567)	5	BC038019	G36087	No description (318 aa)	1
Group B (regulatory/signalling)	Asah1	8	BC003204	G31591	Acid ceramidase[32]	1
	Pcbp2	15	AF236845	G23049	Poly(RC)-binding protein 2[14]	1
	Ptpn2	18	MMPTP	G24539	Phosphotyrosine phosphatase nonreceptor type PTP2[33,34]	1
	Son	16	AF193607	G22961	DNA-binding protein[15]	1
	Tebp	10	AB024935	G40078	Telomerase-binding protein[51]	1
	Unr	3	BC016898	G27853	RNA chaperone[11–13]	1
Group C (frequent DPs)	Klrc1	6	AF106008	G30167	NKG2A[16,19–22]	5
	Klrd1	6	AF030312	G30165	CD94	4
Group D (miscellaneous genes)	C6·1a	X	S68002	G31201	Unknown	1

Group E Unassigned Bpp19 matches chromosomes Ensembl/EMBL.

chr1: AZ590900, BB637539; chr2: AC084288, AF084363, AC091241, AC102705, AF146213; chr3: AC095992, BH174734; chr4: AC073667, AC096022, AC097294, AR038762, BC471007; chr5: AC005817, AC096966, AK020884, AZ433912, BE447421, BI793839; chr6: AC024913, AC069142, AL450397, BB450138, BI963367; chr7: AC079535; chr 8: BG624207; chr9: BB008092, BB148570; chr10: AC007961 (× 2), AL606932; chr11: AC023084 (× 2), AC027641, AL645602; chr12: BB217335, BM658972, chr14: AC079533, AF335420; chr15: AC025528, AC079490, AZ505762; chr16: AB049357, AC096378, AK018679, BH040139, E36258, X78692; chr 18: AL590866, BB444511, BE372342; chr X: AC096104.

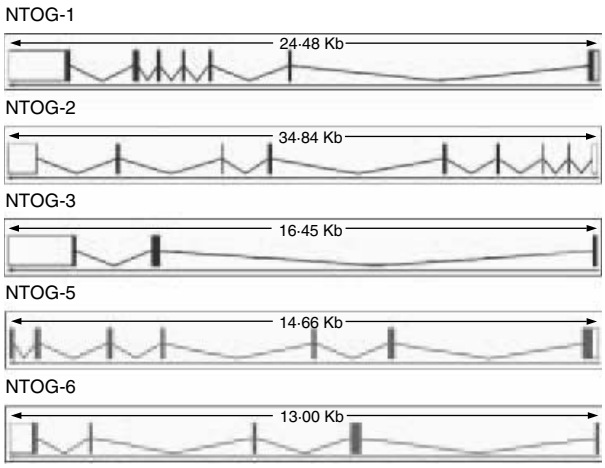

Figure 1. Predicted transcript structure (Ensembl).

transcript structures are given in Fig. 1(a,d) for clarity of presentation novel genes have been designated NTOG-1 to -10.

Candidate membrane proteins. For Bpp9 we found the repeat occurrence of three transcripts that matched the gene NTOG-1 (NM_133718), a putative transmembrane protein from chromosome 9. All three transcripts corresponded to a single region in the 3′ UTR. One transcript from Bpp19 also matched the same gene (NTOG-1), found in Bpp9. However, for Bpp19, the isolated sequence corresponded to a region 2 kb downstream of the Bpp9 products. This was the only occurrence of a transcript found from both clones.

In addition, for Bpp19, two further transcripts were found that matched distinct membrane-coding sequences. The first, NTOG-2 (NM_172514), was found on chromosome 15, protein analysis revealed no functional motifs. The third gene coding for a membrane protein, NTOG-3 (T5841), was present on chromosomes 3 and was predicted by consensus annotation (100%) to be a member of the group of 'uncharacterized haemopoietic/stem progenitor cells protein MD5029 homologues' of which there are two Ensembl gene members.

Candidate regulatory proteins. In the Bpp19 library we found several transcripts for candidate regulatory proteins. One transcript matched gene NTOG-4 (1110019I14Rik). This gene has five predicted transcript forms, which we were not able to distinguish from the short sequence generated. Computer analysis of the protein sequence, however, found the motif DKTGTLT, characteristic of an E1-E2 type ATPase, and was predicted to be putative ampipathic transporter fragment.

One transcript matched gene NTOG-5 (T27763), which the Ensembl database shows as having two predicted gene structures 919 bp (132 aa) or 1063 bp (285 aa), which were not able to be distinguished by our short sequence. Protein functional analysis of the predicted protein sequence showed it contained one region with the classic C2C12H3H (CIICSATITRRDMLGHVKRH) zinc-finger motif, a nucleic acid domain, found in numerous nucleic acid binding proteins.[9]

Another transcript matched gene NTOG-6 (T35738). For this, Ensembl gives three predicted transcript structures on chromosome 16 based on ESTs; however, these could not be distinguished by our sequence. A homologue of the sequence was also found on mouse chromosome 6. Protein analysis revealed a PEST motif, indicative of a nuclear protein with a possible role in transcriptional regulation. Consensus annotation gave a 75% confidence of this being a member of a protein family containing five members in total.

Undescribed proteins. Two sequences mapped to genes NTOG-9 (NM_145449) and NTOG-10 (T60641), respectively. Functional analysis revealed no known motifs for these proteins; however, the latter may be related to the group of interferon alpha 6–16 inducible proteins.[10]

Analysis of two other genes NTOG-7 (T36489) and NTOG-8 (NM_153567) each found by a single transcript and both on chromosome 5 also showed no functional motifs.

Group B: known regulatory and signalling DPs

A large number of the DP were identified from the database as known gene sequences that represent proteins implicated in either transcriptional/translational control or as components of a signalling pathway. These included the following.

RNA-, DNA-binding proteins. For Bpp19, sequences corresponded to RNA-binding proteins unr[11–13] and poly(RC)-binding protein 2,[14] known to be expressed in myocardial development, the DNA-binding protein SON [an alternatively spliced form of negative regulatory element (NRE)],[15] together with a telomerase-binding protein. The zinc-finger protein 313 sequence was identified from Bpp9[9].

Cell signalling. For Bpp9, the DP represent components of signalling cascades that are closely associated with the cell surface, e.g. pellino or Numb or kinases and hydrolases, or a GTP-binding protein, Rap1b.

Group C: frequently expressed DPs

One group dominated from each of the two clones.

CD94/NKG2A. For clone Bpp19, multiple transcripts corresponding to CD94 (4/76) and alignment showed that the sequences spanned the whole gene including the 3′ UTR and 43 bases of 5′ UTR region (Fig. 1). In contrast all transcripts for NKG2A (5/76) spanned only the 672–1917 bp region of the gene sequence despite the presence of a *Rsa*I site.

Granzymes. For clone Bpp9 a high frequency of transcripts (21/81) had cDNA sequences corresponding to serine proteases: mast cell protease 8 and granzymes. Because of the high degree of homology between different granzymes, these sequences transcripts were aligned using the CLUSTALW program and could be divided phylogenetically into granzymes A, B, C and G.

Bpp9 cDNA was found to contain multiple inserts for two novel transcripts, one with homology to the interferon-inducible family of proteins, IFI 6–16[10] (see Group A), again consistent with an antivirus response by the Th memory population.

Group D: miscellaneous DPs

A varied assortment of known genes for which a candidate role in Th clonal diversity is not immediately apparent are listed in Tables 3 and 4 and are discussed later.

Group E: unassigned DP

Bpp9. Twenty-two transcripts had identity with ESTs, HTG clones, GSS libraries or pPGK vectors. Several transcripts were found more than once, matching AL596383 (× 2), AZ497263 (× 2) and BE138264 (× 2).

Bpp19. Fifty-three transcripts matched unassigned regions. Again several transcripts were found more than once, i.e. for AC007961 (× 2) and AC023084 (× 2).

These difference products although presently unassigned may represent yet to be defined gene sequences.

Tissue distribution of novel genes

The findings in this survey (Table 5) are of interest in the broader context of developmental biology since several transcripts exhibit restricted expression in non-lymphoid tissues, both of embryo and adult.

Oligonucleotide primers (Table 2) amplified transcripts from an RNA master panel of representative tissues, using G3PDH and actin primers as positive controls, and correct predicted size of transcripts (100–600 bp) was confirmed by gel analysis.

For the novel genes (NTOG-7 and NTOG-10) with no known functional motifs or homologues or the putative membrane protein (NTOG-2), these transcripts are absent from all tissues tested, including spleen, and indicate that they are restricted to a minor population of lymphoid cells, presumably a subset of Th.

In contrast, the membrane protein (NTOG-1) is developmentally restricted to 11-day-old embryo, brain and heart and absent from 7-day-old and 15-day-old embryo, and all other adult tissues. Similarly NTOG-9, the putative interferon alpha-induced gene, is also developmentally restricted and was found in only in 15-day-old embryo, and in heart, submaxillary gland, smooth muscle and testes tissues.

The PEST protein (NTOG-6) has a distinctive pattern in development and is absent from embryonic tissues and all adult tissues with the exception of liver and testis and membrane protein NTOG-3 was seen only in smooth muscle and testis.

The remaining transcripts had a more ubiquitous tissue distribution. The putative regulatory proteins were the most

Table 4. Transcriptional profile of T cell clone Bpp9

	Gene name	Chromosome	EMBL	Ensembl	Gene group[(Ref.)]	Repeat frequency
Group A (novel genes)	NTOG-1 (NM_133718)	9	BE034992	G32328	Membrane protein (364 aa)	3
	NTOG-9 (NM_145449)	12	BE138264	G21208	Interferon-induced precursor 6–16 FAM14A (215 aa)[10]	3
	NTOG-10 (T60641)	17	BE570229	G48915	No description (122 aa)	2
Group B (regulatory/signalling)	Ctla2b	13	MMCTLA2A	G442S8	Cysteine protease inhibitor 1[52]	1
	MAPKKKK3	17	BC005781	G24242	Similar to mitogen-activated protein kinase kinase kinase kinase 3[53]	
	m-Numb	12	MU70674	G21224	Notch antagonist[31]	1
	Peli1	11	AC091421	G20134	Pellino 1[29,30]	1
	Rap1b	10	BF162089	G20200	GTP-binding protein[35–38]	1
	Zfp313	2	AL589870	G6418	Zinc-finger protein 313[9]	1
Group C (frequent DPs)	Gzma	13	AW494114	G23132	Granzyme A[25–28]	5
	Gzmb	14	BC002085	G15437	Granzyme B	8
	Gzmc	13	MMB10	G15441	Granzyme C	1
	Gzmg	14	MMGRAG	G40284	Granzyme G	4
	Mcpt8	14	MMCP8SP	G22157	Mast cell protease 8 precursor	3
Group D (miscellaneous genes)	Esm-1	16	AK002913	G42379	Endothelial cell-specific molecule 1 precursor[44]	1
	Ftl1	13	MMFERLA	G50708	Ferritin light chain 1[40]	1
	L37A	2	HSPD27388	G50421	60S ribosomal protein L37A[48]	1
	Lum	10	S79461	G36446	Lumican precursor[27,41,42]	1
	Mt1	8	MM1265505	G31765	Metallothionein-1[43]	1
	Prg	10	BI080584	G20077	Serglycan[54]	1
	Pth	7	RNCPTHRA	G44579	Parathyrin[39]	1
	RL44	6	BE137621	G49751	Ribosomal protein[49,50]	2
	Rpn2	2	AK004968	G27642	Ribophorin II[46,47]	1
	Sdf4	4	BC005715	G29076	Calcium binding precursor Cab45[45]	1
	Slc17ab	7	AK013772	G30500	Solute carrier family 17 member 6[55]	1
	Tcb1	6	MMTCBXH	G29878	T cell receptor beta-2 chain C region	2
	Ubiquitin	4	BM123337	G45373	Ribosomal protein[56]	3

Group E: Unassigned Bpp9 matches to chromosomes Ensembl/EMBL.

chr1: AL645950; chr2: AC116592, AK004968, AL589870, AL590373; chr 6: AI653760, AQ974933, MMPPC3, TVB6; chr7: BC002085; chr 8: BM123337; chr 9: AL596383 (× 2), AZ497263 (× 2); chr 10: AZ996521; chr 11: AZ017265; chr 12: AC055772, BE138264 (× 2); chr 17: AC008823 AF092750.

distributed widely: NTOG-4, the transporter fragment, was found in 7-day-old embryo, brain, heart, kidney, smooth muscle and testis; NTOG-5, the zinc-finger protein, amplified from all tissues except 15-day-old embryo and eye; and NTOG-8 was found in heart, kidney, smooth muscle and testis samples.

DISCUSSION

The main drawback of subtractive analysis has been frequent amplification of 'housekeeping' genes due, presumably, to an imbalance in metabolic and/or cell cycle status of the subtractive pair. This has not been our finding, as many DP were either novel or sequences for known regulatory/signalling proteins not implicated before, in Th effector function. The close matching of Th clones (in a quiescent G_0 state), with only a brief exposure to virus peptide before subtraction, may have contributed to our findings. It is striking that from this initial subtractive trawl of a sibling Th pair, we have identified three putative membrane proteins and two regulatory proteins (a zinc-finger containing- and a nuclear protein with a PEST motif) and an amphipathic transporter fragment. In addition, a large number of DP could not be assigned, based on homology to known sequences and will be the subject of further study. However, the aim of this current report has been to place in the public domain predicted sequences and gene assignments of transcripts that, due to their differential expression by Th clones, may provide a candidate list for further functional studies of Th effector diversity and function.

The genes encoding cytokines are in an inactive form in resting T cells, and T-cell triggering promotes the up-regulation of gene expression by the resulting signalling. For the system described here, using TCR-matched clones and synchronization of stimulation as an on/off system, we hoped to create a narrow window for difference products eliminating the vast background of constitutively active, and housekeeping genes. Therefore, we were pleased to see the absence of those genes, such as thymidine kinase, in our

Table 5. Tissue distribution of novel genes by primer amplification of an RNA master panel

	Tissues													
Gene	7d embryo	11d embryo	15d embryo	brain	eye	heart	kidney	liver	lung	spleen	submax gland	smooth muscle	testis	Bpp19 + peptide
NTOG-1	–	+	–	+	–	+	–	–	–	–	–	–	–	+
NTOG-2	–	–	–	–	–	–	–	–	–	–	–	–	–	+
NTOG-3	–	–	–	–	–	–	–	–	–	–	–	+	+	+
NTOG-4	+	–	–	+	–	+	+	–	–	–	–	+	+	+
NTOG-5	+	+	–	+	–	+	+	+	+	+	+	+	+	+
NTOG-6	–	–	–	–	–	–	–	+	–	–	–	–	+	+
NTOG-7	–	–	–	–	–	–	–	–	–	–	–	–	–	+
NTOG-8	–	–	–	–	–	+	–	+	–	–	–	+	+	+
														Bpp9 + peptide
NTOG-9	–	–	+	–	–	+	–	–	–	–	+	+	+	+
NTOG-10	–	–	–	–	–	–	–	–	–	–	–	–	–	+

transcripts, indicating that the subtractive hybridization analysis had been successful.

In addition to a diverse array of known genes, several DP sequences that matched novel genes were identified. These included genes involved in regulatory functions. Firstly a Bpp19 gene consisting of five exons containing a PEST motif, which is predicted to translate to a 178 aa protein and that was also found only from liver and testis. Secondly, we found a zinc-finger protein with a wide tissue distribution. Zinc-finger proteins are known to control trancription, the presence of a single motif suggests that it may be involved in either RNA- or DNA-binding.

Three novel membrane proteins were picked out by sequencing. The first gene transcript has three exons, is predicted to translate to a 135 aa protein containing one transmembrane region and has a wide tissue distribution.

The second transcript consists of seven exons, translates to a 364 aa protein containing two transmembrane regions, and was found in 11-day-old embryo and smooth muscle. Interestingly, this gene matched a single transcript from Bpp19 but multiple transcripts from the Bpp9 T cell. All products mapped to the 3′ UTR, but whereas the Bpp19 product matched the far end of the 3′ UTR (at 82372791 bp) the Bpp9 difference products all matched a region 2 kb upstream (at 882374896 bp), suggesting that the subtraction had discriminated two forms of one protein, possibly using distinct polyadenylation sites.

The third membrane protein consists of 10 exons and translates to a 287 aa protein containing two transmembrane regions; the distribution of this DP was limited to our stimulated Bpp19 cell clone and this suggests therefore that it may be found only on activated Th1 cells. Further studies will be need to characterize all of these membrane proteins and evaluate their usefulness as markers of T cell differentiation.

A striking feature of the DP profile for clone Bpp19 was the finding of multiple transcripts of cDNA sequences of CD94 and NKG2A, which is now being proposed as a marker for Th1 versus Th2 cells. Previous studies have shown that these two proteins form a heterodimer, the CD94/NKG2A receptor, originally described on NK cells,[16] but which has been characterized recently on T cells. These proteins are part of the NKG2 family which includes NKGD2A/C/D and E[17] and which bind to MHC class I molecules.[18] The CD94/NKG2A heterodimer has been shown to negatively regulate CD69-triggered ERK activation and functions.[19–21] It is interesting that of the three known NKG2 forms, A/C/E, that pair with CD94, only NKG2A was found here. Surface expression of the CD94/NKG2 receptor as a marker of murine Th1 versus Th2 cells complements the activation molecules SLAM and TIM[22–24] and our results are consistent with the proposed differential expression of this receptor on activated Th1 cells.

Similarly, for clone Bpp9, there was a high frequency of transcripts with cDNA sequences corresponding to serine proteases: mast cell protease 8 and granzyme A, B, C and G proteases. In contrast to the constitutive expression found in NK cells, granzymes in T lymphocytes are synthesized *de novo* upon antigenic restimulation.[25] Granzymes A, B and C have been shown previously to be expressed differentially in T cells, with granzyme A in murine Th1, granzyme B in murine and human Th1 and granzyme C in murine Tcl.[26–28] Although granzyme G is present in mature T cells, and is up-regulated by IL-2 and IL-5, as far as we are aware there is no report of its differential expression by Th subsets.

A large number of the DPs that could be identified from the database as known gene sequences represent proteins that are implicated in either transcriptional/translational control or as components of a signalling pathway. Sequences corresponded to RNA-binding proteins unr[11–13] and poly(RC)-binding protein 2,[14] known to be expressed in myocardial development. The DNA-binding protein SON [an alternatively spliced form of negative regulatory element (NRE)],[15] together with a telomerase-binding protein were present in Bpp19 while the zinc-finger protein 313 sequence was identified in Bpp9.

Cell signalling DPs represent components of signalling cascades that are associated closely with the cell surface (pellino[29,30] or Numb[31] for Bpp9), or kinases/hydrolases

(acid ceramidase,[32] shown to activate the NF-kappaB and MAPK kinase pathways, phosphotyrosine phosphatase non-receptor type PTP2,[33,34] and similar to MAPKKKK3 for Bpp9) or a GTP-binding protein (Rap1b[35–37] for Bpp9). Interestingly, Numb, a phosphotyrosine-binding antagonist of Notch signalling, is required for cell-fate decisions in other developmental systems while the role of Notch in T-cell development is well documented. The role of the Rap1 family in T-cell activation has recently been the focus of considerable attention.[38]

The function of several DPs, such as the parathyroid hormone precursor (parathyrin)[39] or ferritin light chain, role in Th clonal diversity[40] is not immediately apparent. Other involvement, however, may be inferred from known protein function or association with cytokines. Lumican[27,41,42] expression has been shown to be related to IL-5 levels. The stress-induced expression of metallothionein-1[43] is of common occurrence in natural infection and it has pleiotropic functions. Esm-1 is known to interfere with immune cell migration by binding to adhesion molecules and its expression in endothelial cells is regulated by cytokines.[44] Cab45 is a member of the CREC family of EF-hand proteins that localize to the secretory pathway of mammalian cells,[45] and ER protein ribophorin II[46] has been reported to be overexpressed in colorectal cancer.[47] The ribosomal protein, L37A, has been identified as being expressed differentially in mice differing at the If1 interferon regulatory locus;[48] attention is now being placed on the role of resident ribosomal proteins in translational regulation.[49,50]

In conclusion, this subtractive hybridization study has identified transcripts for both novel and known regulatory genes that are expressed differentially by sibling Th clones differing in lymphokine secretion phenotype. The transcription profiles that we have presented may provide a candidate list, therefore for future 'gain or loss of function' studies relating to Th development and diversity.

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